From the Department of Physiology and Pharmacology,
Karolinska Institutet, Stockholm, Sweden

and

The Swedish School of Sport and Health Sciences, Stockholm,
Sweden

INTRACELLULAR SIGNALING IN HUMAN SKELETAL MUSCLE FOLLOWING DIFFERENT MODES OF EXERCISE

Henrik Mascher

Stockholm 2010

Published by Karolinska Institutet. Printed by Universitetsservice US-AB, Stockholm 2010

ISBN 978-91-7457-144-8

ABSTRACT

Resistance and endurance exercise when performed regularly will cause specific adaptations in human skeletal muscle. Resistance exercise is known to increase strength and muscle mass while endurance training increases vascularisation and mitochondrial density which results in enhanced oxidative capacity. To understand how these adaptations occur, it is important to examine the molecular signaling events in muscle. The Akt-mTOR pathway has been shown to have an important function in the stimulation of protein synthesis.

This pathway is stimulated following resistance exercise in human muscle. During the work included in this thesis it has become clear that endurance exercise also stimulates Akt-mTOR signaling in human skeletal muscle. Study (I) revealed an increased phosphorylation of mTOR, Akt and GSK3 and a marked decrease in eEF2 phosphorylation indicating a stimulatory response on elongation and initiation of protein synthesis in the early recovery phase. Furthermore, as shown in study (II), this stimulatory response is followed by an increase in the fractional synthetic rate (FSR), which was progressively increased when measured up to 3 h following endurance exercise.

It is usually recommended that resistance exercise is performed 2-3 times per week. In study (III), markers for anabolic (Akt, mTOR, p70S6k, rpS6, eEF2 and GSK-3β) as well as catabolic (MAFbx and MuRF-1) processes were investigated following two sessions of resistance exercise separated by 48 hours. From this study it appears that anabolic signaling is slightly enhanced following the second exercise session, and furthermore, the changes in gene expression related to muscle protein degradation (MAFbx and MuRF-1) is attenuated during the second exercise session.

Endurance exercise can compromise the adaptive response of strength training. On the other hand, there is some evidence suggesting that combining endurance training with resistance exercise may have beneficial effects on endurance exercise performance. The final study was designed to evaluate whether resistance exercise can enhance the muscle adaptive response to endurance exercise with respect to molecular signaling related to increased protein synthesis and specific markers for mitochondrial biogenesis. An enhanced signaling response was actually found in the combined exercise protocol. Specifically, expression of genes related to increased mitochondrial biogenesis and oxidative metabolism (PGC-1α, PRC and PDK-4 mRNA) as well markers for anabolic signaling (mTOR, p70S6k), was enhanced when endurance exercise was followed by a session of heavy resistance exercise. This data support the notion that including resistance exercise in endurance training may be beneficial.

In summary, mixed muscle FSR is gradually increased following endurance exercise when measured during the first 3 h of recovery and this increase is accompanied by stimulation of mTOR signaling. Resistance exercise enhances effects on anabolic signaling and attenuates expression of genes involved in muscle protein breakdown and inhibition of muscle growth during a second exercise session performed two days after the first. Finally, combining endurance and heavy resistance exercise can enhance acute adaptive responses and indicates that combined exercise may be superior to endurance exercise alone.

LIST OF PUBLICATIONS

This thesis is based upon the following articles, referred to by their roman numbers (I, II, III, IV)

I. **Henrik Mascher**, Helena Andersson, Per-Anders Nilsson, Björn Ekblom and Eva Blomstrand. Changes in signalling pathways regulating protein synthesis in human muscle in the recovery period after endurance exercise. *Acta Physiol* 191: 67-75, 2007.

II. **Henrik Mascher**, Björn Ekblom, Olav Rooyackers and Eva Blomstrand. Enhanced rates of muscle protein synthesis and elevated mTOR signaling following endurance exercise in human subjects. Submitted, *Acta Physiol* 12:th of august, 2010.

III. **Henrik Mascher**, Jörgen Tannerstedt, Thibault Brink-Elfegoun, Björn Ekblom, Thomas Gustafsson and Eva Blomstrand. Repeated resistance exercise training induces different changes in mRNA expression of MAFbx and MuRF-1 in human skeletal muscle. *Am J Physiol Endocrinol Metab* 294: 43-51, 2008.

IV. Li Wang, **Henrik Mascher***, Niklas Psilander*, Eva Blomstrand and Kent Sahlin. Resistance exercise enhances the effect of endurance training on molecular signaling in human skeletal muscle *Manuscript.*
** These authors have contributed equally*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

4EBP-1	4E binding protein 1
ADP	Adenosine diphosphate
Akt/PKB	Protein kinase B
AMPK	Adenosine monophosphate activated protein kinase
APE	Atom percent excess
BCAA	Branched chained amino acids
cMyc	Avian myelocytomatosis viral oncogene homolog
CT	Cycle threshold
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
eIF2B	Eukaryotic initiation factor 2B
eIF3	Eukaryotic initiation factor 3
eIF4E	Eukaryotic initiation factor 4E
eIF4G	Eukaryotic initiation factor 4G
EGTA	Ethylene glycol tetra acetic acid
ERK1/2	Extracellular signal regulated kinase 1/2
FOXO	Forkhead box, class O
FSR	Fractional synthetic rate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatograph mass spectrometer
GDP	Guanosine diphosphate
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hVps34	mammalian vacuolar protein sorting 34
JNK	c-Jun N-terminal kinases
MAFbx	Muscle atrophy F-box
mRNA	Messenger ribonucleic acid
MuRF-1	Muscle RING Finger 1
MyoD	Myogenic factor D
p21	Cyclin dependent kinase inhibitor 1
P38 MAPK	p38 mitogen-activated protein kinase
p70S6k	p70 S6 kinase
PCR	Polymerase chain reaction
PDK4	Pyruvate dehydrogenase kinase 4
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PLD1	Phospholipase D1
PMSF	Phenylmethanesulfonylfluoride
PRC	PGC-1 related coactivator
PVDF	Polyvinylidene fluoride
REDD1/2	Regulated in development and DNA damage responses 1/2

Rheb	Ras-homolog enriched in brain
RM	Repetition maximum
RNA	Ribonucleic acid
rpS6	Ribosomal protein S6
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SSA	Sulphosalicylic acid
TRIS	Tris(hydroxymethyl)aminomethane
tRNA	Transfer ribonucleic acid
TSC	Tuberous sclerosis protein
Vo_{2max}	Maximal oxygen uptake

1 INTRODUCTION

The skeletal muscle tissue encompasses a unique plasticity to undergo large changes in weight and size. In the steady state, proteins are subjected to continuous synthesis and breakdown for repair, growth and replacement in the muscle. Protein turnover refers to these simultaneous processes of breakdown and synthesis. It has been estimated that around 400 g of proteins are broken down and the same amount of proteins are synthesized each day in a healthy individual. Approximately 75% of the proteins broken down are reutilized. By the use of isotopically labeled amino acids as tracers, the rate of turnover can be estimated. Rates of protein synthesis vary in different tissues. In human skeletal muscle, the protein turnover is reported to be around 1-2% per 24 hours. However, the balance between the rate of synthesis and degradation can be altered by different stimuli such as changes in hormonal levels, injury, disease, nutritional intake and muscle contractions. In a state when the rate of synthesis exceeds the rate of breakdown there is a positive net balance, whereas in a catabolic state, the rate of breakdown exceeds the rate of synthesis. The intracellular signaling events regulating muscle protein turnover in response to exercise are not completely understood, but the Akt-the mammalian target of rapamycin (Akt-mTOR) signaling pathway seems to play a crucial role in stimulation of muscle protein synthesis while the regulatory mechanisms for protein breakdown remains more elusive.

1.1 EFFECTS OF EXERCISE ON THE RATE OF PROTEIN TURNOVER

Skeletal muscle has the capacity to adapt to the type of work demanded such as endurance or resistance exercise. For example, regularly performed endurance exercise will increase vascularisation, mitochondrial protein density and oxidative capacity which results in improved fat oxidation and endurance (Holloszy & Booth 1976, Henriksson 1977, Saltin & Gollnick 1983) while resistance exercise is commonly known to increase muscle mass and strength. The introduction of stable isotope tracers in the research on human muscle protein metabolism during the 1980s has improved the knowledge of these processes greatly. Research on changes in muscle protein turnover following exercise has focused mainly on resistance exercise. However, in 2005, Miller and colleagues reported a large increase in the fractional synthetic rate (FSR) of myofibrillar and sarcoplasmic proteins following endurance-like exercise (1 h leg kicking at 67% of maximum workload). Following this, several studies have recently been conducted on the subject (Howarth et al. 2009, Wilkinsson et al. 2008,

Harber et al. 2009 and 2010, Durham et al. 2010). Generally, protein synthesis is thought to be repressed during exercise and increased in the recovery period following exercise. Protein breakdown is probably unchanged or increased during exercise and elevated in the recovery period (See Kumar et al. 2009). The magnitude of changes in protein metabolism in skeletal muscle in relation to exercise may be dependent on several factors such as the subject's nutritional status, mode of exercise, duration and intensity.

1.1.1 Resistance exercise

Resistance exercise when performed regularly is known to increase muscle mass over time. This results in an increased myotube diameter (hypertrophy) due to accretion of contractile proteins, mainly actin and myosin. It is well established that resistance exercise can alter the balance between muscle protein synthesis and breakdown. An acute bout of resistance exercise can increase the FSR in human skeletal muscle during the first hours and is still present up to 48 h in the recovery period following resistance exercise (Chesley et al. 1992, MacDougall et al. 1995, Phillips et al. 1997). However, in the fasted state the rate of protein breakdown is also enhanced but to a lesser degree. This leads to an improved net protein balance compared to rest but it still remains negative without nutritional supply (Biolo et al. 1995 and 1997, Phillips et al. 1997, Tipton et al. 2001). Studies on protein turnover during exercise are much more difficult since the muscle is not in steady state and the duration of exercise is usually too short for such measurements. When attempts are made to measure muscle protein turnover during exercise, the data indicate decreased or unchanged rate of synthesis (Durham et al. 2004, Dreyer et al. 2006, Drummond et al. 2008, Fujita et al. 2009), while muscle protein breakdown is unchanged (Tipton et al. 2003, Durham et al. 2004). Measurements on FSR in skeletal muscle are usually done on mixed muscle (including all proteins in muscle) but can be divided into myofibrillar, sarcoplasmic and mitochondrial fractions. Interestingly and somewhat unexpectedly, it was recently shown that the FSR of mitochondrial proteins is elevated following resistance exercise in untrained subjects (Wilkinsson et al. 2008). Changes in the rate of protein synthesis and possibly to some extent protein breakdown in skeletal muscle is likely to be mediated through a network of signaling pathways where the Akt-mTOR pathway seems to play a key role (Dreyer et al. 2006, Dreyer et al. 2008, Drummond et al. 2009, Kumar et al. 2009).

1.1.2 Endurance exercise

Acute alterations in protein turnover following endurance exercise in skeletal muscle are less well characterized in comparison to resistance exercise. Endurance exercise is commonly associated with increased mitochondrial density and capillarisation in muscle, hence, adaptations related to this type of exercise is likely to mainly involve synthesis of mitochondrial proteins. However, with few exceptions (Tipton et al. 1996) increased FSR in mixed muscle have been found in human skeletal muscle following this mode of exercise in both the post absorptive and fasted state (Carraro et al. 1990, Sheffield-Moore et al. 2004, Durham et al. 2010, Harber et al. 2010). Increased FSR has been reported following both low intensity (40% of Vo_{2max}) (Carraro et al. 1990, Sheffield-Moore et al. 2004, Durham et al. 2010) and moderate intensity exercise (~70% of Vo_{2max}) (Harber et al. 2010) with no obvious difference in magnitude at these intensities. Chronic endurance exercise has also been reported to increase resting FSR in mixed muscle by approximately 22% (Short et al. 2004). Most of these studies are based on measurements in mixed muscle, however, in the study by Miller and co-workers (2005), increased FSR was found in both myofibrillar and sarcoplasmic protein fractions that persisted up to 72 h in the myofibrillar fraction following 1 h of one-legged kicking exercise at approximately 70% of maximum workload. There is little information regarding protein breakdown following endurance exercise. However, protein breakdown assessed by arteriovenous differences of phenylalanine concentrations over the leg following low intensity exercise (40% of Vo_{2max}) suggests that protein breakdown is elevated immediately after but not 1 h after exercise (Sheffield-Moore et al. 2004).

A decrease in the rate of protein synthesis *during* endurance exercise has been shown on rodents (Dohm et al. 1982, Gautsch et al. 1998), but whether this is the case in human muscle is not known. The only study on FSR in human muscle during endurance exercise showed no change in muscle protein synthesis during exercise (Carraro et al. 1990). This study was done at low intensity (40% of Vo_{2max}), at higher intensity changes in molecular signaling speak in favor of an attenuated protein synthesis during intense endurance exercise. For example, findings of elevated eukaryotic elongation factor 2 (eEF2) phosphorylation and reduced phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4EBP-1) during exercise (Rose et al. 2005 and 2009) may reflect an attenuation of protein synthesis.

1.1.3 Combined exercise

Studies on combined exercise (resistance and endurance exercise performed simultaneously) indicate a compromised muscular adaptation on resistance exercise training. On the other hand, strength training may actually improve endurance performance (Hickson 1980, Marcinik et al. 1991, Kraemer et al. 1995, Hoff et al. 1999, Chtara et al. 2008, Rønnestad et al. 2010). A possible interference on resistance training when combined with endurance exercise has been suggested to be mediated by the so called adenosine monophosphate activated protein kinase (AMPK)-switch, as suggested by Atherton and colleagues (2005). AMPK activity is increased during exercise presumably due to an increase in the AMP:ATP ratio. It appears that activation of AMPK can decrease protein synthesis and hypertrophy in rodents (Bolster et al. 2002, Thomson and Gordon, 2005). However, in humans, increased AMPK activity concomitantly with increased protein synthesis has been found (Dreyer et al. 2006), so the inhibitory role of AMPK on protein synthesis in humans is yet to be determined. A molecular mechanism behind improved endurance performance when adding resistance exercise to an endurance exercise protocol is yet unknown, however, improved neuromuscular function and/or changes in the proportion of fiber types has been suggested. For example, resistance exercise is known to increase the proportion of type IIa fibers at the expense of type IIx fibers, which may be beneficial during endurance exercise (Andersen and Aagaard 2000). In a recent study by Coffey et al. (2009) the acute molecular signaling response following combined exercise in humans was investigated. The aim was to assess whether there was a difference in the molecular response depending on the order of exercise. No clear evidence for difference in order-dependent stimulation or interference of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) gene expression or mTOR phosphorylation was found.

1.2 REGULATION OF PROTEIN SYNTHESIS

1.2.1 The mTOR signaling pathway

The mammalian target of rapamycin (mTOR) has emerged as a crucial regulator of protein synthesis in muscle. The enzymes in the mTOR pathway are activated or deactivated by phosphorylation at specific threonine or serine residues. Activation of mTOR signaling has been associated with stimulation via hormones such as insulin growth factor 1 (IGF-1) and insulin. IGF-1 is known to stimulate Akt-mTOR signaling

and cause hypertrophy in myotubes (Bodine et al. 2001). Akt activates mTOR by phosphorylation at Ser 2448 which promotes mTOR signaling through phosphorylation of p70S6 kinase (p70S6k) and the translational repressor 4EBP-1 (Navé et al. 1999). When phosphorylated at Thr 389, p70S6k activates ribosomal protein S6 (rpS6) by phosphorylation at Ser 235/236 which is believed to facilitate protein translation. When 4EBP-1 is phosphorylated it will not be able to sequester eIF4E which then makes eIF4E available for complex formation with eIF4G promoting initiation of translation. In addition, Akt also regulates protein synthesis via glycogen synthase kinase 3 beta (GSK-3β) (Rommel et al. 2001). GSK-3β inhibits protein translation initiated by the eukaryotic intiation factor 2B (eIF2B) protein (Hardt and Sadoshima, 2002), a guanine nucleotide exchange factor that facilitates the activation of eIF2 by exchange of GDP bound to eukaryotic initiation factor 2 (eIF2 with GTP (Hershey and Merrick, 2000). The active complex interacts with charged initiator methionyl-tRNA and the 40S ribosomal subunit (Merrick 1992, Pavitt et al. 1998) facilitating the elongation process (Welsh et al. 1998, Jefferson et al. 1999, Wang et al. 2001). Thus, inhibition of GSK-3 might promote translation initiation (Welsh et al. 1998, Jefferson et al. 1999, Wang et al. 2001). Indeed, myotubes expressing a non functional (dominant-negative) GSK-3β protein have been shown to develop hypertrophy (Rommel et al. 2001).

Another protein which seems to be connected to the mTOR pathway is eEF2. This factor mediates translocation of the ribosome during the elongation process of protein translation. Its activity is regulated by reversible phosphorylation at Thr 56 (Redpath et al. 1996). When phosphorylated at this residue, eEF2 becomes inactive and is not able to attach to ribosomes and the translation process is thereby inhibited (Browne and Proud 2002). Phosphorylation of eEF2 is mediated by the elongation factor 2 kinase (eEF2k). This kinase is known to be phosphorylated and thereby inhibited by mTOR connecting the regulation of elongation to the mTOR pathway (Redpath et al. 1996). Another mechanism for Akt regulation of mTOR activity is through the tuberous sclerosis complex (TSC1/2). Tuberous sclerosis protein 2 (TSC2, also termed tuberin) is a negative regulator of mTOR and its activity is inhibited by Akt phosphorylation in mammalian cells (Inoki et al. 2002). The inhibitory effect on mTOR by TSC2 is believed to be mediated through blocking of protein Ras-homolog enriched in brain (Rheb) activity (Tee et al. 2003). This protein was identified as a positive regulator of cell growth in *Drosophila* (Saucedo et al. 2003) and in a recent study overexpression of Rheb in transgenic mice was sufficient to induce muscle hypertrophy (Goodman et al.

2010). Indeed activated Rheb seems to positively regulate mTOR signaling *in vitro* (Long et al. 2005). Since TSC2 can increase GTP hydrolysis of Rheb *in vitro* and thereby converting Rheb to an inactive form (Inoki et al. 2003, Tee et al. 2003) mTOR activity is decreased. During conditions of energy starvation, the AMPK kinase (AMPKk) phosphorylates and activates TSC2 (Inoki et al. 2003), which inhibits mTOR activity and thus conserves energy by inhibiting the energy consuming process of protein translation. mTOR is also inhibited by other mechanisms such as myostatin and REDD1/2.

In addition to Akt dependent stimulation of mTOR, it has also been shown that mTOR can be activated in response to other stimuli such as mechanical load and amino acids (Nobukuni et al. 2005, Byfield et al. 2005, Hornberger et al. 2006). In recent years the complexity of the regulatory mechanisms has become evident, mTOR can be activated independently of Akt and several new mechanisms of regulation has been identified such as nutrient sensing by hVps34 and through phospholipase D1 (PLD1) and phosphatidic acid which is suggested to mediate the exercise signaling via mechanotransduction (Hornberger et al. 2006).

A protein that has been established as an important regulator of mitochondrial biogenesis and angiogenesis in rodents is the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Geng et al. 2010). Overexpression of the PGC-1α protein in mice improved exercise performance in concert with a simultaneous increase in mitochondrial gene expression, mitochondrial DNA and mitochondrial enzyme activity (Lin et al. 2002, Calvo et al. 2008).

1.3 REGULATION OF PROTEIN BREAKDOWN

Muscle protein breakdown is regulated mainly by the ubiquitin-proteasome system (Taylor et al. 1995, Solomon and Goldberg, 1996). Proteins that are destined for degradation by this system are tagged by ubiquitin. This ATP dependent process is divided into three steps; ubiquitin is first bound to an ubiquitin activating enzyme (E1) and then transferred to the ubiquitin carrier enzyme (E2) and subsequently, ubiquitin is attached to a protein by a substrate specific ubiquitin ligase (E3). In skeletal muscle, two muscle specific E3 ligases, Muscle RING Finger 1 (MuRF-1) and Muscle Atrophy F-box (MAFbx, also called Atrogin-1), seem to have important functions during the muscle atrophy process. Gene expression of these ligases is markedly upregulated in

various models of atrophy such as denervation, unweighting and sepsis (Bodine et al. 2001, Li et al. 2005). Furthermore, knockout MAFbx and MuRF1 mice showed partial protection against denervation-induced atrophy compared to wild-type mice (Bodine et al. 2001). Recently, MuRF-1 has been shown to be important during the degradation process of myosine light chains 1 and 2 which are components of the thick filament while MAFbx seems to be involved in the breakdown of the muscle specific transcription factor myogenic factor D (MyoD) and the eukaryotic Initiation Factor 3 (eIF3f) which have been shown important in mTOR signaling (Lagirand-Cantaloube et al. 2008 and 2009, Cohen et al. 2009, Csibi et al. 2010). The Forkhead box, class O (FOXO, also called "forkhead") family of transcription factors can regulate the transcription of MuRF-1 and MAFbx and its activity is dependent on phosphorylation. When phosphorylated, FOXO is translocated from the nucleus preventing transcription of MuRF-1 and MAFbx, however, in its dephopshorylated state, it is translocated into the nucleus and stimulates gene transcription of these two E3 ligases. Apart from its function in stimulating protein translation via mTOR, Akt can also phosphorylate FOXO and thereby inhibit gene expression of MuRF1 and MAFbx which provides a link between the stimulation of translation and the protein breakdown. However, the significance and function of these ubiquitin ligases in connection to exercise is still not known.

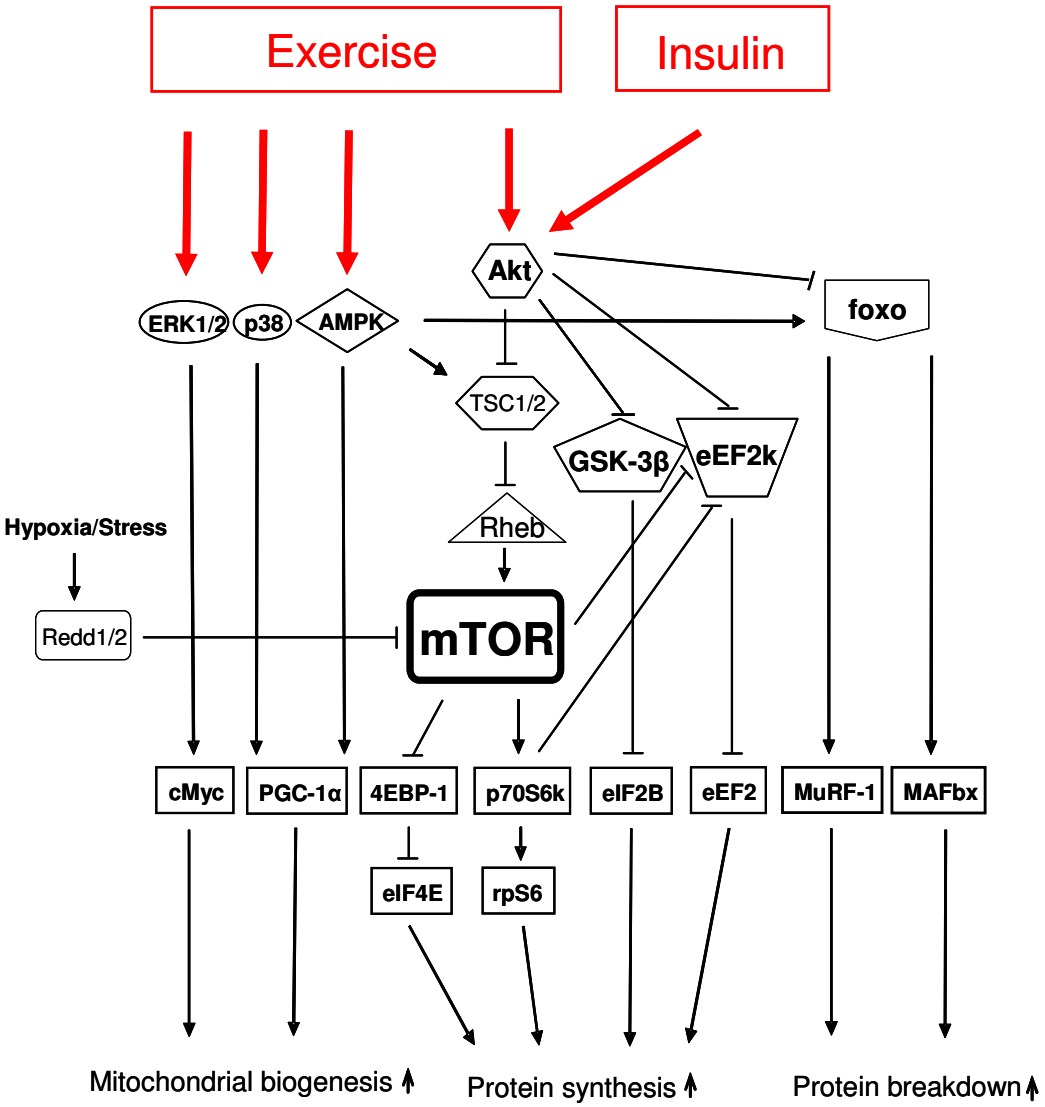

Figure 1. Simplified figure depicting factors influencing the mTOR signaling pathway.

1.4 THE EFFECT OF EXERCISE ON SIGNALING PATHWAYS REGULATING PROTEIN SYNTHESIS AND BREAKDOWN

1.4.1 The mTOR pathway

In the early 1990s p70S6k was known to be activated by mitogenic stimuli, but the upstream activators were not known. However, p70S6k was soon considered to have an important function in translational regulation. In 1999, Baar and Esser found a correlation between acute increase in phosphorylation of p70S6k and change in wet muscle mass after 6 weeks of training by electric stimulation of muscle (Baar and Esser, 1999). This study was followed by several studies on laboratory animals

demonstrating activation of the mTOR pathway following exercise (Hernandez et al. 2000, Nader et al. 2001, Parkington et al. 2003). The first data on p70S6k stimulation in relation to exercise in humans was presented in the study by Karlsson et al. (2004). This study reported increased phosphorylation of p70S6k in the recovery period following resistance exercise when BCAA were ingested. However, in later studies increased phosphorylation of p70S6k has also been found without BCAA supplementation (Dreyer et al. 2006, Terzis et al. 2008, Kumar et al. 2009, Camera et al. 2010). These findings have been extended by Drummond and co-workers (2009) who discovered that increases in signaling through the mTOR pathway and protein synthesis following resistance exercise is attenuated by administration of the potent mTOR inhibitor rapamycin. Although most studies investigating this pathway are done in relation to resistance exercise, it seems now that this pathway is stimulated following endurance exercise as well (Mascher et al. 2007, Benziane et al. 2008, Coffey et al. 2009, Camera et al. 2010).

1.4.2 PGC-1α

PGC-1α has emerged as an important regulator of mitochondrial biogenesis. The mRNA levels of PGC-1α, are elevated after both endurance exercise (Pilegaard et al. 2003, Norrbom et al. 2004, Mahoney et al. 2005, Wang et al. 2009) and resistance exercise (Deldicque et al. 2008) in humans. The gene expression of PGC-1α as well as the activity and stability of the protein has been shown to be stimulated by AMPK and p38 mitogen-activated protein kinase (p38 MAPK) which both can be activated by exercise (Puigserver et al. 2001, Akimoto et al. 2005, Jørgensen et al. 2005).

1.4.3 Ubiquitin ligases

The gene expression of the muscle specific E3 ubiquitin ligases MuRF-1 and MAFbx appears to be altered following exercise in most studies (Coffey et al. 2006, Raue et al. 2007, Mascher et al. 2008). Levels of MuRF-1 mRNA are acutely increased after both resistance and endurance exercise (Louis et al. 2007, Mascher et al. 2008, Harber et al. 2010). MAFbx gene expression is usually decreased or unchanged following resistance exercise (Louis et al. 2007, Deldicque et al. 2008, Mascher et al. 2008). Reports on effects of endurance exercise on MAFbx gene expression is scarcer, but MAFbx mRNA has been reported to increase following endurance exercise (Coffey et al. 2006).

2 AIMS

1. To investigate whether endurance exercise elicit changes in phosphorylation of proteins involved in regulating protein synthesis as well as the time-course for such changes during the first 3 h following endurance exercise in human skeletal muscle.

2. To investigate the fractional synthesis rate (FSR) as well as markers for increased protein synthesis during the early recovery period in human skeletal muscle following intensive endurance exercise.

3. To evaluate whether a second training session performed 48 h after a first one would enlarge the anabolic response in terms of signaling related to protein synthesis and breakdown.

4. To investigate if resistance exercise can enhance the muscle adaptive response to endurance exercise with respect to molecular signaling related to protein synthesis and specific markers of mitochondrial biogenesis in human skeletal muscle.

3 MATERIALS AND METHODS

3.1 SUBJECT CHARACTERISTICS

All subjects included in the studies (37 men and 3 women) were healthy volunteers, recreationally active and did not perform physical exercise more than twice a week. Participants were fully informed about the possible risks associated with the study before they volunteered to participate. The Ethical Committee at the Karolinska Institutet approved the study protocols.

Table 1. Subjects characteristics (± SE) for participants in study I-IV.

	Age (years)	Height (cm)	Weight (kg)	Vo_{2max} (L/min)
Study I (n=6)	23 ± 1	182 ± 3	72 ± 2	3.86 ± 0.16
Study II (n=16)	24 ± 1	182 ± 1	79 ± 4	3.49 ± 0.09
Study III (n=8)	23 ± 1	181 ± 1	74 ± 3	3.91 ± 0.15
Study IV (n=10)	26 ± 1	177 ± 3	72 ± 4	3.62 ± 0.25
Mean	24 ± 1	180 ± 1	75 ± 2	3.66 ± 0.12

All participants were instructed to refrain from exercise and alcohol consumption two days prior to the experiments. In study (II) and (III) the participants followed a dietary schedule two days prior to the test, the diet consisted of 17 energy percent (E%) protein, 26 E% fat and 57 E% carbohydrate in total approximately 3000 kcal per day. In study (IV) the participants maintained a normal diet and physical activity patterns between the two exercise sessions and the food intake the evening before the first experiment was recorded and then duplicated during the evening before the second experiment.

3.2 EXERCISE PROTOCOLS

Study I

Six subjects performed exercise for 1 h at a work rate demanding 75% of their Vo_{2max}. The oxygen consumption was measured at 15 and 45 min of exercise and the heart rate was monitored continuously throughout the exercise period. Muscle samples were taken from the *vastus lateralis* before exercise, immediately after, 30 min, 1, 2 and 3 h

after exercise. Blood samples were drawn from a venous catheter after 20, 40 and 60 min of exercise and in the recovery period at 15, 30, 60, 90 and 120 min following exercise.

Study II

Sixteen male subjects were randomly divided into two groups of either 90 or 180 min recovery. A catheter was inserted into the antecubital veins of both arms, one for blood sampling and the other for infusion of the tracer (L-[$^2H_5$] phenylalanine). The subjects then performed one-legged cycling exercise for 1 h at approximately 65-70% of their one-legged Vo_{2max} with the other leg kept rested in a strap. Oxygen uptake was measured at approximately 15 and 45 min of exercise and heart rates were monitored continuously during exercise. Infusion of tracer was started after exercise in the 90 min recovery group, for the subjects in the 180 min recovery group infusion was started 90 min following exercise. Muscle biopsies were withdrawn from the *vastus lateralis* in both legs before and immediately after exercise as well as 90 or 180 min following exercise in the 90 and 180 min recovery group, respectively. Blood samples were collected before exercise and at 20, 40 and 60 min during exercise in both groups. In the 180 min recovery group blood samples were also collected every 15 min until start of infusion. In both recovery groups, plasma was collected at 0, 5, 10, 15, 30, 40, 50, 70 and 90 min after start of infusion for measurement of tracer enrichment in plasma.

Study III

Eight male subjects performed resistance exercise (leg press) twice with 48 h between the sessions. The protocol consisted of four sets of 10 repetitions at a work load corresponding to approximately 80% of 1RM. Muscle biopsies were taken from the *vastus lateralis* before, 15 min, 1 and 2 h following exercise on both occasions. Blood samples were taken before exercise, after the second set during exercise, immediately after exercise, and following 15, 30, 60, 90 and 120 min of recovery. The same protocol as during the first exercise session was followed during the second session.

Study IV

Ten subjects (7 men and 3 women) performed cycling exercise for 1 h at a work rate corresponding to approximately 65% of their Vo_{2max} at two occasions separated by at least 2 weeks for male and 4 weeks for female subjects. The subjects performed only cycling exercise (E) or cycling followed by 6 sets of leg press exercise (ER) in a

randomized design. During the leg press exercise, the workload was increased from 70 to 80% of their 1RM in the leg. Subjects were asked to do as many repetitions they could but stop at 15 repetitions. Biopsies were taken from the *vastus lateralis* before, 1 h (approximately 20 min after resistance exercise in the ER group) and 3 h (approximately 2 h and 20 min after resistance exercise in the ER group) following cycling in both protocols. Blood samples were collected at the time when biopsies were taken.

3.3 BIOPSY PROCEDURE

Following administration of local anesthesia to the skin, subcutaneous tissue and fascia, muscle biopsies were withdrawn during study (I-III) using a Weil-Blakesley conchotome (AB Wisex, Mölndal, Sweden). In study (IV) a Bergström percutaneous needle with suction was employed. Each biopsy sample was taken from a new incision approximately 2–4 cm proximal to the previous one. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C.

3.4 MEASUREMENTS IN PLASMA

3.4.1 Glucose, lactate and insulin

For glucose and lactate measurements blood samples were collected in heparinized tubes. In study (I) EDTA tubes were used for insulin measurements. Blood samples were centrifuged at 9000 g and analysis performed on plasma. Glucose and lactate concentrations were analyzed according to Bergmeyer (1974). The plasma insulin concentration was measured in study (I) using a radioimmunoassay kit according to the manufacturer's protocol.

3.4.2 Determination of L-[$^2H_5$] phenylalanine enrichment

For measurement of L-[$^2H_5$] phenylalanine enrichment in plasma, GC-MS analysis was performed. Plasma was mixed with equal volume of 15% sulphosalicylic acid (SSA) and put on ice to precipitate plasma proteins. Samples were centrifuged and the supernatant containing the non protein-bound phenylalanine was purified on a column. Samples were then dried with vacuum centrifugation and derivatized for measuring the ions *m*/*z* 336 and *m*/*z* 341 by GC-MS.

3.5 MEASUREMENTS IN MUSCLE TISSUE

Before further analysis, all muscle samples were freeze dried and dissected free from blood and connective tissue in a climate controlled room at 20°C and less than 30% humidity.

3.5.1 Glycogen

In study (I), (II) and (IV) glycogen concentrations were determined in approximately 2 mg of the dissected muscle following incubation in alkaline solution to dissolve the tissue as described by Leighton et al. (1989).

3.5.2 General protocol for Western blot

The following protocol was used with minor modifications.

Approximately three mg of dissected muscle was homogenized in ice cold buffer containing 20 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM B-glycerolphosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% Triton X-100, 20 µg/ml Leupeptin, 50 µg/ml Aprotinin, 40 µg/ml PMSF and 1% phosphatase inhibitor cocktail (Sigma P-2850). Samples were centrifuged at 10.000 g for 10 min at 4°C. Protein concentration was determined in the supernatants using BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). The supernatant from the homogenates were mixed with Laemmli buffer and heated at 95°C for 5 min to denature proteins. To separate proteins with respect to their size, 40 µg (Study I) or 30 µg of protein was loaded onto an SDS-PAGE gel and run on ice for 2 h at 200 V. Samples from each subject were run on the same gel and then incubated approximately 30 min in transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol) before transferring the proteins to a PVDF or nitrocellulose membrane at 300 mA for 3h on ice in a cold room (4°C). Following incubation in transfer buffer the membranes were blocked for 1h at room temperature in blocking buffer containing 5% fat free dry milk (TBS 10 mM Tris pH 7.6, 100 mM NaCl). The membranes were then incubated over night in a cold room at 4°C with the commercially available primary antibody raised against the phosphorylated form of respective protein. Membranes were then washed in TBS (10 mM Tris pH 7.6, 100 mM NaCl) containing 2.5% fat free dry milk and incubated for 1 h at room temperature with a secondary antibody and washed again before quantification of phosphorylated proteins by densitometric scanning. Equal loading of proteins where checked by washing the membranes in Restore Western blot stripping

buffer followed by the same procedure as described above by incubation with polyclonal antibodies raised against respective protein. A Gel Doc 2000 apparatus with the Quantity One software was used for quantification of the phosphorylation.

3.5.3 Measurement of fractional protein synthesis

3.5.3.1 Protocol for analysis of muscle protein FSR

Precipitation of proteins

Approximately 8 mg of freeze dried and dissected muscle was used for homogenization in 1 ml 4% SSA using a minibeadbeater. Homogenized samples were then kept on ice for 30 min to allow precipitation of proteins. Samples were then centrifuged at 16,600 *g* and pellets washed in 4% SSA. The pellet was incubated with 1 ml 0.3 M NaOH in a water bath at 37°C for 1 h to dissolve the pellet. Thereafter, 130 µl of 40% SSA was added and proteins were precipitated on ice.

Hydrolysis of proteins

Following precipitation of proteins, samples were centrifuged 10 min at 16,600 *g* and washed with 4% SSA before breaking down the proteins into amino acids during 24 h with 6 M HCl at 110°C. The hydrolyzed samples were then dried by vacuum centrifugation over night.

Decarboxylation

The protein hydrolysates were dissolved in 450 µl of 0.5 M trisodium citrate (pH 6.3) and filtered through a 0.22 mm filter tube. Then a suspension containing 2 mg of tyrosine decarboxylase (Sigma, T-7927) and 0.25 mg pyridoxalphosphate (Sigma, P-9255) was added per sample before incubation over night at 50°C to convert phenylalanine to phenyl ethylamine.

Ether extraction of phenyl ethylamine

100 µl 6 M NaOH was added and samples were mixed and spun 10 min at 10,000 *g*. To extract phenyl ethylamine from the supernatant 500 µl ether was added. After vigorously shaking the tubes were placed in an ethanol bath with dry-ice. The bottom layer was frozen and the ether phase was transferred to an eppendorf tube containing 100 µl of 0.1 M HCl in order to back-extract the phenyl ethylamine from the ether

phase. The tubes were then placed in the ethanol with dry-ice and when the bottom phase was frozen the ether phase was discarded.

Derivatization

Samples were transferred to GC-MS vials and dried by vacuum centrifugation. Phenylalanine was derivatized for GC-MS analyses during 1 h at 60°C by adding equal volume of N-Methyl-N- (Tert Butyldimethylsilyl) trifluoroacetamide and ethyl acetate (1:1).

Enrichment of tracer in muscle protein

The ratio of labeled and unlabelled phenyl ethylamine was obtained by analyses with a GC–MS (Agilent 5973n; Agilent Technologies, Stockholm, Sweden). GC was done utilizing a HP-5MS column (30 m, 0.25 mm inner diameter, 1 mm film; J&W Scientific, Agilent Technologies). Ions *m/z* 180 (m+2) and *m/z* 183 (m+5) were analyzed. A phenylalanine standard containing 0 - 0.11 APE (atom percentage excess) of L-[$^2H_5$] phenylalanine was dissolved in 200 µl trisodium citrate buffer treated as the samples following hydrolysis.

Calculations of protein synthesis

The enrichment of L-[$^2H_5$] phenylalanine in protein was calculated from the ratio of m+5/m+2 using standard curves. The FSR was calculated as

$$FSR\ (\%/24\ h) = (E_m/A) \times 1440 \times 100$$

where E_m is the muscle protein-bound enrichment of L-[$^2H_5$] phenylalanine during 90 min. A is the area under the curve for L-[$^2H_5$] phenylalanine enrichment in plasma during 90 min following exercise.

3.5.4 mRNA analyses

The total cellular RNA was extracted from freeze-dried muscle biopsy samples using a standard TRIzol protocol. The amount of RNA obtained was measured using a spectrophotometer. In study (III) the RNA quality was visualized by running the RNA on a 1% agarose gel. The RNA was not degraded since the samples of each well produced two clear bands (ribosomal proteins 18S and 26S) with no visible smearing effect. Two micrograms in study (III) or one microgram of the RNA in study (IV) was

synthesized into cDNA using reverse transcriptase and random primers. In study (III), the integrity of the synthesized cDNA was checked by running the samples on a 1% agarose gel. The mRNA was measured using real-time PCR. Both GAPDH and 18S were used as endogenous controls to correct for any variation in the RNA loading in study (III). In study (IV) GAPDH was used as a reference gene. All reactions were performed in 96-well plates. Control experiments revealed approximately equal efficiencies over different starting template concentrations for target genes and the two endogenous controls used. For each individual, all samples were simultaneously analyzed in one assay run. Measurements of the relative distribution of each target gene were performed for each individual; a cycle threshold (CT) value was obtained by subtracting 18S rRNA or GAPDH CT values from respective target CT values. The expression of each target gene was then evaluated by $2^{-\Delta CT}$(Wong et al. 2005).

3.6 METHODOLOGICAL CONSIDERATIONS

3.6.1 The flooding dose protocol

When calculating the fractional synthetic rate in muscle, incorporation of tracer into muscle tissue over time is divided by the tracer labeling of the precursor pool for protein synthesis. Hence, it is of great importance to have a reliable estimate of the precursor pool. The true precursor pool in skeletal muscle is assumed to be the tRNA bound to its amino acid. However, this precursor is rarely used due to its very poor stability and low concentration (Watt et al. 1991). Therefore, surrogates of the true precursor pool are commonly used. When employing the flooding dose protocol, a flood of labeled amino acids (usually phenylalanine) is given, this will give a similar enrichment in the plasma and intracellular compartment so the plasma concentration of tracer can be used as the precursor for protein synthesis (McNurlan et al. 1991). On the other hand, the protocol utilizing the flooding dose with phenylalanine has been suggested to stimulate protein synthesis (Smith et al. 1998).

In a pilot study the effect of a phenylalanine flood on the rate of proteins synthesis was investigated in resting subjects.

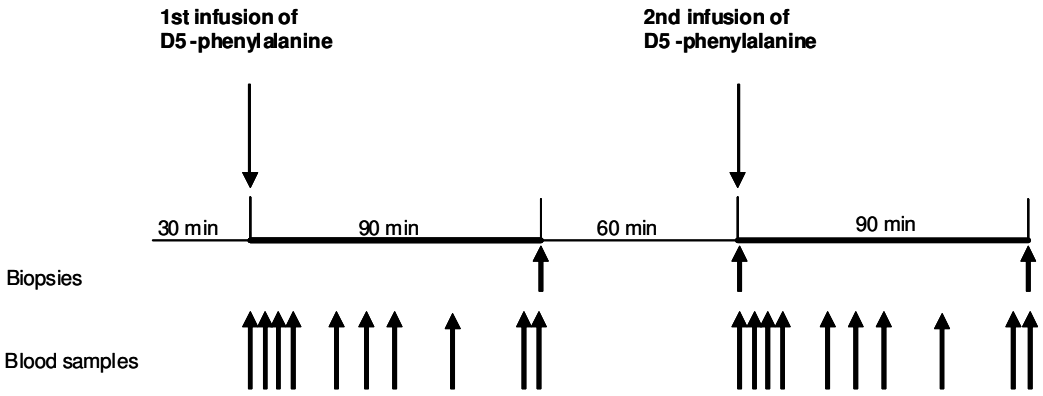

Figure 2. Protocol for two flooding doses given one hour between each dose.

Four healthy male subjects were included, their average (±SE) age, height and weight were 26 ± 3 years, 187 ± 5 cm, 78 ± 5 kg. Subjects were given two flooding doses of L-[$^2H_5$] phenylalanine (40 mg/kg body weight) separated by one hour. Muscle biopsies were withdrawn in both legs 90 min after the first flooding dose as well as before and 90 min after the second flooding dose (Figure 2). The FSR in muscle was determined from enrichment in muscle samples taken at 90 min following the first infusion of tracer. The fractional synthetic rate after the second infusion was calculated as the difference in enrichment in the sample taken 90 min after the second flooding dose and the enrichment in muscle before the second flooding dose.

Table 2. Fractional synthetic rates (FSR) (%/24) h in both legs in four subjects during the first and second flood (L=Left leg, R=Right leg).

	1st flood	2nd flood
S1 (L)	1.62	2.49
(R)	1.54	2.31
S2 (L)	1.89	1.79
(R)	1.98	2.04
S3 (L)	1.51	1.77
(R)	1.39	2.09
S4 (L)	2.04	1.98
(R)	1.61	2.66
Mean	1.70	2.14

Three subjects out of four had higher FSR values following the second flood. Therefore, a stimulatory effect of a flooding dose of phenylalanine can not be excluded. In study (II) FSR was measured in both resting and exercising muscle so any possible stimulatory effect of the phenylalanine flood would be the same in both legs.

The advantage of using the flooding dose technique is that this method allows measurements to be made during shorter periods of time compared to the continuous infusion protocol and it is less affected by changes in the amino acid precursor pool.

The coefficient of variation (CV) in FSR measurements between right and left leg was calculated according to the formulas below:

$S^2 = \Sigma d^2 / 2n$

$CV = S / x \cdot 100$ (%)

Where S is the standard deviation, d is the difference between right and left leg, n is the number of duplicate determinations and x is the sample mean. The coefficient of variation was 10% during the first and 14% during the second flood. In average, the coefficient of variation was found to be 12%, which is similar to previous findings (Olav Rooyackers, unpublished data).

3.6.2 Effects of repeated biopsies

3.6.2.1 Gene expression

The possibility that the procedure of biopsy taking itself can influence gene expression and/or phosphorylation of enzymes investigated should not be neglected. When biopsies were taken from the same incision, Vissing and co-workers found that the expressions of several genes increased 1 h and up to 8 h after biopsy withdrawal, amongst them pyruvate dehydrogenase kinae 4 (PDK-4), cyclin-dependent kinase inhibitor 1 (p21) and MyoD, while some, such as citrate synthase and PGC-1α did not change (Vissing et al. 2005). However, when biopsies were taken separated by 1 h at a new incision, no such effects were found for a number of genes, including PDK-4 (Lundby et al. 2005).

3.6.2.2 Kinase phosphorylation

The MAPK kinase pathways regulate gene transcription and are considered to be activated by stress factors. Phosphorylation and activation of the MAPK kinase signaling pathway following repeated muscle biopsies was investigated by Aronson and co-workers in 1998. A biopsy was taken from *vastus lateralis* and 30 min later the second biopsy was obtained from the same incision. The findings from his study clearly showed that injury, such as biopsy taking, could increase phosphorylation of ERK1/2 as well as the activity of JNK and p38 in human muscle. Importantly, these effects was not seen when a biopsy was taken 5 cm proximal to the first incision indicating a local rather than a systemic response.

4 RESULTS

4.1 PAPER I

In paper (I) the acute effects of an endurance exercise session on the mTOR signaling pathway were studied. Akt phosphorylation at Ser^{473} was elevated 1 and 2 h post exercise. Furthermore, we found an increase in phosphorylation of mTOR at Ser^{2448} immediately after, 30 min and 2 h after exercise. However, p70S6k seemed to be only partially activated since increased levels of phosphorylation was found at $Ser^{424/}Thr^{421}$ but not at the site Thr^{389} which is believed to be crucial for activating the enzyme. Phosphorylation of GSK-3α/β at Ser^{21} and Ser^{9}respectively, was increased immediately after (GSK-3β) or 30min after (GSK-3α) exercise and throughout the 3 h recovery period. Reduced phosphorylation of eEF2 at Thr^{56} was found 30 min and up to 3 h.

4.2 PAPER II

To investigate the effects of endurance exercise on mixed muscle protein synthesis the FSR was measured following exercise utilizing a tracer (L-[$^{2}H_{5}$] phenylalanine) which is incorporated into muscle proteins. In addition, activation of the mTOR signaling pathway in skeletal muscle was studied. Two recovery periods were investigated; 90 or 90-180 min. Measurements of the rate of protein synthesis revealed a tendency for increased rate in the first 90 min following exercise (P=0.10). However, during 90-180 min recovery, there was a 22% higher (P = 0.038) FSR in the exercised leg as compared to the rested leg (Table 3).

Table 3. Individual values of fractional synthetic rates (FSR) (%/24 h) and differences (Diff) between resting (RLeg) and exercising leg (ELeg).

	90 min recovery				180 min recovery		
	RLeg	ELeg	Diff		RLeg	ELeg	Diff
S1	1.57	1.65	0.08	S9	1.27	1.69	0.42
S2	1.52	1.64	0.12	S10	1.18	1.23	0.05
S3	1.25	1.14	-0.1	S11	1.68	1.45	-0.24
S4	1.60	1.80	0.20	S12	2.19	2.22	0.03
S5	1.74	2.21	0.48	S13	1.27	1.64	0.36
S6	1.26	1.27	0.01	S14	1.58	2.40	0.82
S7	1.30	1.24	-0.06	S15	1.23	1.72	0.49
S8	1.32	1.61	0.30	S16	1.05	1.53	0.48
Mean	1.44	1.57	0.13	Mean	1.43	1.74	0.30

In addition, we also found significantly elevated phosphorylation of mTOR at Ser^{2448} and p70S6k at Thr^{389} directly after exercise in the exercised leg. Interestingly, there was a tendency towards elevated phosphorylation in the resting leg as well at this time-point ($P = 0.05$). Phosphorylation of mTOR was elevated in both legs at 90 min after exercise while no effect was seen on p70S6k in either leg. However, at 180 min following exercise, phosphorylation of p70S6k was elevated in both legs while no effect was found in any of the legs for mTOR at this time-point. eIF2Bε phosphorylation was unchanged immediately after but decreased at 90 min after exercise in the exercising leg while an increase was found in the non-exercising leg (due to low initial value) at this time-point. There was no change in phosphorylation of AMPK and eEF2 at any time-point after exercise.

4.3 PAPER III

It is usually recommended that resistance exercise is performed 2-3 times per week. The aim was to examine the anabolic signaling and gene expression before and after resistance exercise at two occasions separated by 48 h. Phosphorylation of mTOR at Ser^{2448} was increased at all time-points following exercise in both exercise sessions although mean values were in average 18% higher (N.S.) after the second session (Figure 3, left). Phosphorylation levels of p70S6k at Thr^{389} were increase at all time-points after exercise with mean values 39 and 30% higher (N.S.) at 15 min and 1 h respectively following the second session (Figure 3, right).

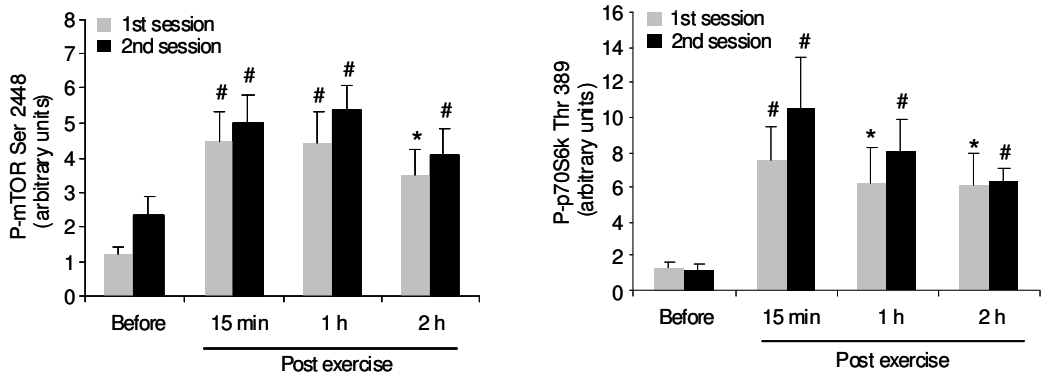

Figure 3. Phosphorylation of mTOR (left) and p70S6k (right) before, 15 min, 1 h and 2 h following exercise, measured at two occasions with 48 h in between. Values are arbitrary units (means ± SE for 8 subjects). $^{*}P < 0.05$, $^{\#}P < 0.01$ *vs. before exercise.*

Elevated phosphorylation of rpS6 at $Ser^{235/236}$ was seen at all time-points following the second exercise session but only at 1 h following the first session. Although not statistically different, mean values of rpS6 phosphorylation after 2nd exercise session were in average 56% higher compared to the first session. eEF2 phosphorylation at Thr^{56} was reduced on both occasions. However, no changes in Akt or GSK-3α/β were observed, indicating Akt-independent activation of mTOR, p70S6k, rpS6 and eEF2.

Furthermore, a divergent regulation of the muscle-specific ubiquitin ligases MAFbx and MuRF-1 was found. The level of MuRF-1 expression was similar before the start of each exercise session; however, there was an acute increase 2 h after exercise on both occasions, but the mRNA level was 26% lower after the second compared to the first occasion (Figure 4, right). MAFbx gene expression did not change significantly during exercise but was reduced by approximately 40% at the start of the second exercise session (Figure 4, left). Reduced expression of myostatin was found 2 h following the first exercise session and remained reduced throughout the rest of the study.

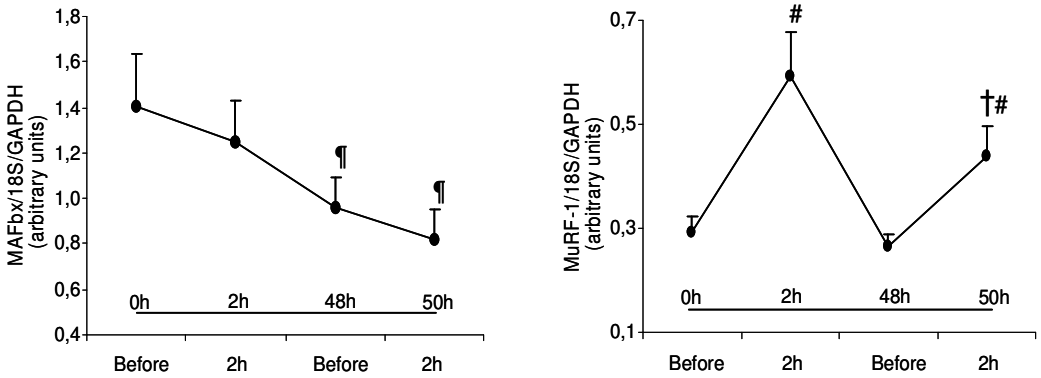

Figure 4. Changes in gene expression of MAFbx (left) and MuRF-1 (right) normalized to the geometric mean of 18S and GAPDH. Values in the graph are arbitrary units (means ± SE for 8 subjects). $^{\#}P < 0.01$ *vs. before each session,* $^{¶}P < 0.01$ *vs. before in the first session,* $^{†}P < 0.05$ *vs. same time-point in the first session.*

4.4 PAPER IV

It is generally believed that combined endurance and resistance exercise will compromise results compared to resistance exercise alone. On the other hand, improvement in short term endurance has been reported when endurance training is combined with strength training. The effect of combined exercise on intramuscular signaling is largely unknown. In paper (IV) we examined the change in mTOR-signaling after endurance exercise alone (E) or endurance exercise followed by resistance exercise (ER). Phosphorylation of Akt at Ser^{473} and AMPK at Thr^{172} were elevated 1 h after exercise in a similar manner in both protocols (Figure 5A).

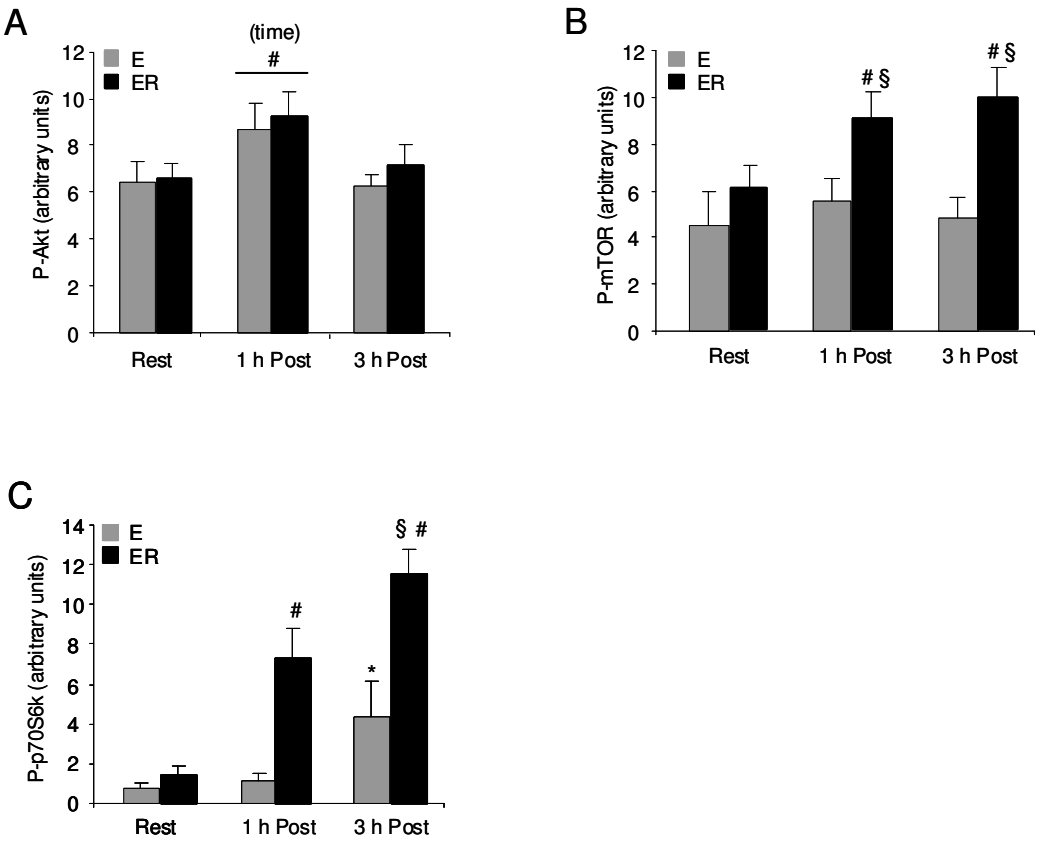

Figure 5. Phosphorylation of Akt at Ser^{473} (A), mTOR at Ser^{2448} (B) and p70S6k at Thr^{389} (C) at rest, 1 h and 3 h following endurance exercise (E) or endurance exercise followed by resistance exercise (ER), values in the graphs are arbitrary units of the mean (± SE for 10 subjects). $^{*}P < 0.05$, $^{\#}P < 0.01$ *vs. Rest,* $^{\S}P < 0.01$ *vs. the same time-point in the other condition.*

However, mTOR phosphorylation at Ser^{2448} was increased at 1 and 3 h after exercise only in the ER protocol (Figure 5B). Phosphorylation of p70S6k at Thr^{389} was elevated 1 and 3 h following exercise in ER and furthermore, to a larger extent in the ER protocol 3 h following exercise compared to in E. In the E protocol phosphorylation of p70S6k was only elevated 3h Post (Figure 5C). Reduced phosphorylation of eEF2 at Thr^{56} was found in the ER protocol up to 3 h into the recovery. In the E protocol however, reduced phosphorylation was seen only at 1 h after exercise. There was a trend (p=0.054) towards increased phosphorylation of p38 MAPK1 h after exercise.

Furthermore, mRNA expression of several genes, which are believed to have important roles in regulating mTOR and mitochondrial biogenesis were analyzed. PGC-1α and PRC gene expression were increased in both conditions at 1 h, the mRNA levels increased further at 3 h post ER and was higher than that of E at this time-point (Figure 6, upper left and lower right).

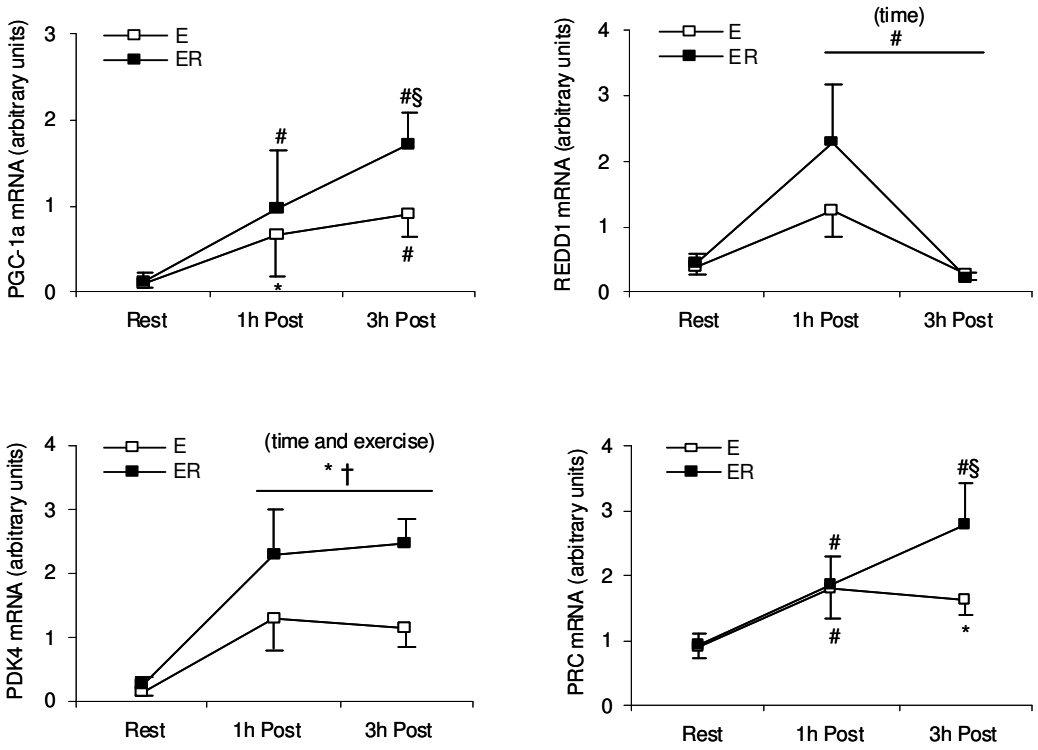

Figure 6. mRNA expression of PGC-1α (upper left), REDD1 (upper right), PDK4 (lower left) and PRC (lower right) normalized to GAPDH at rest, 1 and 3 h post endurance exercise in the two groups E (endurance exercise) and ER (endurance followed by resistance exercise). Values are arbitrary units of the mean (± SE for 10 subjects). $^{*}P < 0.05$, $^{\#}P < 0.01$ *vs. Rest,* $^{\dagger}P < 0.05$, $^{\S}P < 0.01$ *vs. same time-point in the other condition.*

Cellular homologue of avian myelocytomatosis viris oncogene (cMyc) and PDK4 gene expressions were increased in both situations up to 3 h post exercise, however, the increase was larger at all time-points following ER compared to E (Figure 6, lower left). Gene expression of Rheb was increased in a similar manner 1 and 3 h post exercise with both exercise protocols.

5 DISCUSSION

Within the field of exercise physiology, effects on muscle protein turnover following endurance exercise are relatively understudied compared to resistance exercise. However, in recent years this topic has gained more attention. Measurements of FSR following endurance exercise have so far only been made during the time immediately after and up to 3-6 h after exercise with one exception. The only study investigating the early recovery period was done following low intensity exercise (40% of Vo_{2max}) in the study by Sheffield-Moore and colleagues (2004). A transient increase in mixed muscle FSR was found when measured immediately after and up to 1 h, but not during 1-3 h following exercise. The FSR during the early recovery period was further investigated although at moderate intensity in study (II).

Study (II) revealed that the rate of mixed muscle protein synthesis increased progressively when measured up to 3 h following endurance exercise (1 h, demanding ~65% of their estimated one-legged Vo_{2max}). The magnitude of increase in FSR observed in study (II) was 0.0124%/h, which is less than observed in other studies following endurance exercise. At low intensity (40% of Vo_{2max}) increases in fractional synthetic rates that are approximately three times higher have been reported (0.036%/h in Sheffield-Moore et al. 2004 and 0.037%/h in Durham et al. 2010) in both the fed and post absorptive state. Increases of approximately the same magnitude are observed at higher intensity (~70% of Vo_{2max}) (0.041%/h fasted and 0.058%/h fed state in Harber et al. 2010). In the recent study by Harber and colleagues (2010), measurements included the period 2-6 h following exercise, hence, it could be speculated that the rate of protein synthesis is increased even further at later time-points during the recovery period, which may explain the larger increase found in the study by Harber et al. 2010. However, methodological differences can not be excluded; in the mentioned studies a continuous infusion protocol was utilized, while the flooding dose technique was employed in study (II). This may influence the estimate of the precursor pool and thus the FSR.

Resistance exercise is known to increase strength and muscle mass while endurance exercise is known to increase adaptive responses related to enhanced endurance. Hence, somewhat surprisingly, the magnitude of increase in the rate of protein

synthesis following endurance exercise is found to be comparable to what is observed following resistance exercise. Despite a large increase (~150%) in fractional synthetic rates of mitochondrial proteins following endurance exercise (Wilkinson et al. 2008), it is not likely that the increase in mixed muscle protein synthesis following endurance exercise can be attributed to an elevated synthesis of mitochondrial proteins since mitochondrial protein content only accounts for about 5% of the total muscle mass. The increase in muscle protein synthesis following endurance exercise may also be related to structural changes such as alterations in fiber type composition or a compensatory effect for attenuated protein synthesis during exercise. From the few studies available, there seem to be no difference in the magnitude of increase between low or moderate intensity endurance exercise.

In study (I) the time course of signaling related to increased protein translation and elongation was investigated following an acute bout of endurance exercise (1 h at ~75% of Vo_{2max}). A divergent time-course between changes in phosphorylation of Akt and mTOR support the notion that mTOR can be activated independently of Akt. These findings were partially confirmed in study (IV), where 1 h of cycling exercise (~65% of Vo_{2max}) elicited a stimulatory response on the Akt-mTOR pathway. Furthermore, increased signaling through the Akt-mTOR pathway was found in study (II) following 1 h of one-legged cycling exercise (~65% of Vo_{2max}). Activation of the mTOR signaling pathway following endurance exercise has now been confirmed in most other studies on humans (Benziane et al. 2008, Wilkinson et al. 2008, Coffey et al. 2009). These findings support the notion that the mTOR pathway is involved in the regulation of muscle protein synthesis following endurance exercise. Whether there is specificity with respect to the type of proteins being synthesized during stimulation of the mTOR pathway following resistance and endurance exercise is not known. A very interesting challenge for future studies would be to identify the regulatory signaling mechanisms behind synthesis of specific proteins following endurance and resistance exercise. Interestingly, stimulation of PGC-1α gene expression has been linked to mTOR activation (Cunningham et al. 2007) which implies that mTOR stimulates synthesis of mitochondrial proteins in addition to contractile proteins.

Another interesting finding in study (II) was a tendency towards increased phosphorylation of p70S6k (P=0.05) in the resting leg. In line with these findings, increased phosphorylation of mTOR has been found following one-legged resistance

exercise (Apro and Blomstrand, 2010). There is no obvious explanation for this but it may involve a yet unknown systemic effect. Muscle activity in the resting leg is not likely to explain this, there was for example no change in glycogen content in the resting muscle at any time-point.

It is usually recommended that resistance exercise is performed 2-3 times per week. In study (III) anabolic and catabolic pathways were investigated before and after two sessions of resistance exercise separated in time by 48 h. Results from study (III) indicated a slightly enhanced signaling through the mTOR pathway in the recovery period during the second session. Similar to study (I), (II) and (IV) it seemed that mTOR and some of its downstream targets could be activated independently of Akt. Furthermore, a divergent regulation of the gene expression for two muscle specific ubiquitin ligases, MAFbx and MuRF-1, was found. The reduction in MAFbx mRNA is in agreement with findings of reduced MAFbx gene expression up to 24 h following one bout of resistance exercise (Churchley et al. 2007, Kostek et al. 2007, Coffey et al. 2009). Increased mRNA abundance of MuRF-1 is also in agreement with most studies (Yang et al. 2006, Raue et al. 2007). The divergent regulation of these two ubiquitin ligases suggest specific functions in the acute and late phases of recovery, such as repair due to structural damages and/or other proteolytic events that may occur in the post exercise period. In recent years substrates for these two ubiquitin ligases have been identified. The translation initiation factor eIF3 is recognized as a substrate for MAFbx (Lagirand-Cantaloube et al. 2008). Hence, the observed decrease in MAFbx mRNA expression during the second exercise session in study (III) may be related to enhanced translation initiation over time. One substrate for MuRF-1, is the myosin heavy chain, attenuated mRNA expression of MuRF-1 following the second exercise session may therefore be related to decreased proteolysis of myosine heavy chains (Clarke et al. 2007). Taken together, increased anabolic signaling and attenuated gene expression of proteolytic markers (MAFbx and MuRF-1) as well as myostatin (inhibitor of muscle growth) during the second exercise session may all be indications of a more anabolic milieu during the second exercise session.

In general, endurance exercise is believed to inhibit gains in muscle mass and strength. On the other hand, some studies report that resistance exercise may be beneficial for endurance performance (See Aagard et al. 2010). Most studies conducted on combined exercise are long term studies investigating physiological parameters, whereas only a

few studies have measured the acute responses of molecular signaling. In the study by Atherton et al. (2005), a divergent signaling response to endurance and resistance exercise was indicated when isolated and incubated rat muscles were stimulated to contract electrically to mimic the two forms of exercise. Endurance-like exercise stimulated AMPK-PGC-1α while resistance-like exercise stimulated Akt-mTOR signaling and protein synthesis. This has led to speculations of inhibitory crosstalk between these two signaling pathways. However, the findings in study (IV) contradict such a mechanism in human skeletal muscle. Furthermore, increased synthesis of mitochondrial proteins in untrained subjects following resistance exercise (Wilkinsson et al. 2008) has been observed. The only study on molecular signaling following combined exercise was done recently by Coffey and co-workers (2009). The main aim of this study was to investigate if the order of exercise had an effect on the acute molecular signaling response. Subjects were divided into two groups, each performing two bouts of exercise, either endurance or strength training in the first session. In both groups, biopsies were taken at rest, 15 min after each exercise session as well as 3 h into the recovery period. In this cross over design, comparisons of anabolic signaling after single mode exercise with combination exercise could only be made 15 min following exercise. There was no enhanced effect on signaling in the combined exercise protocol compared to the single mode exercise at this time-point. The lack of effect may be related to differences in the exercise protocol such as intensity of exercise, timing of the biopsies, also the subjects reported to be participating in regular resistance and aerobic training for more than one year while subjects in study (IV) were less active. The novel findings of enhanced molecular signaling in the combined exercise protocol strengthen the notion of an enhanced, rather than a diminished effect on endurance training when combining the two forms of exercise in the same session. Further studies on this topic are warranted to clarify for example whether the enhanced signaling response found in study (IV) is present following chronic exercise.

The molecular signaling mechanism behind the enhanced signaling response in the combined protocol is not obvious. However, p38 MAPK has been suggested to stimulate PGC-1α gene expression (Puigserver et al. 2001, Akimoto et al. 2005). Phosphorylation of this enzyme tended to be increased 1 h post exercise in the ER protocol which could explain enhanced levels of PGC-1α mRNA in ER. Other factors such as levels of blood lactate (Hashimoto, et al. 2007), reactive oxygen species (ROS)

(St-Pierre et al. 2006) and Ca^{2+}(Kusuhara et al. 2007) may also have contributed to elevate PGC-1α gene expression. There is currently no obvious explanation for the enhanced response on mTOR signaling in the combined protocol. Since the response of resistance exercise alone was not investigated it is unclear whether the enhanced signaling response is due to an additive effect or simply related to the type of exercise as such. Resistance exercise recruits more type II fibers, and therefore it is possible that the enhanced signaling response is the result of a larger number of fibers recruited when performing combined exercise**.**

In conclusion, endurance exercise appears to gradually increase mixed muscle protein synthesis in the early recovery period. Furthermore, this increase coincides with stimulation of the Akt-mTOR pathway, indicating that this pathway is involved in the observed stimulation of protein synthesis. Recent observations of surprisingly large increases in mixed muscle protein synthesis following endurance exercise, comparable to what is reported after resistance exercise, warrants further investigation. One session of resistance exercise provides a more anabolic milieu 2 days later when performing a second session. Finally, combining endurance and resistance exercise enhance the acute signaling response related to increased protein synthesis as well as specific markers for mitochondrial biogenesis. From the last study it seems that combined exercise may be beneficial for endurance training. However, further studies are warranted to determine whether chronic training employing the currently used protocol is an effective strategy to improve muscle oxidative capacity.

6 ACKNOWLEDGEMENTS

I wish to thank all people involved in the work during my time as a PhD student at the Åstrandlaboratory. Especially I would like to thank the following people:

Eva Blomstrand my main supervisor, for good advice and always taking time for discussing projects and results and for guiding my first steps in the field of exercise physiology.

Björn Ekblom for invaluable advice and help conducting these studies.

Kent Sahlin for good advice and help during the last project.

Olav Rooyackers at the Department of Anesthesiology and Intensive Care at Karolinska University Hospital for good advice and guidance, as well as for the opportunity to conduct measurements of fractional synthesis rates in muscle.

Jan Henriksson my co-supervisor at Karolinska Institutet, Department of Physiology and Pharmacology.

My colleagues in the "protein group" at Åstrandaboratoriet: **William Apro** and **Jörgen Tannerstedt** as well as the newcomer **Marcus Borgenvik** for fun times and hard times in and outside the lab during the years.

Marjan Pontén our happy and energetic laboratory assistant, for invaluable help in the laboratory as well as for bringing a sense of "party mood" to the Åstrandlaboratory!

Li Wang for help and interesting discussions during our collaboration as well as fun times in and outside the laboratory.

Niklas Psilander for help in the final study.

Phoung Philträd my former colleague and friend for enjoyable discussions and occasional wine drinking.

Toomas Tiivel for advice and lots of interesting talks in and outside the office.

Christina Hebert, Maria Klaude, Eva Nejman-Skoog, **Towe Jakobsson** at the Department of Anesthesiology and Intensive Care, Karolinska University Hospital for guidance and help in my work as well as for an enjoyable time at the laboratory when measuring fractional synthesis rates in muscle.

Thibault Elfegoun-Brink, **Filip Larsen** and **Mikael Mattson** for help with the oxygen uptake equipment.

P-A Nilsson and **Helena Andersson** for help with the first study.

Mona Esbjörnsson, Ted Österlund, Tomas Rundqvist, Makis Terzis, Spyros Methenetis for fruitful collaborations beside the scope of this thesis.

Forskings- och utbildningsnämden (FUN) and **Centrum för idrottsforskning (CIF)** at GIH for funding my research.

All brave subjects who volunteered to participate in these studies.

My parents and my family **Helene** and **Saga Wahlstedt** for putting up with many late evenings and long hours in front of the computer.

7 REFERENCES

Aagaard P, Andersen JL. Effects of strength training on endurance capacity in top-level endurance athletes. *Scand J Med Sci Sports* Suppl 2:39-47, 2010.

Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams RS, Yan Z. Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. *J Biol Chem* 280: 19587-19593, 2005.

Andersen JL, Aagaard P. Myosin heavy chain IIX overshoot in human skeletal muscle. *Muscle Nerve* 23: 1095-1104, 2000.

Apró W, Blomstrand E. Influence of supplementation with branched-chain amino acids in combination with resistance exercise on p70(s6) kinase phosphorylation in resting and exercising human skeletal muscle. *Acta Physiol (Oxf)* [Epub ahead of print], 2010.

Aronson D, Wojtaszewski JF, Thorell A, Nygren J, Zangen D, Richter EA, Ljungqvist O, Fielding RA, Goodyear LJ. Extracellular-regulated protein kinase cascades are activated in response to injury in human skeletal muscle. *Am J Physiol* 275: C555-561, 1998.

Atherton PJ, Babraj J, Smith K, Singh J, Rennie MJ, Wackerhage H. Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J* 19: 786-788, 2005.

Baar K, Esser K. Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol* 276: C120-127, 1999.

Benziane B, Burton TJ, Scanlan B, Galuska D, Canny BJ, Chibalin AV, Zierath JR, Stepto NK. Divergent cell signaling after short-term intensified endurance training in human skeletal muscle. *Am J Physiol Endocrinol Metab* 295: 1427-1438, 2008.

Bergmeyer HU. Methods in Enzymatic Analysis. *Academic Press*, New York, 1974.

Biolo G, Maggi SP, Williams BD, Tipton KD, Wolfe RR. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am J Physiol* 268: 514-520, 1995.

Biolo G, Tipton KD, Klein S, Wolfe RR. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am J Physiol* 273: 122-129, 1997.

Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 3: 1014-1019, 2001.

Bolster DR, Crozier SJ, Kimball SR, Jefferson LS. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J Biol Chem* 277: 23977-23980, 2002.

Browne GJ, Proud CG. Regulation of peptide-chain elongation in mammalian cells. *Eur J Biochem* 269: 5360-5368, 2002.

Byfield MP, Murray JT, and Backer JM. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J Biol Chem* 280: 33076-33082, 2005.

Calvo JA, Daniels TG, Wang X, Paul A, Lin J, Spiegelman BM, Stevenson SC, Rangwala SM. Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *J Appl Physiol* 104: 1304-1312, 2008.

Camera DM, Edge J, Short MJ, Hawley JA, Coffey VG. Early time course of Akt phosphorylation after endurance and resistance exercise. *Med Sci Sports Exerc* 42: 1843-1852, 2010.

Carraro F, Stuart CA, Hartl WH, Rosenblatt J. and Wolfe RR. Effect of exercise and recovery on muscle protein synthesis in human subjects. *Am J Physiol* 259: E470-E476, 1990.

Chesley A, MacDougall JD, Tarnopolsky MA, Atkinson SA, Smith K. Changes in human muscle protein synthesis after resistance exercise. *J Appl Physiol* 73: 1383-1388, 1992.

Chtara M, Chaouachi A, Levin GT, Chaouachi M, Chamari K, Amri M, Laursen PB. Effect of concurrent endurance and circuit resistance training sequence on muscular strength and power development. *J Strength Cond Res* 22: 1037-1045, 2008.

Churchley EG, Coffey VG, Pedersen DJ, Shield A, Carey KA, Cameron-Smith D, Hawley JA. Influence of preexercise muscle glycogen content on transcriptional activity of metabolic and myogenic genes in well-trained humans. *J Appl Physiol* 102: 1604-1611, 2007.

Clarke BA, Drujan D, Willis MS, Murphy LO, Corpina RA, Burova E, Rakhilin SV, Stitt TN, Patterson C, Latres E, Glass DJ. The E3 ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle. *Cell Metab* 6: 376-385, 2007.

Coffey VG, Jemiolo B, Edge J, Garnham AP, Trappe SW, Hawley JA. Effect of consecutive repeated sprint and resistance exercise bouts on acute adaptive responses in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 297: 1441-1451, 2009.

Coffey VG, Zhong Z, Shield A, Canny BJ, Chibalin AV, Zierath JR, Hawley JA. Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *FASEB J* 20: 190-192, 2006.

Cohen S, Brault J, Gygi S, Glass D, Valenzuela D, Gartner C, Latres E, Goldberg A. During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitinylation. *J Cell Bio* 185: 1083-1095, 2009.

Csibi A, Cornille K, Leibovitch MP, Poupon A, Tintignac LA, Sanchez AM, Leibovitch SA. The translation regulatory subunit eIF3f controls the kinase-dependent mTOR signaling required for muscle differentiation and hypertrophy in mouse. *PLoS One* 5: e8994, 2010.

Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 450: 736-740, 2007.

Deldicque L, Atherton P, Patel R, Theisen D, Nielens H, Rennie MJ, Francaux M. Effects of resistance exercise with and without creatine supplementation on gene expression and cell signaling in human skeletal muscle. *J Appl Physiol* 104: 371-378, 2008.

Dohm GL, Tapscott EB, Barakat HA, Kasperek GJ. Measurement of in vivo protein synthesis in rats during an exercise bout. *Biochem Med* 27: 367-373, 1982.

Dreyer HC, Drummond MJ, Pennings B, Fujita S, Glynn EL, Chinkes DL, Dhanani S, Volpi E, Rasmussen BB. Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *Am J Physiol Endocrinol Metab* 294: E392-400, 2008.

Dreyer HC, Fujita S, Cadenas JG, Chinkes DL, Volpi E, Rasmussen BB. Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. *J Physiol* 576: 613-624, 2006.

Drummond MJ, Dreyer HC, Pennings B, Fry CS, Dhanani S, Dillon EL, Sheffield-Moore M, Volpi E, Rasmussen BB. Skeletal muscle protein anabolic response to resistance exercise and essential amino acids is delayed with aging. *J Appl Physiol* 104: 1452-1461, 2008.

Drummond MJ, Fry CS, Glynn EL, Dreyer HC, Dhanani S, Timmerman KL, Volpi E, Rasmussen BB. Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. *J Physiol* 587: 1535-1546, 2009.

Durham WJ, Casperson SL, Dillon EL, Keske MA, Paddon-Jones D, Sanford AP, Hickner RC, Grady JJ, Sheffield-Moore M. Age-related anabolic resistance after endurance-type exercise in healthy humans. *FASEB J* 24: 4117-4127, 2010.

Durham WJ, Miller SL, Yeckel CW, Chinkes DL, Tipton KD, Rasmussen BB, Wolfe RR. Leg glucose and protein metabolism during an acute bout of resistance exercise in humans. *J Appl Physiol* 97: 1379-1386, 2004.

Fujita S, Dreyer HC, Drummond MJ, Glynn EL, Volpi E, Rasmussen BB. Essential amino acid and carbohydrate ingestion before resistance exercise does not enhance postexercise muscle protein synthesis. *J Appl Physiol* 106: 1730-1739, 2009.

Gautsch TA, Anthony JC, Kimball SR, Paul GL, Layman DK, Jefferson LS. Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. *Am J Physiol Cell Physiol* 274: C406-C414, 1998.

Geng T, Li P, Okutsu M, Yin X, Kwek J, Zhang M, Yan Z. PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. *Am J Physiol Cell Physiol* 298: 572-579, 2010.

Goodman CA, Miu MH, Frey JW, Mabrey DM, Lincoln HC, Ge Y, Chen J, Hornberger TA. A phosphatidylinositol 3-kinase/protein kinase B-independent activation of mammalian target of rapamycin signaling is sufficient to induce skeletal muscle hypertrophy. *Mol Biol Cell* 21: 3258-3268, 2010.

Harber MP, Crane JD, Dickinson JM, Jemiolo B, Raue U, Trappe TA, Trappe SW. Protein synthesis and the expression of growth-related genes are altered by running in human vastus lateralis and soleus muscles. *Am J Physiol Regul Integr Comp Physiol* 296: 708-714, 2009.

Harber MP, Konopka AR, Jemiolo B, Trappe SW, Trappe TA, Reidy PT. Muscle Protein Synthesis and Gene Expression During Recovery From Aerobic Exercise in the Fasted and Fed States. Am J Physiol Regul Integr Comp Physiol. 2010 Aug 18. [Epub ahead of print]

Hardt SE, Sadoshima J. Glycogen synthase kinase-3beta: a novel regulator of cardiac hypertrophy and development. *Circ Res* 90: 1055-1063, 2002.

Hashimoto T, Hussien R, Oommen S, Gohil K, Brooks GA. Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis. *FASEB J* 21: 2602-2612, 2007.

Henriksson J. Training induced adaptation of skeletal muscle and metabolism during submaximal exercise. *J Physiol* 270: 661-675, 1977.

Hernandez, JM, Fedele MJ, Farrell PA. Time course evaluation of protein synthesis and glucose uptake after acute resistance exercise in rats. *J Appl Physiol* 88: 1142-1149, 2000.

Hershey JWB, Merrick WC. Pathway and mechanism of initiation of protein synthesis. In *Translational Control of Gene Expression*, Sonenberg N, Hershey JWB, Mathews MB (eds), pp 33–88. Cold Spring Harbor, USA: Cold Spring Harbor Laboratory Press, 2000.

Hickson RC. Interference of strength development by simultaneously training for strength and endurance. *Eur J Appl Physiol Occup Physiol* 45: 255-263, 1980.

Hickson RC, Rosenkoetter MA, Brown MM. Strength training effects on aerobic power and short-term endurance. *Med Sci Sports Exerc*12: 336-339, 1980.

Hoff J, Helgerud J, Wisløff U. Maximal strength training improves work economy in trained female cross-country skiers. *Med Sci Sports Exerc* 31: 870-877, 1999.

Holloszy JO, Booth FW. Biochemical adaptations to endurance exercise in muscle. *Annu Rev Physiol* 38: 273-291, 1976.

Hornberger TA, Chu WK, Mak YW, Hsiung JW, Huang SA, Chien S. The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. *Proc Natl Acad Sci U S A* 103: 4741-4746, 2006.

Howarth KR, Moreau NA, Phillips SM, Gibala MJ. Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. *J Appl Physiol* 106: 1394-1402, 2009.

Inoki K, Li Y, Zhu T, Wu J, Guan, KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 4: 648-657, 2002

Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* 17: 1829-1834, 2003.

Jefferson LS, Fabian JR, Kimball SR. Glycogen synthase kinase-3 is the predominant insulin-regulated eukaryotic initiation factor 2B kinase in skeletal muscle. *Int J Biochem Cell Biol* 31: 191-200, 1999.

Jørgensen SB, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA, Pilegaard H. Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. *FASEB J* 19: 1146-1148, 2005.

Karlsson HK, Nilsson PA, Nilsson J, Chibalin AV, Zierath JR, Blomstrand E. Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. *Am J Physiol Endocrinol Meta* 287: 1-7, 2004.

Kostek MC, Chen YW, Cuthbertson DJ, Shi R, Fedele MJ, Esser KA, Rennie MJ. Gene expression responses over 24 h to lengthening and shortening contractions in human muscle: major changes in CSRP3, MUSTN1, SIX1, and FBXO32. *Physiol Genomics* 31: 42-52, 2007.

Kraemer WJ, Patton JF, Gordon SE, Harman EA, Deschenes MR, Reynolds K, Newton RU, Triplett NT, Dziados JE. Compatibility of high-intensity strength and endurance training on hormonal and skeletal muscle adaptations. *J Appl Physiol* 78: 976-989, 1995.

Kumar V, Atherton P, Smith K, Rennie MJ. Human muscle protein synthesis and breakdown during and after exercise. *J Appl Physiol* 106: 2026-2039, 2009.

Kumar V, Selby A, Rankin D, Patel R, Atherton P, Hildebrandt W, Williams J, Smith K, Seynnes O, Hiscock N, Rennie MJ. Age-related differences in the dose-response relationship of muscle protein synthesis to resistance exercise in young and old men. *J Physiol* 587: 211-217, 2009.

Kusuhara K, Madsen K, Jensen L, Hellsten Y, and Pilegaard H. Calcium signalling in the regulation of PGC-1alpha, PDK4 and HKII mRNA expression. *Biol Chem* 388: 481-488, 2007.

Lagirand-Cantaloube J, Cornille K, Csibi A, Batonnet-Pichon S, Leibovitch MP, Leibovitch SA. Inhibition of Atrogin-1/MAFbx Mediated MyoD Proteolysis Prevents Skeletal Muscle Atrophy In Vivo. *PLoS ONE* 4: e4973, 2009

Lagirand-Cantaloube J, Offner N, Csibi A, Leibovitch MP, Batonnet-Pichon S, Tintignac LA, Segura CT, Leibovitch SA. The initiation factor eIF3-f is a major target for atrogin1/MAFbx function in skeletal muscle atrophy. *EMBO J* 27: 1266-1276, 2008.

Leighton B, Blomstrand E, Challiss RA, Lozeman FJ, Parry-Billings M, Dimitriadis GD, Newsholme EA. Acute and chronic effects of strenuous exercise on glucose metabolism in isolated, incubated soleus muscle of exercisetrained rats. Acta *Physiol Scand* 136: 177-184, 1989.

Li YP, Chen Y, John J, Moylan J, Jin B, Mann DL, Reid MB. TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB J* 19: 362-370, 2005.

Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418: 797–801, 2002.

Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J. Rheb binds and regulates the mTOR kinase. *Curr Bio*l 15: 702-713, 2005.

Louis E, Raue U, Yang Y, Jemiolo B, Trappe S. Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *J Appl Physiol* 103: 1744-1751, 2007.

Lundby C, Nordsborg N, Kusuhara K, Kristensen KM, Neufer PD, Pilegaard H. Gene expression in human skeletal muscle: alternative normalization method and effect of repeated biopsies. *Eur J Appl Physiol* 95: 351-360, 2005.

MacDougall JD, Gibala MJ, Tarnopolsky MA, MacDonald JR, Interisano SA, Yarasheski KE. The time course for elevated muscle protein synthesis following heavy resistance exercise. *Can J Appl Physiol* 20: 480-486, 1995.

Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA. Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. *FASEB J* 19: 1498-1500, 2005.

Marcinik EJ, Potts J, Schlabach G, Will S, Dawson P, Hurley BF. Effects of strength training on lactate threshold and endurance performance. *Med Sci Sports Exerc* 23: 739-743, 1991.

Mascher H, Andersson H, Nilsson PA, Ekblom B, Blomstrand E. Changes in signalling pathways regulating protein synthesis in human muscle in the recovery period after endurance exercise. *Acta Physiol (Oxf)* 191: 67-75, 2007.

Mascher H, Tannerstedt J, Brink-Elfegoun T, Ekblom B, Gustafsson T, Blomstrand E. Repeated resistance exercise training induces different changes in mRNA expression of MAFbx and MuRF-1 in human skeletal muscle. *Am J Physiol Endocrinol Metab* 294: E43-51, 2008.

McNurlan MA, Essen P, Heys SD, Buchan V, Garlick PJ, Wernerman J. Measurement of protein synthesis in human skeletal muscle: further investigation of the flooding technique. *Clin Sci (Lond)* 81: 557-564, 1991.

Merrick WC. Mechanism and regulation of eukaryotic protein synthesis. *Microbiol Rev* 56: 291-315, 1992.

Miller BF, Olesen JL, Hansen M, Dossing S, Crameri RM, Welling RJ, Langberg H, Flyvbjerg A, Kjaer M, Babraj JA, Smith K, Rennie MJ. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol* 567: 1021-1033, 2005.

Nader GA, Esser KA. Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J Appl Physiol* 90: 1936-1942, 2001.

Navé BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* 344: 427-431, 1999.

Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, Gustafsson T. PGC-1αlpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* 96: 189-194, 2004.

Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkruis FJ, Thomas G. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci* 102: 14238-14243, 2005.

Parkington JD, Siebert AP, LeBrasseur NK, Fielding RA. Differential activation of mTOR signaling by contractile activity in skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 285: 1086-1090, 2003.

Pavitt GD, Ramaiah KV, Kimball SR, Hinnebusch AG. eIF2 independently binds two distinct eIF2B subcomplexes that catalyze and regulate guanine-nucleotide exchange. *Genes Dev* 12: 514-526, 1998.

Phillips SM, Tipton KD, Aarsland A, Wolf SE, Wolfe RR. Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol* 273: 99-107, 1997.

Pilegaard H, Saltin B, Neufer PD. Exercise induces transient transcriptional activation of the PGC-1αlpha gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB, Spiegelman BM. Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. *Mol Cell* 8: 971-982, 2001.

Raue U, Slivka D, Jemiolo B, Hollon C, Trappe S. Proteolytic gene expression differs at rest and after resistance exercise between young and old women. *J Gerontol A Biol Sci Med Sci* 62: 1407-1412, 2007.

Redpath NT, Foulstone EJ, Proud CG. Regulation of translation elongation factor-2 by insulin via a rapamycin-sensitive signalling pathway. *EMBO J* 15: 2291-2297, 1996.

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* 3: 1009-1013, 2001.

Rose AJ, Bisiani B, Vistisen B, Kiens B, Richter EA. Skeletal muscle eEF2 and 4EBP1 phosphorylation during endurance exercise is dependent on intensity and muscle fiber type. *Am J Physiol Regul Integr Comp Physiol* 296: 326-333, 2009.

Rose AJ, Broholm C, Kiillerich K, Finn SG, Proud CG, Rider MH, Richter EA, Kiens B. Exercise rapidly increases eukaryotic elongation factor 2 phosphorylation in skeletal muscle of men. *J Physiol* 569: 223-228, 2005.

Rønnestad BR, Hansen EA, Raastad T. Effect of heavy strength training on thigh muscle cross-sectional area, performance determinants, and performance in well-trained cyclists. *Eur J Appl Physiol* 108: 965-975, 2010.

Saltin B, Gollnick P. Skeletal muscle adaptability: significance for metabolism and performance. In: L.D. Peachey, R.H. Adrian & S.R. Geiger (eds) *Handbook of Physiology*, Section 10, Skeletal Muscle, pp. 555-631. American Physiological Society, Bethesda, MD, USA, 1983.

Saucedo LJ, Gao X, Chiarelli DA, Li L, Pan D, Edgar BA. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol* 5: 566-571, 2003.

Sheffield-Moore M, Yeckel CW, Volpi E, Wolf SE, Morio B, Chinkes DL, Paddon-Jones D, Wolfe RR. Postexercise protein metabolism in older and younger men following moderate-intensity aerobic exercise. *Am J Physiol Endocrinol Metab* 287: E513-E522, 2004.

Short KR, Vittone JL, Bigelow ML, Proctor DN, Nair KS. Age and aerobic exercise training effects on whole body and muscle protein metabolism. *Am J Physiol Endocrinol Metab* 286: 92-101, 2004.

Smith K, Reynolds N, Downie S, Patel A, Rennie MJ. Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. *Am J Physiol* 275: E73-78, 1998.

Solomon V, Goldberg AL. Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *J Biol Chem* 271: 26690-26697, 1996.

St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jäger S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 127: 397-408, 2006.

Taylor RG, Tassy C, Briand M, Robert N, Briand Y, Oulali A. Proteolytic activity of proteasome on myofibrillar structures. *Mol Biol Rep* 21: 71-73, 1995.

Tee AR, Fingar DC, Manning BD, Kwiatkowski DJ, Cantley LC, Blenis J. Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc Natl Acad Sci* 99: 13571-13576, 2003.

Terzis G, Georgiadis G, Stratakos G, Vogiatzis I, Kavouras S, Manta P, Mascher H, Blomstrand E. Resistance exercise-induced increase in muscle mass correlates with p70S6 kinase phosphorylation in human subjects. *Eur J Appl Physiol* 102: 145-152, 2008.

Thomson DM, Gordon SE. Diminished overloadinduced hypertrophy in aged fast-twitch skeletal muscle is associated with AMPK hyperphosphorylation. *J Appl Physiol* 98: 557-564, 2005.

Tipton KD, Borsheim E, Wolf SE, Sanford AP, Wolfe RR. Acute response of net muscle protein balance reflects 24-h balance after exercise and amino acid ingestion. *Am J Physiol Endocrinol Metab* 284: 76-89, 2003.

Tipton KD, Ferrando AA, Williams BD, Wolfe RR. Muscle protein metabolism in female swimmers after a combination of resistance and endurance exercise. *J Appl Physiol* 81: 2034-2038, 1996.

Tipton KD, Rasmussen BB, Miller SL, Wolf SE, Owens-Stovall SK, Petrini BE, Wolfe RR. Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise. *Am J Physiol Endocrinol Metab* 281: E197-E206, 2001.

Wang X, Paulin FE, Campbell LE, Gomez E, O'Brien K, Morrice N, Proud CG. Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the epsilon-subunit and their functions in vivo. *EMBO J* 20: 4349-4359, 2001.

Wang L, Psilander N, Tonkonogi M, Ding S, Sahlin K. Similar expression of oxidative genes after interval and continuous exercise. *Med Sci Sports Exerc* 41: 2136-2144, 2009.

Watt PW, Lindsay Y, Scrimgeour CM, Chien PA, Gibson JN, Taylor DJ, Rennie MJ. Isolation of aminoacyl-tRNA and its labeling with stable-isotope tracers: Use in studies of human tissue protein synthesis. *Proc Natl Acad Sci U S A* 88: 5892-5896, 1991.

Welsh GI, Miller CM, Loughlin AJ, Price NT, Proud CG. Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett* 421: 125-130, 1998.

Wilkinson SB, Phillips SM, Atherton PJ, Patel R, Yarasheski KE, Tarnopolsky MA, and Rennie MJ. Differential effects of resistance and endurance exercise in the fed state on signalling molecule phosphorylation and protein synthesis in human muscle. *J Physiol* 586: 3701-3717, 2008.

Vissing K, Andersen JL, Schjerling P. Are exercise-induced genes induced by exercise? *FASEB J* 19: 94-96, 2005.

Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *Biotechniques* 39: 75-85, 2005.

Yang Y, Jemiolo B, Trappe S. Proteolytic mRNA expression in response to acute resistance exercise in human single skeletal muscle fibers.
J Appl Physiol 101: 1442-1450, 2006.