STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH
133

Juha Hulmi

# Molecular and Hormonal Responses and Adaptation to Resistance Exercise and Protein Nutrition in Young and Older Men

JYVÄSKYLÄN YLIOPISTO

STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH 133

Juha Hulmi

# Molecular and Hormonal Responses and Adaptation to Resistance Exercise and Protein Nutrition in Young and Older Men

Esitetään Jyväskylän yliopiston liikunta- ja terveystieteiden tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Liikunnan salissa (L304)
maaliskuun 28. päivänä 2009 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Sport and Health Sciences of the University of Jyväskylä,
in Auditorium L 304, on March 28, 2009 at 12 o'clock noon.

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2009

# Molecular and Hormonal Responses and Adaptation to Resistance Exercise and Protein Nutrition in Young and Older Men

STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH 133

Juha Hulmi

# Molecular and Hormonal Responses and Adaptation to Resistance Exercise and Protein Nutrition in Young and Older Men

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2009

Editors
Harri Suominen
Department of Health Sciences, University of Jyväskylä
Pekka Olsbo, Marja-Leena Tynkkynen
Publishing Unit, University Library of Jyväskylä

URN:ISBN:978-951-39-3524-5
ISBN 978-951-39-3524-5 (PDF)

ISBN 978-951-39-3495-8 (nid.)
ISSN 0356-1070



Jyväskylä University Printing House, Jyväskylä 2009

*“If it weren't for the fact that the TV set and the refrigerator are so far apart, some of us wouldn't get any exercise at all.”*

Joey Adams, comedian, (1911-1999)

# ABSTRACT

Hulmi, Juha Jarmo Tapio
Molecular and hormonal responses and adaptation to resistance exercise and protein nutrition in young and older men
Jyväskylä: University of Jyväskylä, 2009, 109 p.
(Studies in Sport, Physical Education and Health,
ISSN 0356-1070; 133)
ISBN 978-951-39-3524-5 (PDF), 978-951-39-3495-8 (nid.)
Diss.

The aim of the present study was to investigate the mechanisms leading to muscle hypertrophy in humans by studying local muscle molecular and systemic hormonal responses to a single bout of heavy resistance exercise (RE) and to long-term resistance training (RT) with or without protein supplementation in a randomized controlled double-blinded design. In line with earlier studies, heavy RT for 21 weeks led to muscle hypertrophy and an increase in muscle strength in both young and older men. Whey protein ingestion before and after each RE workout increased vastus lateralis (VL) muscle cross-sectional area and seemed to accelerate the increase in VL muscle thickness as well as the increase in body mass. However, no effect of protein was seen in muscle fiber size or in muscle force. A bout of RE in both young and older men decreased myostatin and its receptor gene expression. However, when protein was ingested before and after the RE bout, the decrease in myostatin mRNA and protein content did not occur and also the increase in serum testosterone during the RE bout was attenuated. The results of the older men suggest that the latter was not explained by a significant difference in the expression of androgen receptors. Protein ingestion increased the gene expression of the positive cell cycle regulator cdk2 after the RE bout and RT. The RE bout rapidly and favourably increased the phosphorylation of mTOR signaling pathway proteins, and whey protein ingestion strengthened and prolonged this response. RT did not consistently change the acute mRNA response to the single bout of RE or change the basal level of mRNA expression. However, the basal individual changes in androgen receptor concentration during the RT period seemed to explain, in part, the muscle hypertrophy observed during RT. Altogether, this thesis provides new information about the molecular events that possibly regulate muscle hypertrophy during resistance training with or without protein ingestion. These results could be applied when planning proper training and nutrition protocols with the objective of increasing or at least maintaining good muscle size and functional capacity.

Keywords: resistance exercise, protein supplementation, muscle hypertrophy, myostatin, cdk2, mTOR signaling, testosterone

**Author's address**

Juha Hulmi
Department of Biology of Physical Activity
Neuromuscular Research Center
P.O. Box 35
40014 University of Jyväskylä, Jyväskylä, Finland

**Supervisors**

Professor Antti Mero, PhD
Department of Biology of Physical Activity
Neuromuscular Research Center
University of Jyväskylä, Jyväskylä, Finland

Docent Vuokko Kovanen, PhD
Department of Health Sciences
Finnish Centre for Interdisciplinary Gerontology
University of Jyväskylä, Jyväskylä, Finland

**Reviewers**

Professor Marcas Bamman, PhD
Department of Physiology and Biophysics
The University of Alabama, Birmingham, USA

Professor Jay Hoffman, PhD
Department of Health and Exercise Science
The College of New Jersey, USA

**Opponent**

Professor Jeffrey Stout, PhD
Department of Health and Exercise Science
University of Oklahoma, Norman, USA

# ACKNOWLEDGEMENTS

The work described in this dissertation was carried out by the author in the Department of Biology of Physical Activity at the University of Jyväskylä. The facilities of the department and the faculty provided excellent tool to do the reseach.

I express my deepest gratitude to my supervisors, Professor Antti Mero and Docent Vuokko Kovanen. They provided me the opportunity for doctoral studies. Antti guided me into the field of research already in the early phases of my master studies and gave me a big freedom to do the research. Vuokko was important especially in biological aspects of muscle and she taught me the importance of patience and fidelity. The clinician of the project, Dr Harri Selänne, was always available and his schedules were flexible and therefore made the demanding muscle biopsy work possible. The nice humorous atmosphere we always had together helped us all to survive nicely through the tough project.

I also wish to express big gratitude to the chair of the Department and also collaborator, Professor Keijo Häkkinen, who was an important advisor in my work.

I also like to mention Dr Juha Ahtiainen with whom I started my first research work in 2003 doing my exercise physiology bachelor thesis in his PhD project. Following that we have collaborated in my thesis.

The laboratory personnel were extremely important in my work. Special thanks go for Mr Risto Puurtinen, Ms Aila Ollikainen and Ms Tuovi Nykänen. The secretaries Ms Minna Herpola and Ms Katja Pylkkänen were indispensable in lots of paperwork.

I would like to thank also other co-authors not mentioned above: Mr Tuomas Kaasalainen, Ms Eija Pöllänen, Ms Inna Lisko, Professor Markku Alen, Professor Heikki Kainulainen, Mr Jörgen Tannerstedt, Professor Jeff Volek, Professor William Kraemer, Dr Maarit Lehti, Docent Jyrki Komulainen and Mr Kai Nyman for their contribution during this work.

I gratefully acknowledge also all the other colleagues, office workers as well as laboratory and technical staff and students working in this project for fruitful collaboration and nice atmostphere to enjoy working.

I owe my special thanks to all the subjects who volunteered to participate in this study; without their blood and sweat this work would never have been done.

I wish to address my sincere thanks to the referees of my thesis, Professors Marcas Bamman and Jay Hoffman, for their thorough and fair review and valuable advice for finishing this work.

Mr Michael Freeman and Mr Simon Walker are acknowledged for language revision of this thesis. Michael Freeman and Mr Matthew Stults-Kolehmainen provided language revision for original articles contributing to this thesis.

I warmly thank Anna for her love and patience. My parents Jarmo and Arja and brother Juuso have always supported me and they have also emphasized the importance of hard work. They, and friends and close relatives have made my life enjoyable also outside the world of research.

Finally, I wish to acknowledge the Ministry of Education, Finnish Cultural Foundation, Ellen and Artturi Nyyssönen Foundation, University of Jyväskylä Rectoral Grant and the Department of Biology of Physical Activity, University of Jyväskylä who have financially supported this work.

Juha Hulmi

Jyväskylä, March 2009

# CONTENTS

## ORIGINAL PAPERS

This thesis is based on the following original research articles, which will be referred to by their Roman numerals.

I Hulmi JJ, Volek JS, Selänne H, Mero A. Protein ingestion prior to strength exercise affects blood hormones and metabolism. Medicine and Science in Sport and Exercise 37: 1990-1997, 2005.

II Hulmi JJ, Ahtiainen JP, Kaasalainen T, Pöllänen E, Häkkinen K, Alen M, Selänne H, Kovanen V, Mero AA. Post-exercise myostatin and activin IIb mRNA levels: effects of strength training. Medicine and Science in Sport and Exercise 39: 289-297, 2007.

III Hulmi JJ, Kovanen V, Lisko I, Selänne H, Mero AA. The effects of whey protein on myostatin and cell cycle related gene expression responses to a single heavy resistance exercise bout in older men. European Journal of Applied Physiology 102: 205-213, 2008.

IV Hulmi JJ, Ahtiainen JP, Selänne H, Volek JS, Häkkinen K, Kovanen V, Mero AA. Androgen receptors and testosterone in men - effects of protein ingestion, resistance exercise and fiber type. The Journal of Steroid Biochemistry and Molecular Biology 110: 130-137, 2008.

V Hulmi JJ, Kovanen V, Selänne H, Kraemer WJ, Häkkinen K, Mero AA. Acute and long-term effects of resistance exercise with or without protein ingestion on muscle hypertrophy and gene expression. Amino Acids, published online 2008. DOI: 10.1007/s00726-008-0150-6.

VI Hulmi JJ, Tannerstedt J, Selänne H, Kainulainen H, Kovanen V, Mero AA. Resistance exercise and whey protein ingestion affects mTOR signaling pathway and myostatin in men. Journal of Applied Physiology, minor revision accepted 2009.

VII Ahtiainen JP, Hulmi JJ, Kraemer WJ, Lehti M, Nyman K, Pakarinen A, Selänne H, Alen M, Komulainen J, Kovanen V, Mero A, Häkkinen K. Effects of resistance training on skeletal muscle IGF-I and androgen receptor expression in young and elderly men. Submitted, 2009.

## ABBREVIATIONS AND DEFINITIONS

| | |
|---|---|
| AcvrIIb | Activin receptor IIb |
| Akt | Also called Protein kinase B (PKB) |
| AR | Androgen receptor |
| BCAA | Branched chain amino acids |
| Cdk2 | Cyclin-dependent kinase 2 |
| CHO | Carbohydrates |
| CSA | Cross-sectional area |
| eEF2 | Eukaryotic elongation factor 2 |
| FLRG | Follistatin-related gene (protein) |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| IGF-I | Insulin-like growth factor I |
| MAFbx | Muscle Atrophy F-Box / atrogin-1 |
| MGF | Mechano growth factor |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger RNA |
| mTOR | Mammalian target of rapamycin |
| MyoD | Myogenic differentiation factor |
| PI3K | Phosphoinositide 3-kinase |
| PKB | Protein kinase B (also called Akt) |
| $p70^{S6K}$ | Ribosomal protein S6 kinase (S6K1, size 70 kDa) |
| QF | Quadriceps femoris (muscle) |
| RE | Resistance exercise (bout) |
| rpS6 | Ribosomal protein S6 |
| RT | Resistance training |
| RT- PCR | Reverse transcriptase-polymerase chain reaction |
| Ser | Serine (amino acid) |
| Thr | Threonine (amino acid) |
| TORC1 / TORC2 | Target of rapamycin complex 1 or 2 (see mTOR) |
| tRNA | Transfer RNA |
| US | Ultrasonography |
| VL | Vastus lateralis (muscle) |
| 4E-BP1 | 4E-binding protein |

# 1 INTRODUCTION

Muscle weakness and atrophy occurs with age and is characterized by loss of muscle mass, maximal strength and power, and strength endurance. It has been estimated that on average 5% of muscle mass is lost per decade after the age of 40, and that this decrease may be even more rapid after age 65 (Greenlund and Nair 2003). Muscle atrophy occurs as a consequence of aging, especially if the person is inactive, has poor nutrition and/or has a chronic disease. Many young individuals with a present inactive lifestyle, especially those with chronic diseases, have a low muscle mass which, in turn decreases their ability to engage in many normal activities. Fortunately, skeletal muscle has a high capacity for contractile activity-induced growth (Morpurgo 1879; Goldberg 1967; Goldberg et al. 1975). Resistance training in particular has well established beneficial effects on skeletal muscle size and body composition, physical fitness, and injury prevention and rehabilitation (Häkkinen et al. 2001b; Kraemer et al. 2002; Kraemer and Ratamess 2004). Because of these positive effects, resistance training has become extremely popular during the last few years. The mechanisms behind the resistance training adaptations found in humans remain unclear, hence the reason for embarking on the present study. This study was also prompted by the fact that advances in molecular biology have enabled us during recent years to better understand the molecular and cellular mechanisms involved both in the development and growth of tissues, and adaptation to physical activity and nutrition. Thus, we are now beginning to understand the mechanisms that underlie the changes in muscle mass that are seen with resistance training, protein nutrition, and aging.

Protein, derived from the Greek word *proteos,* means 'primary' or 'most important'. The legendary Greek wrestler Milo is said to have gained an advantage over his competitors during his five successive Olympic victories by eating enormous amounts of beef (Tipton and Witard 2007). In the sports domain, the importance of nutrition, especially protein first interested bodybuilders; however, nowadays it is a major issue among all athletes and "habitual trainers". However, the precise mechanisms underlying protein

ingestion in the context of a bout of resistance exercise in humans remain unclear.

It is known that even a single resistance exercise session starts the processes of adaptation in skeletal muscle; however, only when exercise bouts are repeated do more noteworthy adaptations occur. After a bout of resistance exercise muscle protein synthesis increases at greater rate than protein degradation, but without amino acids and/or protein intake before or after exercise the protein net balance remains negative (Biolo et al. 1995b; Biolo et al. 1997; Tipton et al. 1999; Pitkänen et al. 2003). Some studies have suggested that the most efficient way to induce a positive protein balance in skeletal muscle after a resistance training session is to ingest nutrition containing essential amino acids soon after, and possibly also before and during, each resistance exercise workout (Esmarck et al. 2001; Tipton et al. 2004; Andersen et al. 2005). Whey, which accounts for 20% of the total proteins of bovine milk (Ha and Zemel 2003; Etzel 2004; Krissansen 2007), is considered to be a high-quality protein source containing large amounts of essential amino acids important in protein synthesis (Borsheim et al. 2002), and is also fast acting (Boirie et al. 1997; Dangin et al. 2002). However, more data are needed on acute and long-term responses after whey protein ingestion, especially in the context of resistance exercise, to better understand the adaptive mechanisms in human muscles.

The present study investigated the physiological and molecular effects of protein ingestion in the context of a bout of resistance exercise in a randomized controlled design in both young and older men. The study was focused on blood hormones (studies I, III, IV, VI), muscle gene expression in mRNA and protein levels (studies II-VI), phosphorylation of muscle protein kinases (VI), and muscle hypertrophy (V, VI). Moreover, the long-term effects of resistance training with or without protein ingestion were investigated to understand how muscle adapts to resistance training (studies II, V-VII).

# 2 REVIEW OF THE LITERATURE

## 2.1 Effects of resistance training on muscle hypertrophy

It is well known that heavy resistance training, with multiple sets and repetitions, increases muscle cross-sectional area and improves maximal force production, especially in previously untrained subjects (Häkkinen et al. 2001b; Kraemer et al. 2002; Hubal et al. 2005; Wernbom et al. 2007). The terms resistance exercise and training in this thesis refer to a heavy dynamic resistance exercise bout or to months of training, repectively, consisting of multiple set exercise workouts with several repetitions in every set (Tesch et al. 1986; Häkkinen and Pakarinen 1993). Skeletal muscle hypertrophy is characterized by an increase in the size and number of myofibrils (Luthi et al. 1986; Rosenblatt and Woods 1992), which lead to the enlargement of muscle fibers (MacDougall et al. 1980; McCall et al. 1996; Esmarck et al. 2001; Häkkinen et al. 2001b) and whole muscle cross-sectional area and volume (McCall et al. 1996; Esmarck et al. 2001; Häkkinen et al. 2001b; Ahtiainen et al. 2003). The proportion of non-contractile tissue to contractile tissue (Goldspink and Howells 1974; MacDougall et al. 1984) and the number of muscle fibers (Goldspink and Howells 1974; MacDougall et al. 1984; McCall et al. 1996) may not significantly change in normal resistance training induced muscle hypertrophy in humans. Measuring the total number of muscle fibers in living humans is, however, basically impossible with the present techniques. In some (MacDougall et al. 1982; Tesch and Larsson 1982; D'Antona et al. 2006) but not all cross-sectional studies (Eriksson et al. 2005), extremely hypertrophied bodybuilders have not had much larger cross-sectional areas of muscle fibers than individuals with more average-sized muscles. Therefore, an increase in muscle fiber size may not fully account for the hypertrophy of the whole muscle after heavy resistance training for many years.

Muscle hypertrophy is regarded as fiber-type specific with the fast type II fibers increasing in size more after typical resistance training period than the slow type I fibers (Costill et al. 1979; Aagaard et al. 2001; Häkkinen et al. 2001b;

Kosek et al. 2006). However, bodybuilders typically performing more repetitions and having shorter rest periods between sets have more similar increase of their type I and type II fiber sizes (Fry 2004). To date, insufficient evidence on the relative superiority of different resistance training modes and protocols on muscle hypertrophy is available (Fry 2004; Wernbom et al. 2007).

Resistance training induces robust hypertrophy on both the whole muscle and myofiber levels at all ages and in both genders (Fiatarone et al. 1994; Häkkinen et al. 1998a; Ivey et al. 2000; Bamman et al. 2003; Kosek et al. 2006). However, some studies (Moritani and deVries 1980; Welle et al. 1996; Kosek et al. 2006), but not all (Häkkinen et al. 1998a; Roth et al. 2001) suggest that older individuals may have a smaller relative muscle hypertrophy response to resistance training than the younger individuals. The possible existence of age-difference may depend on the training programs used (e.g., frequency and volume of training), the ages compared, or even gender (Ivey et al. 2000; Häkkinen et al. 2001a; Bamman et al. 2003).

## 2.2 Effects of protein supplementation on muscle hypertrophy

In addition to resistance training, protein ingestion may play an important role in recovery from exercise and in muscle size adaptations. Protein or essential amino acid ingestion during resistance training has been shown in many human studies to increase muscle myofiber cross-sectional area (Andersen et al. 2005; Bird et al. 2006; Cribb et al. 2007; Hartman et al. 2007) as well as lean or fat-free body mass (Burke et al. 2001; Candow et al. 2006a; Kerksick et al. 2006; Cribb et al. 2007; Hartman et al. 2007; Willoughby et al. 2007). However, the fact that some studies have not found protein ingestion to have a significant effect on muscle fiber size or lean body mass (Godard et al. 2002; Candow et al. 2006b; Olsen et al. 2006) calls for more studies of this issue.

Only a few studies have compared different protein sources. Hartman et al. (2007) found a larger muscle hypertrophy response to a period of resistance training with ingestion of milk proteins compared to ingestion of soy protein. This result was probably explained by the larger increase observed in muscle protein synthesis after milk compared to soy, as reported by the same group (Wilkinson et al. 2007). However, not all studies have shown differences between milk/whey and other protein sources on gains in muscle size or lean body mass (Brown et al. 2004; Candow et al. 2006a; Kalman et al. 2007). The most important component in whey/milk in increasing protein synthesis and the size of skeletal muscle is probably its high content of the amino acid leucine, because leucine, acting as a signaling molecule in mTOR cascade, has been shown to be the most important amino acid in protein synthesis, and possibly also in protein degradation regulation (Buse and Reid 1975; Smith et al. 1998; Mero 1999; Ha and Zemel 2003; Katsanos et al. 2006; Kimball 2007; Rieu et al. 2007). The effects of other protein sources on muscle hypertrophy than milk or soy proteins have been only rarely studied. One study showed that a meat-

containing diet contributed to greater gains in fat-free mass and skeletal muscle mass during resistance training in older men than a purely lacto-ovovegetarian diet did (Campbell et al. 1999). However, this was not supported by another study (Haub et al. 2002). Meat, such as lean beef, can induce an increase in mucle protein synthesis (Symons et al. 2007), but so can other high quality protein-rich food sources, such as eggs (Moore et al. 2009).

Several studies suggest that the timing of protein intake is more important than the amount. Protein and carbohydrate intake before and/or immediately after a resistance exercise session may be more beneficial, in terms of muscle protein anabolism and long-term hypertrophy, than nutrient ingestion at other times (Suzuki et al. 1999; Esmarck et al. 2001; Cribb and Hayes 2006). One reason for this could be increased circulation in the muscles during exercise and some time after it, and enhanced muscle uptake of the amino acids and glucose (Biolo et al. 1995b; Biolo et al. 1997; Levenhagen et al. 2001; Tipton et al. 2001). Whey proteins, containing different individual proteins e.g. β-lactoglobulin, α-lactalbumin, glycomacropeptide and immunoglobulins (Ha and Zemel 2003; Etzel 2004; Krissansen 2007), have been shown to be advantageous for increasing muscle size (Andersen et al. 2005; Hartman et al. 2007) and for improving muscle protein synthesis after a bout of resistance exercise (Koopman et al. 2005; Tang et al. 2007; Tipton et al. 2007; Wilkinson et al. 2007). This is most probably due to the good amino acid composition of milk proteins (Rutherfurd and Moughan 1998; Ha and Zemel 2003). A question worth examining is whether the timed addition of a high-quality protein, such as whey, to the ingestion of ordinary daily diet improves the muscle net protein synthesis response to exercise without interfering with the response to normal food. Recent results utilizing an acute design without exercise suggest that an essential amino acid and carbohydrate supplement does not interfere acutely with the normal muscle protein synthesis response to a mixed meal (Paddon-Jones et al. 2005). It is thus possible that the addition of whey, when used chronically in conjunction with exercise workouts may be more anabolic for skeletal muscle than the ingestion only of normal mixed meals throughout the day. The present study was designed to search answers to these questions in a situation where the normal food intake was not heavily restricted and whey protein was ingested in the context of an acute exercise bout and over a training period of 21 weeks.

Protein ingestion immediately after a resistance exercise bout increases the protein synthesis response of the muscle starting ~1-3 hours after exercise (Koopman et al. 2005; Tang et al. 2007; Tipton et al. 2007; Wilkinson et al. 2007). If nutrition that contains amino acids is ingested before and/or during a resistance exercise bout compared to exercise in a fasted state, it can prevent the decrease, or may even increase, the protein synthesis during exercise (Beelen et al. 2008a; Beelen et al. 2008b; Fujita et al. 2008). The change in protein synthesis has been shown to contribute more than the change in protein degradation to the net protein balance of muscle (synthesis - degradation) after a bout of resistance exercise with or without nutrition ingestion (Rasmussen and Phillips

2003). Nevertheless, protein ingestion may also slow down the breakdown of muscle protein during resistance exercise (Beelen et al. 2008a). Figure 1 shows the response of muscle protein turnover (synthesis and breakdown) after ingestion of protein with or without RE bout.

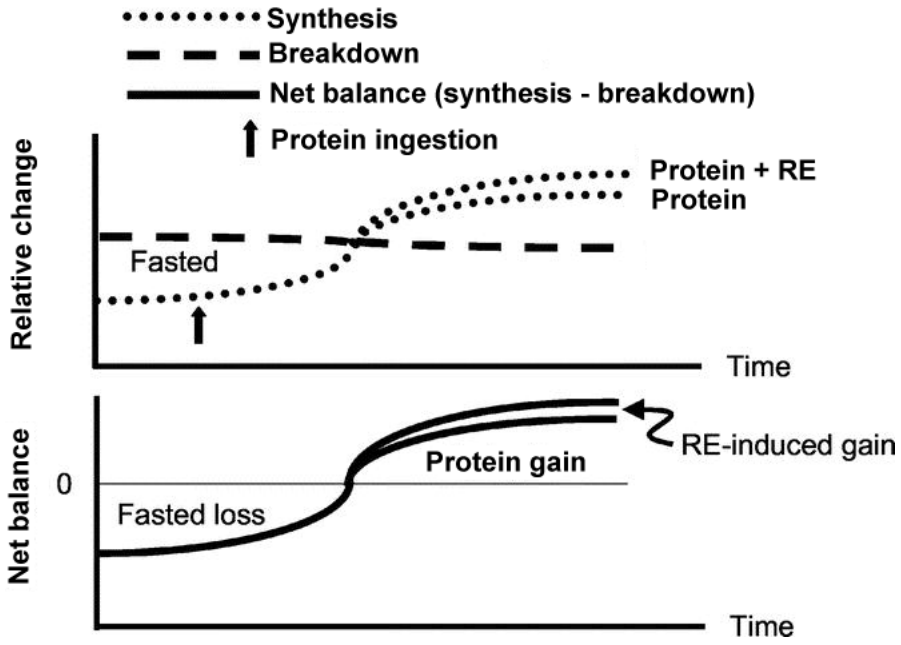


FIGURE 1 Simplified figure showing muscle protein turnover after protein ingestion or with RE bout and protein ingestion. The figure is modified from (Rasmussen and Phillips 2003), with permission from the Exercise and Sport Sciences Reviews.

Different protein sources have different effects on protein synthesis and breakdown. For example, the whey fraction of milk is absorbed fast and is short-acting, increasing quickly and largely protein synthesis compared to e.g. casein proteins. However, whey has a smaller decreasing effect on protein breakdown than the more slowly acting casein (Boirie et al. 1997; Dangin et al. 2001; Dangin et al. 2002). The effect of protein or amino acid ingestion on protein synthesis also seems to be affected by other variables such as aging (Volpi et al. 2000; Dangin et al. 2003; Cuthbertson et al. 2005; Rasmussen et al. 2006).

## 2.3 Mechanisms of muscle hypertrophy

### 2.3.1 Introduction to regulation of muscle hypertrophy

Skeletal muscle is tissue with a high ability to adapt. It consists on average of 20% of proteins, the rest being mostly water (75-80%), carbohydrates and lipids. Muscle hypertrophy, therefore, occurs due to a higher rate of protein synthesis than protein degradation, which eventually leads to an increase in total muscle

protein. The increase in muscle protein mass is regulated by both systemic and local factors, for instance muscle strain with or without possible damage, sarcoplasmic calcium concentrations, energy and nutrient state, intramuscular oxygen concentrations, hormones, growth factors and cytokines, and temperature. These factors either directly or (usually) indirectly increase the amount of myofibrillar proteins of which the protein mass of skeletal muscle is mostly comprised (Rennie et al. 2004; Nader 2005; Baar et al. 2006; Rennie et al. 2006; Wackerhage and Rennie 2006; Frier and Locke 2007; Joulia-Ekaza and Cabello 2007; Vary and Lynch 2007).

Synthesis of the proteins regulating the amount of myofibrillar proteins occurs in a number of steps, including transcription, posttranscriptional processing, nuclear export of messenger RNA (mRNA) and translation of mRNA into protein (Nelson and Cox 2000). All these steps can be regulated with numerous agents and networks of proteins. Protein activity is also regulated via processes other than synthesis. Those include the cellular location of the protein and its three-dimensional configuration. The latter is affected, for instance, by glycosylation, phosphorylation, proteolytic processing and the formation of disulfide bonds (Nelson and Cox 2000). Degradation of the protein can of course also be an important regulator of the total protein concentration, and therefore also of its activity (Goldberg 2003).

Control of the translation of mRNA into protein seems to be an especially important step in acute resistance loading-induced protein synthesis (Wong and Booth 1990; Chesley et al. 1992; Welle et al. 1999). Nevertheless, transcriptional control is also important, as for many proteins the transcription step has been shown to be the most important control point in the increase in muscle protein synthesis after resistance exercise (Chen et al. 2002). Transcription of a new message (mRNA) for the protein synthesis is an important aspect of muscle hypertrophy since inhibition of DNA-dependent mRNA transcription by actinomycin D prevents normal overload-induced muscle hypertrophy (Goldberg and Goodman 1969). Moreover, for some of the proteins regulating muscle size such as myostatin, recent small interfering RNA (siRNA) experiments have suggested that the regulation of these proteins at mRNA level has a large effect on muscle size (Magee et al. 2006).

In addition to the acute increase in muscle protein content after each bout of resistance exercise, the DNA content of muscle fibers, which is important for protein synthesis, increases during the process of muscle hypertrophy (Goldberg 1967; Adams and Haddad 1996). This increase in muscle DNA is thought to be achieved by satellite cells donating their nuclei to existing muscle fibers (Allen et al. 1999; Adams et al. 2002; Kadi et al. 2004b; Petrella et al. 2006). Figure 2 presents a simplified overview of muscle size regulation. Selected molecules that are important in this thesis are presented in figure 3 and reviewed below.

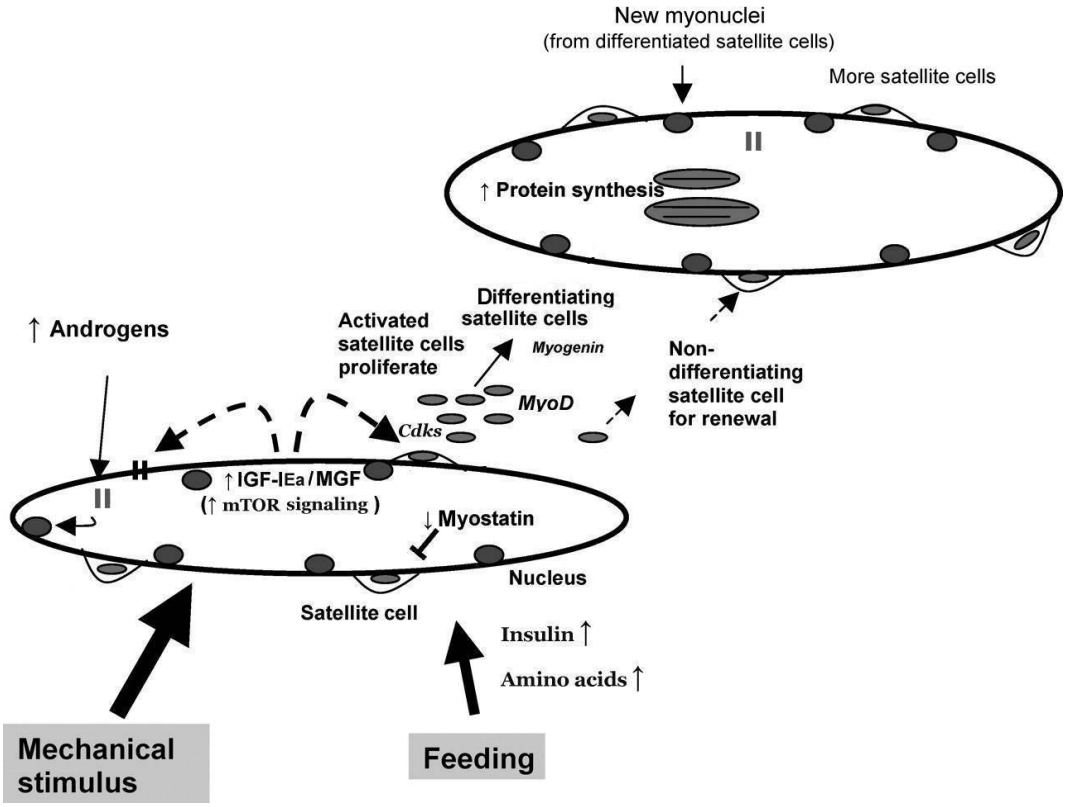


FIGURE 2 Simplified representation of some key mechanisms influencing hypertrophy of a muscle fiber in response to mechanical overload and/or nutrition (feeding). Arrows indicate activation and T-bars inhibition. II = receptor, cdk = cyclin-dependent kinase. The figure is modified from (Harridge 2007), with permission from the Experimental Physiology.

### 2.3.2 Testosterone and its receptors

Testosterone is an androgen secreted primarily from leydig cells of the testis in men that interacts with skeletal muscle cells via binding to androgen receptors (ARs) (Georget et al. 1997; Osipova-Goldberg et al. 2001). Androgens are thought mostly to bind to their receptors in the cytoplasm of the muscle fiber, and after binding ARs translocate into the nucleus to operate as a transcription factor (Georget et al. 1997; Osipova-Goldberg et al. 2001). However, ARs have also been shown to have fast, non-genomic effects (Estrada et al. 2003). Goldberg's early study (Goldberg 1967) showed that work-induced muscle growth did not differ between hypophysectomised rats and normal animals. This suggested that increase in muscle size with overloading does not *require* pituitary gland hormones such as growth hormone, and testosterone through its upstream regulator, luteinizing hormone. However, the importance of endogenous levels of androgens on human skeletal muscle seems to be underscored by recent findings suggesting that physiological levels of androgens are important for normal adaptations in muscle size and strength to occur with resistance training (Kvorning et al. 2006) and that supraphysiological levels increase the adaptive response in muscle size to resistance training (Bhasin et al. 1996). Moreover, the increase in muscle mass

induced by electrical stimulation was effectively suppressed by the androgen receptor blockade in rats (Inoue et al. 1994). The effect of testosterone on muscle size has been shown to be dose-dependent across a range of hypo- to supraphysiological concentrations of testosterone in blood (Bhasin et al. 2001).

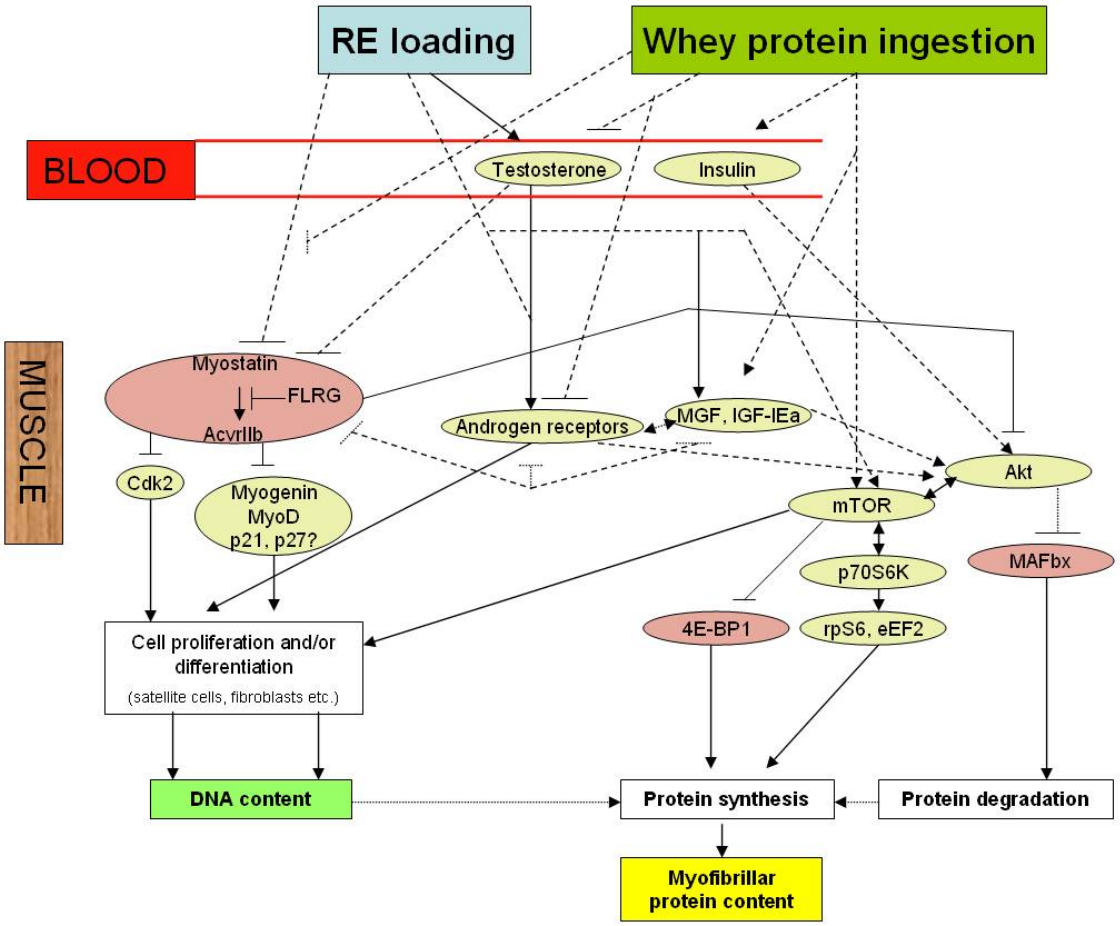


FIGURE 3 Schematic illustration of the complex pathways studied in the present study. These pathways are proposed to affect muscle hypertrophy after resistance exercise (RE) loading and protein ingestion. An arrow means activation, T-bar line without arrow means inactivation/inhibition. The dashed lines demonstrate more uncertain or less strong pathway. AcvrIIb = activin receptor IIb, FLRG = follistatin related gene protein, cdk2 = cyclin-dependent kinase 2, IGF-I = insulin-like growth factor I, MGF = mechano growth factor, mTOR = mammalian target of rapamycin, Akt = protein kinase B (PKB), p70S6K = ribosomal protein S6 (rpS6) kinase, eEF2 = eukaryotic elongation factor 2, 4E-BP1 = eIF4E binding protein 1, MAFbx = muscle atrophy F-box. Light yellow circles are molecules or molecule pathways that are positive for muscle hypertrophy whereas light red circles are negative pathways.

There is evidence to suggest that the exogenous androgen administration-induced increase in muscle size is somewhat larger in slow-twitch type I muscle fibers compared to fast-twitch type II fibers (Hartgens et al. 1996; Kadi et al. 1999; Sinha-Hikim et al. 2003; Ustunel et al. 2003; Eriksson et al. 2005; Sinha-Hikim et al. 2006). Also, the variability in the responsiveness of different skeletal muscles to androgens, shown in some studies, may reflect differential

expression or localization of ARs between different muscles (Kadi et al. 2000; Monks et al. 2006).

Testosterone increases muscle size by increasing muscle protein synthesis (Ferrando et al. 1998) and enhancing satellite cell activation, leading to an increase in the number of myonuclei (Kadi et al. 1999; Sinha-Hikim et al. 2003; Eriksson et al. 2005; Sinha-Hikim et al. 2006). However, long-term testosterone administration may not always increase the number of satellite cells beyond that achievable by training alone (Kadi 2000). Testosterone has also been reported to increase the synthesis of ARs (Kadi et al. 2000; Ferrando et al. 2002; Sinha-Hikim et al. 2004). However, at least in some muscles this effect may be short-term (Kadi et al. 2000; Ferrando et al. 2002).

### 2.3.3 Insulin

Insulin is a polypeptide hormone secreted from the pancreas. It has important functions in macronutrient metabolism, such as glucose uptake (Ganong 2001). Furthermore, insulin can increase muscle protein synthesis (Biolo et al. 1995a), but for that it also needs an increase in amino acids (Drummond et al. 2008). It seems that the role of insulin in the regulation of protein synthesis is to allow the effects of amino acids to occur (Kimball and Jefferson 2006). Insulin rapidly increases protein synthesis through the insulin receptor substrate1-PI3K-Akt pathway but it can also affect through a slower pathway by increasing the amount of ribosomes and, therefore, the capacity for protein synthesis (Proud 2006; Greenhaff et al. 2008). Insulin has other effects on muscle metabolism such as increased uptake of amino acids, increased blood flow in the muscles, and decreased protein degradation (Biolo et al. 1995a; Ganong 2001; Greenhaff et al. 2008).

### 2.3.4 Insulin-like growth factor I

Insulin-like growth factor I (IGF-I) exists as three splice variants, i.e. IGF-IEa, IGF-IEb and IGF-IEc (also called the 'mechano growth factor', MGF), of which MGF is muscle-specific (Yang et al. 1996). IGF-IEa in muscle is similar to the hepatic endocrine type of IGF-I. The second isoform has been classified as IGF-IEb in rats and IGF-IEc or MGF in humans (Yang et al. 1996). The difference in the IGF-I isoforms is that IGF-IEa has exon 6 but not 5, whereas IGF-IEb has exon 5 but not 6, and IGF-IEc (MGF) has exon 6 and part of exon 5 (Hameed et al. 2003; Harridge 2007). Muscle MGF mRNA concentrations are approximately 100-fold lower than those of the IGF-IEa isoform (Hameed et al. 2003). Recently, the MGF peptide has been shown to exist in human skeletal muscle (Philippou et al. 2008). The importance of IGF-I isoforms for muscle growth has been shown in many *in vivo* rodent and *in vitro* cell culture studies (Barton-Davis et al. 1998; Jacquemin et al. 2004; Barton 2006). IGF-I can also affect muscle hypertrophy through the mTOR pathway (Rommel et al. 2001). However, a recent study in mice suggests that mechanical load can induce muscle hypertrophy and activate the mTOR signaling pathway independent of the

functioning of the IGF-I receptor (Spangenburg et al. 2008). It is, however, possible that, at least in part, the local IGF-I in muscle fibers has its effects via intracrine mechanism directly inside the cell or through other receptors. It is also possible that a long period without some cellular mechanism, such as the effects of IGF-I through its receptors (Spangenburg et al. 2008), may lead to adaptation in other systems important in muscle growth. It has been proposed that MGF may be important in starting repair and adaptation processes after a resistance exercise bout by activating satellite cells (Hill and Goldspink 2003).

### 2.3.5 Myostatin

The cytokine myostatin, previously known as Growth and Differentiation Factor 8 (GDF-8), is a member of the TGF-β superfamily. Structurally, myostatin appears to share several features with other members of the TGF- β superfamily. Myostatin is synthesized as a precursor form that is proteolytically processed. Following removal of the 24 amino acid signal peptide from the N-terminus, myostatin is further cleaved into an N-terminal propeptide and a 110 amino acid biologically active C-terminus (Figure 4) (McPherron et al. 1997; Lee and McPherron 2001).

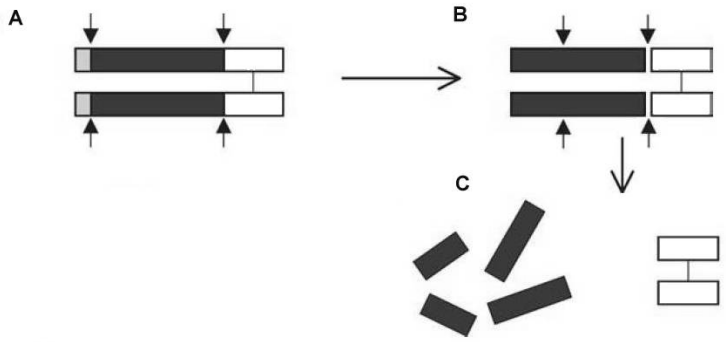


FIGURE 4 Processing of myostatin protein. Two proteolytic processing events occur from the full-sized myostatin (a). The first one is the removal of the N-terminal signal sequence (grey: a to b) and the second a generation of the C-terminal fragment (white: b to c), which is is the activive form of myostatin that can bind the receptors. C-terminal fragment is thought to be as a dimer form and it remains bound to the N-peptide (black) in a latent complex. Activation of this latent myostatin can occur by proteolytically cleavaging the propeptide (c). The figure is modified from (Lee 2004), with permission from the Annual Review of Cell and Developmental Biology.

McPherron et al. (1997) first showed that targeted deletion of the C-terminus of myostatin in mice leads to increased muscle mass. The increase in muscle mass was due to both hypertrophy and hyperplasia of muscle fibers. Furthermore, cattle that have mutations in the myostatin coding sequence have bigger muscle mass than normal cattle (Kambadur et al. 1997; McPherron and Lee 1997). The deficiency of myostatin also leads to a larger proportion of fast type II fibers and a reduced proportion of slow type I fibers (Girgenrath et al. 2005) and also possibly to bigger muscle force and enhanced performance in some sports

(Mendias et al. 2006; Mosher et al. 2007). However, the lack of myostatin may reduce the size and strength of tendons (Mendias et al. 2008).

The first case of a myostatin (point) mutation in a human child has been reported (Schuelke et al. 2004). At birth, and at four years age this boy was exceptionally muscular and strong while he also seemed to be healthy. The first studies in humans suggested that myostatin is expressed in the human skeletal muscle as a ~26 kDa protein (myostatin immunoreactive protein), which is purported to be glycosylated dimer and/or that the monomer of myostatin is strongly bound by some other protein (Gonzalez-Cadavid et al. 1998; Taylor et al. 2001). Myostatin is secreted into plasma and stays there at rather constant levels (Gonzalez-Cadavid et al. 1998; Hill et al. 2002). Serum concentrations of this 26 kDa protein have correlated inversely with fat-free mass, while skeletal muscle concentrations of myostatin were also increased in HIV-infected men going through weight loss in comparison to healthy men (Gonzalez-Cadavid et al. 1998). Serum myostatin has also inversely correlated with total body muscle mass in relation to height (Schulte and Yarasheski 2001; Yarasheski et al. 2002). Consequently, myostatin gene polymorphism (Schuelke et al. 2004) and also blood and muscle myostatin concentrations (Gonzalez-Cadavid et al. 1998; Schulte and Yarasheski 2001; Yarasheski et al. 2002) are thought to regulate muscle size in humans.

Myostatin circulates in blood probably as an inactive latent complex of a disulfide-bonded carboxy-terminal dimer bound to proteins such as its propeptide and/or follistatin related gene protein (FLRG) (Hill et al. 2002) and/or growth and differentiation factor associated protein-1 (GASP-1) (Hill et al. 2003). These binding proteins of myostatin are assumed to inhibit its signaling. It has been observed that adult mice injected with the propeptide of myostatin show increased muscle mass (Wolfman et al. 2003). Follistatin can also inhibit the activity of myostatin (Lee 2007), but its function is unclear as it was not found to be associated with mature C-terminal myostatin in mouse blood (Hill et al. 2002). Expression of FLRG as a transgene in mice substantially increased muscle size growth (Lee 2007), suggesting that by binding to myostatin FLRG inhibits the activity of myostatin. A soluble form of the activin type IIB receptor has also been shown to cause dramatic increases in muscle mass when injected into wild-type mice (Lee et al. 2005). However, the binding of FLRG, follistatin and the activin IIb receptor does not seem to be specific to myostatin alone; thus other ligands closely related to myostatin and capable of suppressing muscle growth probably have to exist (Lee et al. 2005; Lee 2007). Possible candidates are bone morphogenetic protein 11 and activins A, B and AB (Souza et al. 2008).

Myostatin action is not limited to embryonic development but is also an important regulator of adult muscle mass, as demonstrated in adult mice (Bogdanovich et al. 2002; Whittemore et al. 2003). Interestingly, in these studies increased muscle mass resulted only from hypertrophy of the existing muscle fibers, not from both hyperplasia and hypertrophy, as was the case when myostatin was knocked out before birth (McPherron et al. 1997) or in cattle that had a myostatin mutation (Kambadur et al. 1997; McPherron and Lee 1997).

Therefore, in adults, myostatin may regulate only the size of the existing muscle fibers. Alternatively or additionally, this difference may also be due to the amount of myostatin inhibition as the hindering of myostatin by e.g. neutralizing antibodies is partial and transient compared to completely knocking out the gene before birth. Recently the first experiments in humans have started examining intravenously injectable neutralizing MYO-029 antibody to myostatin and a tendency for increased muscle size after six months of its use have already been shown in patients with muscle dystrophy (Wagner et al. 2008; Krivickas et al. 2009).

Myostatin acts possibly through the paracrine or autocrine mechanisms by re-entry through the cell surface receptors (Lee and McPherron 2001) but also possibly systematically (Gonzalez-Cadavid et al. 1998). Activated proteolytically cleaved C-terminal myostatin is thought to first bind to a type IIb serine/threonine kinase receptor which then recruits a type I receptor (Lee 2004). The type II receptor phosphorylates the type I receptor, leading to the phosphorylation of Smad proteins, which translocate to the nucleus to regulate the gene expression (Rebbapragada et al. 2003) (Figure 5). There seems to be also Smad-independent signaling of myostatin through MAPK (Philip et al. 2005; Yang et al. 2006) and mTOR pathways (Amirouche et al. 2009).

Myostatin appears to work in the regulation of muscle size in adults in part by inhibiting the proliferation and differentiation of satellite cells around terminally differentiated adult myofibers (McCroskery et al. 2003). The effect is probably achieved through regulating cell cycle proteins (e.g. p21, cdk2, cdk4 and cyclin D1) and myogenic regulatory factors (e.g. myogenin and MyoD) (Langley et al. 2002; Rios et al. 2002; Joulia et al. 2003; McCroskery et al. 2003; Yang et al. 2007). The present study focuses on myostatin, its receptor and downstream cell cycle regulators and myogenic transcription factors, with the aim of further understanding the molecular mechanisms behind the adaptive mechanisms to resistance exercise and training in human skeletal muscle.

### 2.3.6 mTOR pathway in regulation of protein synthesis

During translation, genetic code in the form of messenger RNA (mRNA) is converted to protein at the ribosome (Nelson and Cox 2000). In translational regulation of protein synthesis, phosphorylation or dephosphorylation (i.e., addition or removal of phosphate ($PO_4$), respectively) of specific protein kinase enzymes are known to be important (Glass 2005). The phosphorylation of an amino acid side chain is the most common, and probably also most important, mechanism in regulating protein function and therefore in controlling signal transduction pathways in muscle cells (Sefton and Shenolikar 2001).

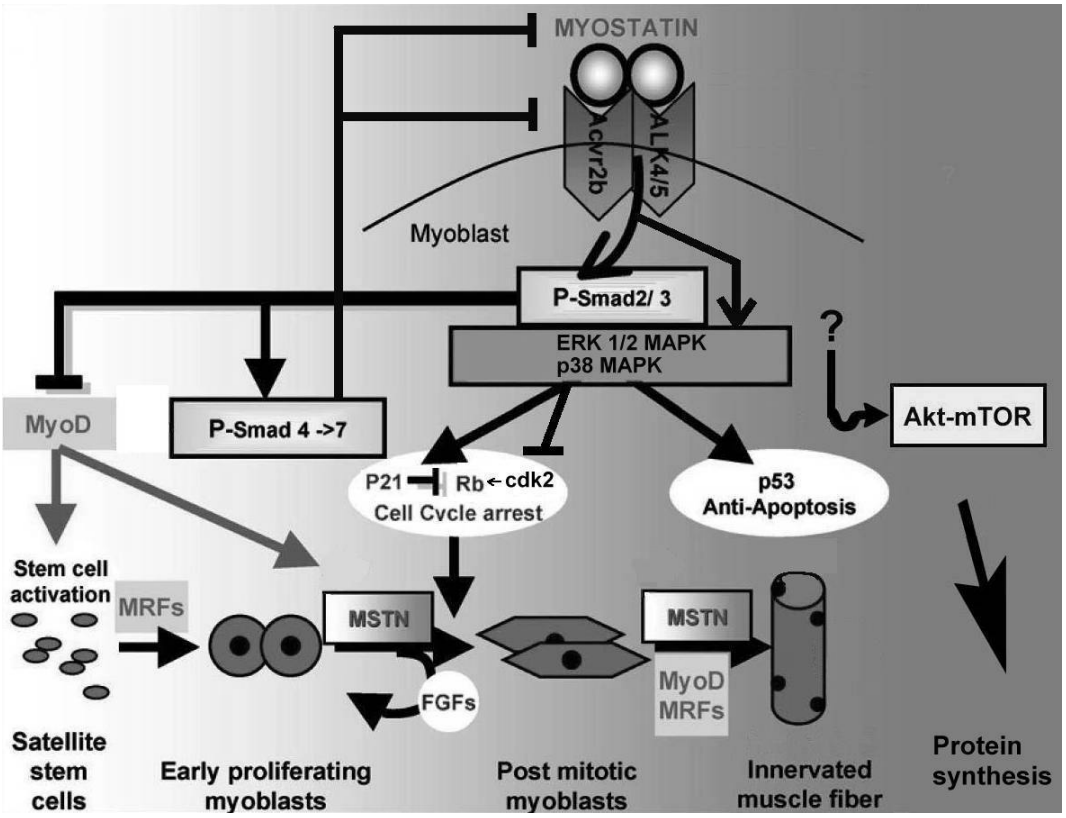


FIGURE 5 Simplified illustration of myostatin (MSTN) mechanism of action. The myostatin pathway is not clear and only some of the suggested pathways are shown. Myostatin signals through activin receptor IIb (Acvr2b) being as a heterodimer with type II receptor ALK 4 or 5. Smads are then activated by phosphorylation. Eventually cell proliferation is arrested and myogenic regulatory factors (MRFs) are inhibited. FGF = fibroblast growth factor. Autoregulatory loop through Smad-7 represses myostatin and its receptors. Arrows indicate activation and T-bars inhibition. The figure is modified from (Bradley et al. 2008), with permission from the Cellular and Molecular Life Sciences.

During translation, it is mostly the amount of total protein synthesis that is regulated. However, a specific groups of proteins can also be selected to translation by selecting the eligible mRNAs (Bolster et al. 2003). Translation of mRNA into protein can be divided into three stages: initiation, elongation and termination. Each of these stages requires many translation factors which are in contact with ribosomes (Proud 2007). Translation initiation is the best known of the three stages and is also the most important in the control of protein synthesis (Proud 2007; Stipanuk 2007). Translation initiation consists of two steps. The first is the formation of a pre-initiation complex where transfer RNA binds to a ribosome, a process regulated in particular by eukaryotic initiation factor 2. The second is the recruitment of a ribosome to mRNA, which is controlled by eukaryotic initiation factor 4 (eIF4) proteins (Proud 2007). Of the eIF4 proteins, eIF4E is the one that binds mRNA and connects mRNA to a ribosome when it is in contact with eIF4G (Proud 2007). Translation can also be repressed by microRNAs which are small ~20 nucleotide-long RNA molecules not translated to protein (Clop et al. 2006).

Many signaling pathways that affect muscle hypertrophy by regulating translation have been identified. Such cascades achieve this affect through e.g. Akt-GSK3, mTOR, ERK-MAPK and calcineurin (Rennie et al. 2004; Glass 2005; Nader 2005; Baar et al. 2006; Vary and Lynch 2007). Of these, the signaling pathway downstream of mTOR has been shown to be especially important (Glass 2005). This pathway, shown as a simplified figure 6 is shortly reviewed below.

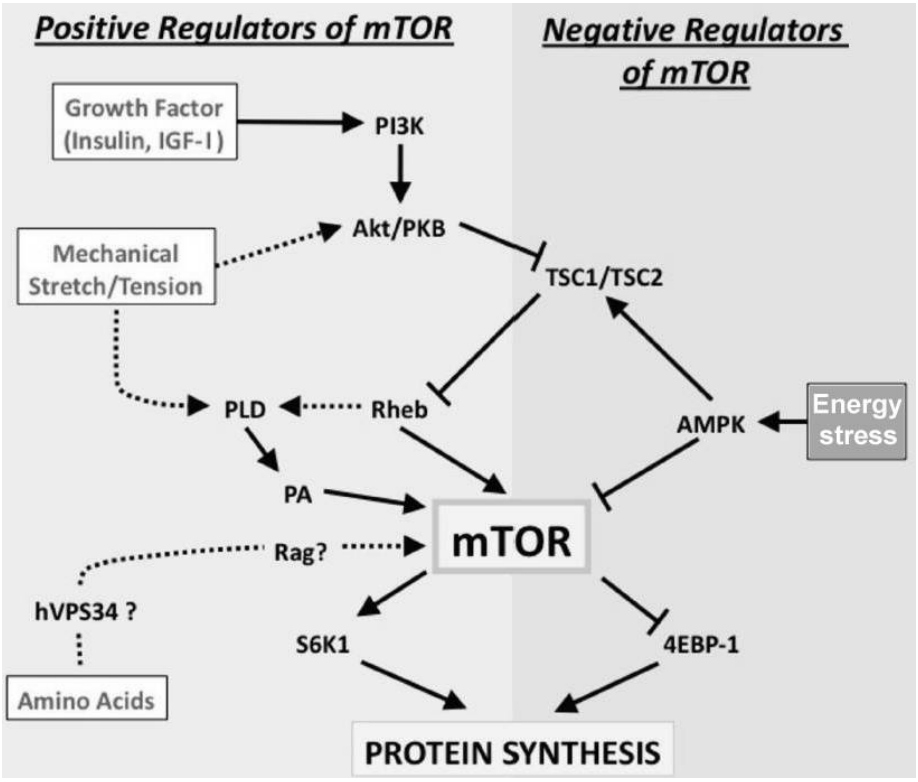


FIGURE 6 A model of a signaling pathway, through which different factors can contribute to mTOR/TORC1 signaling and protein synthesis in skeletal muscle. Activators are written inside the white boxes with black font and inhibitory signal is written inside the gray box with white font. Solid lines depict well studied interactions among molecules while dotted lines are recently suggested interactions. AMPK = 5' AMP-activated protein kinase, the others are mentioned in the text below (Modified from (Miyazaki and Esser 2008)), with permission from the Journal of Applied Physiology.

**Akt.** Protein kinase B (PKB) or Akt is a serine/threonine protein kinase downstream of phosphoinositide kinase-3 (PI3K). Three Akt proteins are known to exist: Akt1, Akt2 and Akt3, of which Akt3 has only low expression in skeletal muscle (Masure et al. 1999). Akt1 is the isoform that increases amino acid transport and protein synthesis (Hajduch et al. 1998). Hereafter, Akt refers to the Akt1 isoform. It is thought that PI3K recruits Akt to the plasma membrane, where Akt is phosphorylated from at least two amino acid sites, $Thr^{308}$ and $Ser^{473}$ (Sarbassov et al. 2005; Frost and Lang 2007). Full activation of Akt probably needs phosphorylation at both of these sites (Alessi et al. 1996). $Thr^{308}$ residue is phosphorylated by PDK1 (Alessi et al. 1997) and $Ser^{473}$ by

mTOR in a complex with the rictor protein (Sarbassov et al. 2005). Akt increases protein synthesis at least by phosphorylating and inactivating downstream substrates glycogen synthase kinase 3 (GSK-3) (Cross et al. 1995) and tuberous sclerosis (TSC) proteins (Inoki et al. 2002), and also possibly by phosphorylating and activating mTOR at $Ser^{2448}$ (Nave et al. 1999). Akt also decreases protein degradation by phosphorylating and thus inactivating the forkhead box O (FoxO) transcription factors. Phosphorylation of FoxOs move them from the nuclei to cytosol rendering them inactive (Stitt et al. 2004).

**mTOR.** Mammalian TOR (target of rapamycin, also called FRAP, RAFT1 and SEP) exists in cells with several regulatory proteins, forming a complex called TORC1 or TORC2. Known regulator proteins in the TORC1 complex are the raptor, Rheb, PRAS40, and LST8/GβL whereas in the TORC2 complex the raptor is replaced by the rictor protein (Kimball 2007). One difference between functions of these complexes is that mTOR seems to phosphorylate 4E-BP1 and $p70^{S6K}$/S6K1 when it is in the TORC1 complex, while when it is in TORC2, it phosphorylates Akt (Inoki et al. 2002; Jacinto et al. 2004; Sarbassov et al. 2004; Sarbassov et al. 2005). However, phosphorylation of 4E-BP1 at some sites may occur also through TORC2 (Wang et al. 2005).

As mentioned above, Akt activates mTOR (Nave et al. 1999). Other molecules that have been shown to bind and/or regulate mTOR signaling include protein kinase C and A, phospholipase C, intracellular calcium, phospholipase D (at least its D1 isoform, PLD1), phosphatidic acid and also $p70^{S6K}$ (Cass et al. 1999; Fang et al. 2001; Willard et al. 2001; Iijima et al. 2002; Fang et al. 2003; Chiang and Abraham 2005; Sun et al. 2008).

**S6K1 / $p70^{s6K}$.** 40S ribosomal protein S6 kinase (S6K1), of size 70 kDa or 85 kD ($70^{S6K}$/$p85^{S6K}$), is a major effector of cell growth. $p70^{S6K}$ is a mitogen activated Ser/Thr protein kinase that is required for cell growth (Pullen and Thomas 1997). It is cytosolic isoform of S6K1 whereas $p85^{S6K}$ is thought to be primarily nuclear (Pullen and Thomas 1997). This difference means that their function probably also differs. $p85^{S6K}$ is derived from the same gene and is identical to $p70^{S6K}$ kinase except for 23 extra residues at the amino terminus (Pullen and Thomas 1997). Both isoforms are downstream of mTOR in the TORC1 complex, a pathway distinct from the Ras/MAP kinase cascade (Pullen and Thomas 1997).

The best-known downstream target of $p70^{S6K}$ is the S6 protein of the 40S ribosomal subunit (rpS6) (Pullen and Thomas 1997). However, many other $p70^{S6K}$ substrates are also known. Of those, eukaryotic elongation factor 2 kinase (eEF2k) and eukaryotic translation initiation factor 4B (eIF4B) may be the most important in relation to protein synthesis (Ruvinsky and Meyuhas 2006). Of the many phosphorylation sites, phosphorylation of $p70^{S6K}$ at $Thr^{389}$ is probably the chief event in the activation of $p70^{S6K}$ (Pullen and Thomas 1997).

Activation of mTOR in the TORC1 complex leads to phosphorylation and activation of $p70^{S6K}$ (Inoki et al. 2002; Jacinto et al. 2004). In mice, S6K1 has been shown to have a positive effect as disrupting the functional S6K1 gene results in small body size (Shima et al. 1998) low muscle mass and smaller cross-sectional

area of muscle fibers (Ohanna et al. 2005). Cell culture experiments further showed that deletion of S6K1 reduced myoblast size to the same extent as that observed with mTOR inhibition by rapamycin (Ohanna et al. 2005) suggesting that the effect of S6K1 on fiber size occurs mostly through TORC1 complex. However, deletion of S6K1 did not have an effect on cell number in contrast to the drug rapamycin that affects mTOR (Ohanna et al. 2005). Therefore, S6K1 is needed for the mTOR effect on cell size but probably not to the effect on cell number. The importance of S6K1 has also been shown in nutrition-induced hypertrophy. Two-days of starvation in mice led to muscle loss in wild-type but not in S6K1-deficient mice (Ohanna et al. 2005). In contrast, two days of refeeding after starvation fully or partially restored muscle mass in the wild-type mice but not in the S6K1-deficient mice. This would suggest that nutrient availability may not be largely responsible for modifying muscle mass when S6K1 is not available (Ohanna et al. 2005).

**rpS6** (ribosomal protein S6) is one of the proteins of the 40S small ribosomal subunit in the higher eukaryotic ribosomes. rpS6 exists both in the nucleus and cytosol (Pende et al. 2004). rpS6 resides in the interface between the two ribosomal subunits 40S and 60S, and interacts with tRNA, the initiation factors and mRNA (Nygard and Nilsson 1990), and therefore it has been suggested that rpS6 binds mRNA and takes part in translation initiation (Ruvinsky and Meyuhas 2006). It has at least five phosphorylation sites of which $Ser^{236}$ is possibly the most important (Flotow and Thomas 1992). In addition to S6K1, S6K2 also phosphorylates rpS6 (Ruvinsky and Meyuhas 2006). Against the earlier hypothesis the rpS6 is responsible for the translation efficiency of mRNAs with a 5'-terminal oligopyrimidine tract, recent evidence suggests that these mRNAs are translationally controlled independently of S6K and rpS6 (Ruvinsky et al. 2005; Ruvinsky and Meyuhas 2006). In fact, the phosphorylation of rpS6 may even be an acute negative regulator of total protein synthesis, at least in some cells, while at the same time it may be a positive regulator of long-term cell size (Ruvinsky and Meyuhas 2006).

**4E-BP1.** Activity of eukaryotic initiation factor eIF4E can be regulated by various eIF4E binding proteins, 4E-BPs (also called PHAS-I). Of those, 4E-BP1 is the best characterized (Kimball and Jefferson 2006). The binding of eIF4E by 4E-BP1 obstructs the interaction of eIF4E with eIF4G, blocking the formation of the translation initiation complex (Mader et al. 1995; Kimball and Jefferson 2006). Upstream of 4E-BP1, mTOR can phosphorylate 4E-BP1, which dissociates 4E-BP1 from eIF4E (Inoki et al. 2002), and therefore increases translation (Pause et al. 1994). Vice versa, dephosphorylation of 4E-BP1 increases association of the inactive complex of 4E-BP1 with eIF4E (Prod'homme et al. 2005). After exercise, formation of this 4E-BP1 complex with eIF4E has been shown to correlate inversely with muscle protein synthesis in contrast to a complex of eIF4E with eIF4G, which correlated positively with protein synthesis (Gautsch et al. 1998). Surprisingly, deletion of 4E-BP1 may not have an effect on muscle mass (Le Bacquer et al. 2007), suggesting that 4E-BP1 may not be mandatory for muscle hypertrophy.

Phosphorylation of 4E-BP1 by mTOR at $Thr^{36/47}$ has recently been shown to be rapamycin insensitive and, therefore, probably occurs through the TORC2 complex (Wang et al. 2005). Vary et al. (2007), however, showed that pretreatment with rapamycin prevented the feeding-induced phosphorylation of mTOR, eIF4G, and S6K1, and also partially attenuated the phosphorylation of 4E-BP1. However, the specific phosphorylation site of 4E-BP1 was not investigated in their study.

**eEF2.** In mammals, the elongation stage of translation requires at least two eukaryotic elongation factors: eEF1 and eEF2. eEF2 is responsible for mediating the translocation step in which ribosome shifts in relation to mRNA (Proud 2007). Phosphorylation of eEF2 by eEF2 kinase interferes with the formation of the complex of eEF2 with ribosome and, therefore, inactivates eEF2 and slows down translation (Ryazanov et al. 1988; Carlberg et al. 1990). In contrast, dephosphorylation of eEF2 restores its activity (Ryazanov et al. 1988). As was mentioned above, $p70^{S6K}$ regulates eEF2 through eEF2 kinase (Ruvinsky and Meyuhas 2006).

### 2.3.7 Muscle satellite cells, cell cycle regulatory factors and myogenic transcription factors

In addition to acute changes in gene transcription and mRNA translation, and therefore to an increase in muscle protein content, muscle fiber DNA content increases during hypertrophy to maintain the capacity of muscle for protein synthesis (Goldberg 1967; Adams and Haddad 1996). Increase in muscle DNA is achieved via the addition of new myonuclei from myogenic stem cells, especially satellite cells, located between the muscle sarcolemma and basal lamina. Satellite cells are needed because muscle fibers are terminally differentiated and therefore incapable of dividing to produce additional DNA to maintain their myonuclear domain size, an important prerequisite for muscle hypertrophy at least in the case of a large increase in fiber area (Allen et al. 1999; Adams et al. 2002; Kadi et al. 2004b; Petrella et al. 2006). In addition to supplying nuclei to muscle fibers to meet their need of higher protein synthesis capacity, satellite cells are also important in muscle fiber repair following muscle damage (Charge and Rudnicki 2004).

*Cell cycle and its regulators.* The cell cycle is series of molecular events that eventually lead to cell division (cytokinesis) and the creation of two identical daughter cells. The cell cycle can be divided into two main stages: interphase and mitosis (somatic cells). The interphase is divided into three stages: RNA and proteins are synthesized in the $G_1$ phase and DNA is synthesised and chromosomes replicated in the S (synthesis) phase. In the $G_2$ phase the cell continues its growth and prepares itself for nuclear division (mitosis). In rapidly replicating human cells, progression through the cell cycle lasts about 24 hours of which mitosis takes only ~30 minutes. Most cells finally exit the cell cycle by entering to the $G_0$ phase (Scott et al. 2004).

One important regulator of the cell cycle is the cyclin protein concentration. Their concentrations change during the progression of the cell

cycle. To be effective enzymes, cyclins need cyclin-dependent kinases (cdks) to be complexed with them. Different cdk-cyclin complexes are expressed in the various phases of the cell cycle (Malumbres et al. 2000; Scott et al. 2004).

*Cell cycle and differentiation in skeletal muscle tissue*. During muscle tissue development, the proliferated mononucleated myoblasts withdraw from the cell cycle and muscle-specific gene expression starts. Eventually, those cells fuse into multinuclear myotubes, which further mature into multinucleated muscle cells (Charge and Rudnicki 2004). The cell cycle and differentiation of satellite cells is regulated, in part, by myogenic transcription factors such as myogenin and MyoD, and by cdks such as cdk2, which in turn are inhibited by cdk inhibitors such as p21 and p27 (Malumbres et al. 2000; Rios et al. 2002; McCroskery et al. 2003). In addition to the satellite cells and some undefined extracellular cells, myogenin and MyoD are also localized in the myonuclei inside the muscle fibers (Ishido et al. 2004; Kadi et al. 2004a).

## 2.4 Resistance exercise induced molecular changes possibly leading to muscle hypertrophy

The increase in muscle protein after a resistance exercise (RE) session is achieved mainly by an increase in muscle protein synthesis, starting from ~1-2 hours after each heavy hypertrophic type of RE bout and remaining elevated ~16-72 hours depending on the exercise protocol and probably also the subject's training status (Chesley et al. 1992; MacDougall et al. 1995; Phillips et al. 1997; Miller et al. 2005; Tang et al. 2008). Some studies suggest that during a RE bout in a fasting state, muscle protein synthesis decreases (Dreyer et al. 2006b; Dreyer et al. 2008) and muscle protein degradation increases (Biolo et al. 1995b; Phillips et al. 1997). However, the long-term fasting situations in the previous studies probably exaggerated this response as ingestion of amino-acid containing nutrition can prevent the decrease or even increase protein synthesis during exercise (Beelen et al. 2008a; Beelen et al. 2008b; Fujita et al. 2008). Resistance loading for some weeks or months can also lead to increased DNA content in muscle fibers during hypertrophy to maintain capacity for protein synthesis (Goldberg 1967; Adams and Haddad 1996) by adding more myonuclei with the aid of muscle myogenic stem cells, especially satellite cells (Allen et al. 1999; Adams et al. 2002; Kadi et al. 2004b; Petrella et al. 2006).

Below, the terms RE bout and RT are used to refer to a bout of heavy hypertrophic type of dynamic resistance exercise or months of training in humans, respectively. In this type of exercise several repetitions are performed in every set in an exercise workout with multiple sets for the muscle group being trained (Tesch et al. 1986; Häkkinen and Pakarinen 1993).

### 2.4.1 Testosterone and androgen receptor

Different short- (Bamman et al. 2001; Willoughby and Taylor 2004; Ratamess et al. 2005; Kraemer et al. 2006) and long-term (Kadi et al. 2000) RE regimens have been shown to affect AR expression at the mRNA and protein levels in the skeletal muscle of young humans, but not in all studies (Kvorning et al. 2007). On the basis of the complex results obtained in the different studies, Ratamess et al. (2005) suggested that AR protein content may initially be down-regulated during or soon after heavy RE due to muscle protein degradation (Biolo et al. 1995b; Phillips et al. 1997), while being up-regulated thereafter on both the AR mRNA and protein levels (Bamman et al. 2001; Willoughby and Taylor 2004). This is, however, only speculation and needs to be proven.

The level of testosterone, the AR ligand in blood, is also affected by exercise. An acute bout of hypertrophic-type RE usually transiently elevates blood testosterone concentrations (Häkkinen and Pakarinen 1993; Chandler et al. 1994; Kraemer et al. 1998; Bloomer et al. 2000; Kraemer et al. 2006).

### 2.4.2 IGF-I

A bout of RE has been shown to increase muscle IGF-IEa mRNA expression in some (Kim et al. 2005b) but not in all studies (Hameed et al. 2003; Psilander et al. 2003). MGF mRNA has been shown to increase more consistantly (Hameed et al. 2003; Kim et al. 2005a; Bamman 2007), although not in all studies (Bickel et al. 2005). Of the IGF-I isoforms, MGF expression in response to a bout of RE or muscle overloading may be blunted with aging (Owino et al. 2001; Hameed et al. 2003). MGF mRNA has been shown to increase after a RE bout earlier than that of IGF-IEa, suggesting that they are differentially regulated after the RE bout (Hameed et al. 2003; Petrella et al. 2006).

### 2.4.3 Gene expression of myogenic and cell cycle regulatory factors in humans

Gene expression of muscle myostatin has been shown either to decrease (Kim et al. 2005a; Coffey et al. 2006a; Raue et al. 2006; Kim et al. 2007; Kvorning et al. 2007; Louis et al. 2007; Mascher et al. 2008) or not to change (Jensky et al. 2007) after a bout of RE. After repeated bouts of RE or long-term RT, myostatin mRNA has been found to decrease (Roth et al. 2003; Costa et al. 2007; Kim et al. 2007), increase (Willoughby 2004), or not change (Kvorning et al. 2007). No changes have been reported for the receptor of myostatin, activin receptor IIb, after a bout of RE or period of RT (Kim et al. 2007).

A bout of RE has been reported to increase the gene expression of myogenin in most (Willoughby and Nelson 2002; Psilander et al. 2003; Bickel et al. 2005; Kim et al. 2005b; Yang et al. 2005; Bamman et al. 2007) but not in all studies (Coffey et al. 2006a). The same is true for MyoD: an increase (Willoughby and Nelson 2002; Psilander et al. 2003; Bickel et al. 2005; Kim et al. 2005b; Yang et al. 2005; Raue et al. 2006) or no change (Coffey et al. 2006a;

Bamman et al. 2007) has been observed. Myogenin mRNA has also been shown to increase after months of RT (Willoughby and Rosene 2003; Bamman et al. 2007) while some have observed no change (Bamman et al. 2003). For MyoD the same is true, a long-term increase (Willoughby and Rosene 2003) or no change (Bamman et al. 2007) has been reported. The myogenin and MyoD mRNA responses may not, however, always lead to an increase in the protein levels of these transcripts (Kosek et al. 2006).

Whereas the cdk2 response to a single bout of RE or long-term RT has not been reported in humans, its regulator p21 mRNA has been shown to either increase (Bickel et al. 2005) or not to change acutely after a RE bout (Kim et al. 2005a; Kim et al. 2007). For p27 mRNA, either a small decrease or no change has been reported after a bout of RE (Kim et al. 2005a; Kim et al. 2007).

The RE-induced changes in the mRNA expression of these and other myogenic and cell cycle regulatory factors may have a complex time course (Psilander et al. 2003; Louis et al. 2007). Therefore, the timing of the biopsy can have a large effect. Another important factor is the 'repeated biopsy effect' that may occur with some proteins such as the myogenic regulatory factors that may be involved in the recovery from biopsy damage (Charge and Rudnicki 2004; Vissing et al. 2005). Therefore, including a control group is always important in muscle biopsy designs to see whether the observed result is really due to the administered treatment.

### 2.4.4 Phosphorylation of mTOR-pathway protein kinases in humans

**Sensing of the mechanical loading.** Currently, the mechanisms that sense mechanical loading and its conversion to a chemical signal is uncertain, but some effectors have been suggested. Activation of mTOR after loading is probably mostly PI3K-Akt independent because PI3K inhibitor worthmannin does not inhibit mechanically induced phosphorylation of $p70^{S6K}$ at $Thr^{389}$, a protein downstream of mTOR (Hornberger et al. 2004; Hornberger and Chien 2006). Inhibition of the phospholipase D (PLD) enzyme, and, therefore, a decrease in its reaction product phosphatidic acid (PA), blocked mechanically induced mTOR activation (Hornberger et al. 2006) (Figure 5). This suggests that PLD and its function of hydrolyzing phosphatidylcholine to generate PA (and choline) (Jenkins and Frohman 2005) is probably an important upstream mediator of mTOR signaling in RE. Rapamycin is a drug that has been shown to inhibit TORC1 signaling. Experiments with rapamycin have shown that mechanically induced phosphorylation of $p70^{S6K}$ at $Thr^{389}$ needs functional mTOR associated with the protein raptor and, therefore, a TORC1 complex (Bodine et al. 2001b; Hornberger et al. 2004). Spangenburg et al. (Spangenburg et al. 2008) created a knock-out mouse line with muscles that cannot respond to IGF-I, at least through IGF-I receptors. The result showed that loading of those muscles increased mTOR signaling, and also muscle hypertrophy, suggesting that IGF-I signaling through its receptors is not a limiting upstream regulator of mTOR signaling. However, intracrine signaling by local muscle IGF-IEa and/or MGF cannot be excluded.

**Exercise-induced responses. Akt.** Conflicting information is available on Akt. Blomstrand et al. (2006) found that a single RE session decreased the phosphorylation of Akt at $Ser^{473}$ immediately after exercise while at 1h and 2h postexercise there was no change compared to the pre-values. Other studies have shown either a decrease (Deldicque et al. 2008; Terzis et al. 2008), increase (Dreyer et al. 2006b; Wilkinson et al. 2008), or no change (Coffey et al. 2006b; Eliasson et al. 2006; Mascher et al. 2008) in Akt phosphorylation at $Ser^{473}$ from 0 to 2 h postexercise in the absence of nutrient supply. When nutrition was supplemented (Cuthbertson et al. 2006), or the exercise was performed in a state with full muscle glycogen (Creer et al. 2005), an increase in the phosphorylation of Akt was seen within 3h after exercise. Also, Akt phosphorylation at $Thr^{308}$ has been studied, and both a decrease (Deldicque et al. 2008) and no change (Coffey et al. 2006b) has been reported after a bout of RE. RT for 8-10 weeks increased the phosphorylation of Akt at $Ser^{473}$ (Leger et al. 2006; Wilkinson et al. 2008).

**mTOR.** A bout of RE has been shown both to increase (Dreyer et al. 2006b; Mascher et al. 2008; Terzis et al. 2008; Wilkinson et al. 2008) or not to change (Karlsson et al. 2004; Creer et al. 2005; Glover et al. 2008) the phosphorylation of mTOR at $Ser^{2448}$. Also, an increase in phosphorylation at $Ser^{2481}$ has been reported (Mascher et al. 2008). RT for 8-10 weeks has been shown to increase (Leger et al. 2006) or not to change (Wilkinson et al. 2008) the phosphorylation of mTOR.

**$p70^{S6K}$.** A single RE session has been shown to increase the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ in a large number of studies (Coffey et al. 2006b; Dreyer et al. 2006b; Dreyer et al. 2008; Glover et al. 2008; Mascher et al. 2008; Terzis et al. 2008; Wilkinson et al. 2008), and also at $Ser^{424}/Thr^{421}$ (Karlsson et al. 2004; Koopman et al. 2006; Koopman et al. 2007; Deldicque et al. 2008; Mascher et al. 2008; Terzis et al. 2008), from 0 to 3h postexercise, even in the absence of protein supply. However, some studies have shown no changes in phosphorylation at $Thr^{389}$ (Karlsson et al. 2004; Koopman et al. 2007; Deldicque et al. 2008). A longer period of RT may not change the phosphorylation of $p70^{S6K}$ as it has remained at resting levels after 8-10 weeks of RT (Leger et al. 2006; Wilkinson et al. 2008).

**rpS6.** A single eccentric-concentric bout of RE has been shown to increase (Glover et al. 2008; Mascher et al. 2008), to decrease (Koopman et al. 2007), or not to change (Karlsson et al. 2004; Koopman et al. 2006) the phosphorylation of rpS6 at $Ser^{235/236}$. An increase in phosphorylation has been reported also at $Ser^{240/244}$ of rpS6 (Glover et al. 2008).

**4E-BP1.** A bout of RE has been shown to decrease the phosphorylation of 4E-BP1 at $Thr^{37/46}$ (Dreyer et al. 2006b; Koopman et al. 2006; Deldicque et al. 2008). A longer period of RT such as 8 weeks may not change the phosphorylation of 4E-BP1 (Leger et al. 2006). For the phosphorylation of **eEF2** at $Thr^{56}$ after a bout of RE, some studies have shown a decrease (Dreyer et al. 2006b; Mascher et al. 2008), and some no change (Deldicque et al. 2008).

The eccentric component of the movement may be especially important in the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ and at $Ser^{424}/Thr^{421}$ as well as rpS6 at

$Ser^{235/236}$, at least *in the absence of added nutritional supply* (Eliasson et al. 2006). The phosphorylation of mTOR at $Ser^{2448}$ (Eliasson et al. 2006) and Akt at $Ser^{473}$ (Cuthbertson et al. 2006; Eliasson et al. 2006) may not be differentially affected by the contraction mode. However, when nutrition containing amino acids is provided after exercise, the contraction mode may not have significant effect on the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ (Cuthbertson et al. 2006). For many of the phosphorylations mentioned above, training state also may affect the exercise response (Coffey et al. 2006b; Wilkinson et al. 2008).

## 2.5 Resistance exercise with protein nutrition and the molecular changes important in muscle hypertrophy

### 2.5.1 Testosterone and androgen receptor

An acute bout of RE transiently elevates the blood testosterone concentration, but this response seems to be attenuated with post-exercise feeding, at least in young men (Chandler et al. 1994; Kraemer et al. 1998; Bloomer et al. 2000; Kraemer et al. 2006), and may especially be due to protein (Chandler et al. 1994). However, more evidence is needed. The mechanisms underlying this response are unknown, but the decrease could be due to a reduction in the synthesis/secretion of testosterone and/or an increase in metabolic clearance such as uptake to the muscle. Nutrition may also affect the ARs since feeding after a RE bout has been shown to increase AR protein content in young men (Kraemer et al. 2006). However, the effects of protein ingestion *per se* are not known.

### 2.5.2 IGF-I

High caloric and protein intake combined with a RE bout may stimulate circulating IGF-I concentration that originates from the liver (Kraemer et al. 1998); however, it is unclear whether a similar effect exists for the local muscle IGF-I expression. A study conducted in a resting state showed that seven days of high-protein diet increased muscle IGF-I mRNA expression (Harber et al. 2005). Furthermore, 20 g of protein consumed before and after each RE workout during 10 weeks of RT resulted in larger IGF-I mRNA compared to carbohydrate placebo (Willoughby et al. 2007). The effects of protein ingestion on acute IGF-I expression after a single bout of RE is not known.

### 2.5.3 Gene expression of myogenic and cell cycle regulatory factors

No published myostatin studies exist on the effects of nutrient ingestion in the context of a RE bout. However, in the resting state, myostatin mRNA decreased after feeding a mixed meal in old men and women (Smith et al. 2008). In various animal species and study settings, the effects of different nutritional

protocols for the expression of myostatin have yielded very contradictory results (Carlson et al. 1999; Jeanplong et al. 2003; Guernec et al. 2004; Nakazato et al. 2006; Terova et al. 2006).

Of the myogenic transcription factors, myogenin mRNA increased after refeeding in fish (Chauvigne et al. 2003). *In vitro* findings also showed that feeding increased myogenin protein levels in satellite cells in culture when compared to a food-deprived state (Halevy et al. 2003). Lack of evidence exists for the other myogenic or cell cycle regulatory factors and nutrition and for all of these regulators in the context of RE and protein nutrition.

### 2.5.4 Phosphorylation of mTOR-pathway protein kinases in humans

**Sensing of amino acids.** Similarly to mechanical loading, the mechanisms underlying the sensing of amino acids are uncertain but some effectors have been suggested. Some amino acids can activate mTOR signaling pathway kinase $p70^{S6K}$ through mTOR in the TORC1 complex (Kimball et al. 1999; Hornberger and Chien 2006), but independently of TSC1/2 (Smith et al. 2005). Unlike insulin, amino acids do not activate mTOR in the TORC1 complex through PI3K and Akt (Kimball et al. 1999; Hornberger and Chien 2006). However, amino acids that can increase serum insulin concentration (van Loon et al. 2000), may possibly also do so partly through PI3K and Akt.

The upstream mechanism activating TORC1, at least in the case of some amino acids, may operate through increasing intracellular $Ca^{2+}$, which in turn activates kinase hVps34 (human vacuolar protein sorting 34) (Gulati et al. 2008) (Figure 5). hVps34 is a class III PI3K, differing from the class I PI3K that is activated by insulin (Gulati and Thomas 2007). The stimulation of mTOR by amino acids may utilize Rag GTPases (Kim et al. 2008; Sancak et al. 2008). Rag proteins promote, in an amino acid-sensitive manner, the localization of mTOR to a compartment that also contains Rheb, which is an activator protein of mTOR (Sancak et al. 2008).

**Resistance exercise-induced responses with protein or amino acid ingestion.** Ingestion of branched chain amino acids (BCAA) during and after a RE bout has been shown to increase the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ and at $Ser^{424}/Thr^{421}$ as well as rpS6 at $Ser^{235/236}$ at 1 and 2h post-RE compared to non-energetic placebo (Karlsson et al. 2004). Furthermore, at 1h after the same exercise bout, the phosphorylation of mTOR at $Ser^{2448}$ was increased with BCAA more than with placebo, while Akt at $Ser^{473}$ and GSK-3 at $Ser^{9}$ were unaffected by BCAA ingestion (Blomstrand et al. 2006). Protein intake together with carbohydrate before, during and 1h after a RE bout increased the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ and rpS6 at $Ser^{235/236}$ at post 0 to 4h compared to carbohydrate only and reduced the decrease in the phosphorylation of 4E-BP1 at $Thr^{37}$ immediately post-RE but did not affect the phosphorylation of $p70^{S6K}$ at $Thr^{421/424}$ (Koopman et al. 2007).

The addition of carbohydrates may also have an effect. Dreyer et al. (2008) studied the effects of essential amino acid ingestion with carbohydrates (EAA+CHO) 1h after a RE bout. EAA+CHO increased, unlike BCAA, as noted

earlier (Blomstrand et al. 2006), also the phosphorylation of Akt at $Ser^{473}$ measured 2h post-RE. In agreement with the previous study (Blomstrand et al. 2006), the phosphorylation of mTOR at $Ser^{2448}$ was also increased as was the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ when EAA+CHO was ingested. EAA+CHO also increased the phosphorylation of 4E-BP1 at $Thr^{37/46}$ but did not affect eEF2 phosphorylation at $Thr^{56}$ (Dreyer et al. 2008).

Intake of EAA+CHO 1h *before* a RE bout when compared to the fasting situation increased the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ and 4E-BP1 at $Thr^{37/46}$ at 0 to 1h post-RE and seemed to decrease the phosphorylation of eEF2 at $Thr^{56}$ immediately pre- as well as post-RE (Fujita et al. 2008). Unfortunately the study was not double-blinded as is the case with all the other studies comparing feeding and fasting. Lack of a placebo group also limits the study by Glover et al. (2008). In that study, the subjects remained either in the fasted state or ingested 10 g of protein and 41 g of CHO at 1.5, 3 and 4.5 h after the RE bout, and the biopsy was taken at 6h post-RE. The combination of exercise and nutrition increased the phosphorylation of $p70^{S6K}$ at $Thr^{389}$ and rpS6 at $Ser^{240/244}$ above that of exercise in the fasting state. While intact protein sources such as whey have gained widespread popularity, the effects of intact protein sources alone on mTOR signaling pathway in human muscle, and especially in the longer term after a bout of RE, i.e., from 12-72 hours, or after months of resistance training, are unknown. Therefore there is a need to investigate these responses to whey protein with a controlled, double blind design. Importantly, the present study compared the effects of protein ingestion with the placebo instead of comparing feeding and fasting situations when seeking to clarify the molecular mechanisms responsible for resistance exercise-induced muscle hypertrophy.

# 3 PURPOSE OF THE STUDY

The overall purpose of the present research was to obtain new information about the mechanisms leading to muscle hypertrophy in men by studying systemic hormonal and local molecular responses both to a bout of heavy resistance exercise and to long-term systematic resistance training with or without protein supplementation. Both young and older men were examined to elucidate a possible role of age on training and protein nutrition responses. Furthermore, the study was designed to clarify the significance of training status on the responses of muscle to an acute bout of resistance exercise in humans.

The specific aims of the present studies were as follows:

1) To investigate the role of protein ingestion in the context of a bout of heavy resistance exercise (RE) on acute systemic hormonal responses in young and older men. The primary hypothesis was that ingestion of protein before RE bout affects blood insulin and testosterone responses during or immediately after exercise (Original papers I, III, IV, VI-VII).

2) To investigate the effects of whey protein ingestion immediately before and after a bout of heavy RE on acute RE-induced gene expression of myostatin, cell cycle and myogenic regulatory factors and androgen receptors (Original papers III-VI). The study also investigated responses and adaptation after 21 weeks of resistance training (RT). The main hypothesis was that whey protein enhances muscle hypertrophy and modifies the gene expression of myostatin and cell cycle proteins following RE and RT (Original papers V-VI).

3) To investigate the role of whey protein ingestion in the context of a RE bout and long-term training on mTOR pathway protein kinase signaling. The main hypothesis was that whey protein strengthens the mTOR signaling responses to a RE bout but that the longer term effects after the training period are small (Original paper VI).

4) To investigate whether the acute molecular responses to a bout of RE are different after long-term training period. The main hypothesis was that for some but not all acute gene expression responses to resistance exercise there are differences depending on the training state of the subject (Original papers II and VII).

# 4 RESEARCH METHODS

## 4.1 Subjects

The study involved both young and older male subjects. The subjects were recruited for the study by advertising in newspapers and through email lists. Physical characteristics of the subjects are presented in table 1.

TABLE 1 Physical characteristics of the subjects (mean ± SD)

| Original paper | Groups | Age (years) | Height (m) | Weight (kg) | Body fat (%) | RT experience |
|---|---|---|---|---|---|---|
| I | C-O: n=10 | 23.9 ± 2.0 | 1.78 ± 0.04 | 78.0 ± 6.0 | 15.9 ± 3.0 | 6 ± 2 years |
| II | RT: n=11 | 60.9 ± 5.0 | 1.77 ± 0.03 | 80.0 ± 1.6 | 20.3 ± 1.4 | - |
| | Cont: n=11 | 63.9 ± 7.4 | 1.72 ± 0.07 | 77.0 ± 14.2 | 18.6 ± 7.7 | - |
| III, IV | Protein: n=9 | 61.4 ± 4.3 | 1.76 ± 0.06 | 85.8 ± 9.4 | 24.0 ± 4.1 | 0.5 years |
| | Placebo: n=9 | 62.1 ± 4.2 | 1.77 ± 0.03 | 79.6 ± 3.2 | 22.1 ± 12.6 | 0.5 years |
| | Cont: n=6 | 66.5 ± 7.1 | 1.70 ± 0.07 | 72.1 ± 9.8 | 19.3 ± 2.8 | - |
| V | Protein: n=11 | 25.2 ± 5.2 | 1.82 ± 0.06 | 76.1 ± 7.6 | 17.0 ± 3.7 | - |
| | Placebo: n=10 | 27.2 ± 3.0 | 1.81 ± 0.06 | 74.7 ± 8.5 | 16.9 ± 4.4 | - |
| | Cont: n=10 | 24.9 ± 2.7 | 1.83 ± 0.05 | 75.5 ± 8.1 | 17.3 ± 3.8 | - |
| VI | Protein: n=9 | 24.7 ± 5.0 | 1.82 ± 0.07 | 76.7 ± 8.1 | 16.8 ± 4.0 | - |
| | Placebo: n=9 | 27.4 ± 3.1 | 1.81 ± 0.06 | 75.9 ± 8.0 | 17.3 ± 3.9 | - |
| | Cont: n=10 | 25.2 ± 2.7 | 1.82 ± 0.05 | 74.5 ± 7.8 | 16.6 ± 3.5 | - |
| VII | Y RT: n=7 | 27.6 ± 5.1 | 1.85 ± 0.03 | 78.6 ± 6.4 | 16.9 ± 4.0 | - |
| | Y Cont: n=10 | 25.2 ± 3.0 | 1.83 ± 0.04 | 75.7 ± 10.8 | 16.3 ± 4.3 | - |
| | O RT: n=10 | 61.0 ± 5.3 | 1.77 ± 0.03 | 79.6 ± 5.5 | 23.6 ± 2.7 | - |
| | O Cont: n=8 | 63.6 ± 8.0 | 1.72 ± 0.08 | 76.4 ± 14.3 | 23.1 ± 3.2 | - |
| | Y SM: n=8 | 28.8 ± 6.9 | 1.81 ± 0.04 | 87.9 ± 12.3 | 16.4 ± 2.8 | 6 ± 3 years |

Y = young, O = older, C-O = cross-over experiment with protein (n = 10) and placebo (n = 10). RT = group with 21-wk resistance training, Cont = control group, SM = strength-trained men

All the subjects went through medical examination (in older men also resting and exercise electrocardiogram recording) and none of them had contraindications to perform heavy resistance training (RT). None of the

subjects had a regular ingestion of nutritional supplements or pharmacological substances that might affect the measured variables. The subjects were moderately active. Their normal habitual activities included walking, jogging, swimming or ball-games. In the training studies, none of the subjects had any regular RT experience. The subjects were carefully informed about the design of the study with special information on the possible risks and discomfort that might result. Thereafter, the subjects signed the written informed consent form to participate in the study, which had been approved by the ethics committee of the University of Jyväskylä, Finland. The study was conducted according to the Declaration of Helsinki.

## 4.2 Experimental design and data collection

The present designs included both acute heavy resistance exercises (RE) and long-term training periods. The total duration of the training studies was 23 weeks from which the first two weeks was a control period in which no experimental RT was carried out but the subjects maintained their normal recreational activities. Part of the studies also included protein ingestion before and after a RE workout. A control group was included and all the measurements (within the subjects) were always carried out at the same time of day to exclude the effects of biopsy sampling or effects of time of year or daily variations (Vissing et al. 2005; Sedliak et al. 2007). All the measurements were preceded by at least two days of rest from physical activity. For studies III and IV, the subjects had trained with supervision heavy whole-body RT for 21 weeks, two times per week. Two weeks after the training, a subsample (age 57-72 years) was identified who were willing to take part in that study. These subjects were matched according to age, body mass and maximal 1RM strength, and then randomly assigned to the groups (table 1). Thus, previous training experience during the previous months was highly standardized among subjects. To study the effects of several years of systematic RT on IGF-I and AR expression, eight recreationally strength trained men (n = 8) were also recruited to study VII. Experimental designs are summarized in table 2 and figure 7.

### 4.2.1 Experimental acute resistance exercise session

In studies II-VII, a bilateral leg press machine (David 210, David Fitness and Medical, Finland) (Figure 8) was used for the single heavy RE bout with similar protocols as described in earlier studies (Häkkinen et al. 2001b). The total number of sets for the leg press was five (each containing 10 repetition maximums). Recovery time between sets was two minutes. In study VII, in addition to leg presses in strength trained men, the RE bout included also four sets of squats from a knee angle of 70° to 180° with a two-minute recovery between the sets and four minutes between the exercises. In these strength trained men the RE bout was performed only once. In study I, RE bout

consisted of five sets of 1 RM squats (2 min recovery between sets), three sets of 10 RM squats (3 min recovery) and four sets of 10 RM leg presses (2 min recovery). The squats were performed in a Smith machine where the barbell moves up and down without any frontal or lateral movement. The depth of the movements was individually controlled for the leg presses and the squats.

TABLE 2 Summary of the experimental designs, acute and long-term exercise loadings, protein ingestion and primary variables measured

| **Orig. paper** | **Acute RE** | **Long-term RT** | **Protein (against placebo)** | **Primary variables** |
|---|---|---|---|---|
| I | 5x1 RM and 3x10 RM squat and 4x10 RM leg press | - | 25 g whey and caseinate | Serum: testosterone, insulin |
| II | 5x 10 RM leg press | 21-wk RT | - | 1) TB m. mass (BI)<br>2) m. mRNA: myostatin, AcvrIIb, myogenin |
| III | 5x 10 RM leg press | - | 15 g whey before and after RE | m. mRNA: cdk2, myostatin, FLRG |
| IV | 5x 10 RM leg press | - | 15 g whey before and after RE | 1) m. protein: AR<br>2) m. mRNA: AR, MGF, IGF-IEa<br>3) m. IH: AR<br>4) serum: testosterone |
| V | 5x 10 RM leg press | 21-wk RT | 15 g whey before and after acute RE bout and each RE workout during RT period | 1) m. CSA (MRI)<br>2) m. strength<br>3) m. mRNA: cdk2, myostatin, AcvrIIb, myogenin, MAFbx |
| VI | 5x 10 RM leg press | 21-wk RT | 15 g whey before and after acute RE bout and each RE workout during RT period | 1) m. thickness (US) and fiber size<br>2) m. phospho- and total proteins: $p70^{S6K}$, mTOR, 4E-BP1, rpS6, Akt, myostatin<br>3) serum: testosterone, insulin |
| VII | 5x 10 RM leg press<br><br>In SM, 5x 10 RM leg press and 4x10 squat | 21-wk RT | - | 1) m. thickness (US) and fiber size<br>2) m. protein: AR<br>3) m. mRNA: AR, MGF, IGF-IEa<br>4) serum: testosterone |

Abbreviations. m. = muscle, RE = resistance exercise bout, RT = resistance training, mRNA = messenger RNA from skeletal muscle biopsy, TB = total body, BI = bioelectrical impedance. AcvrIIb = activin receptor IIb, AR = androgen receptor, IH = immunohistochemistry, FT = fiber-type, CSA = cross-sectional area, MRI = magnetic resonance imaging, US = ultrasound, SM = strength-trained men

The first set started with the 75% 1RM load. The loads in all the studies were adjusted during the course of the RE bout due to fatigue so that each subject

would be able to perform 10 repetitions at each set. If the load was too heavy, the subject was assisted slightly during the last repetitions of the set. In these assisted repetitions, the assistant tried to maintain the same velocity of movement (and thus contraction time) as in the first repetitions of the set. The same assistant was in attendance at every RE performed.

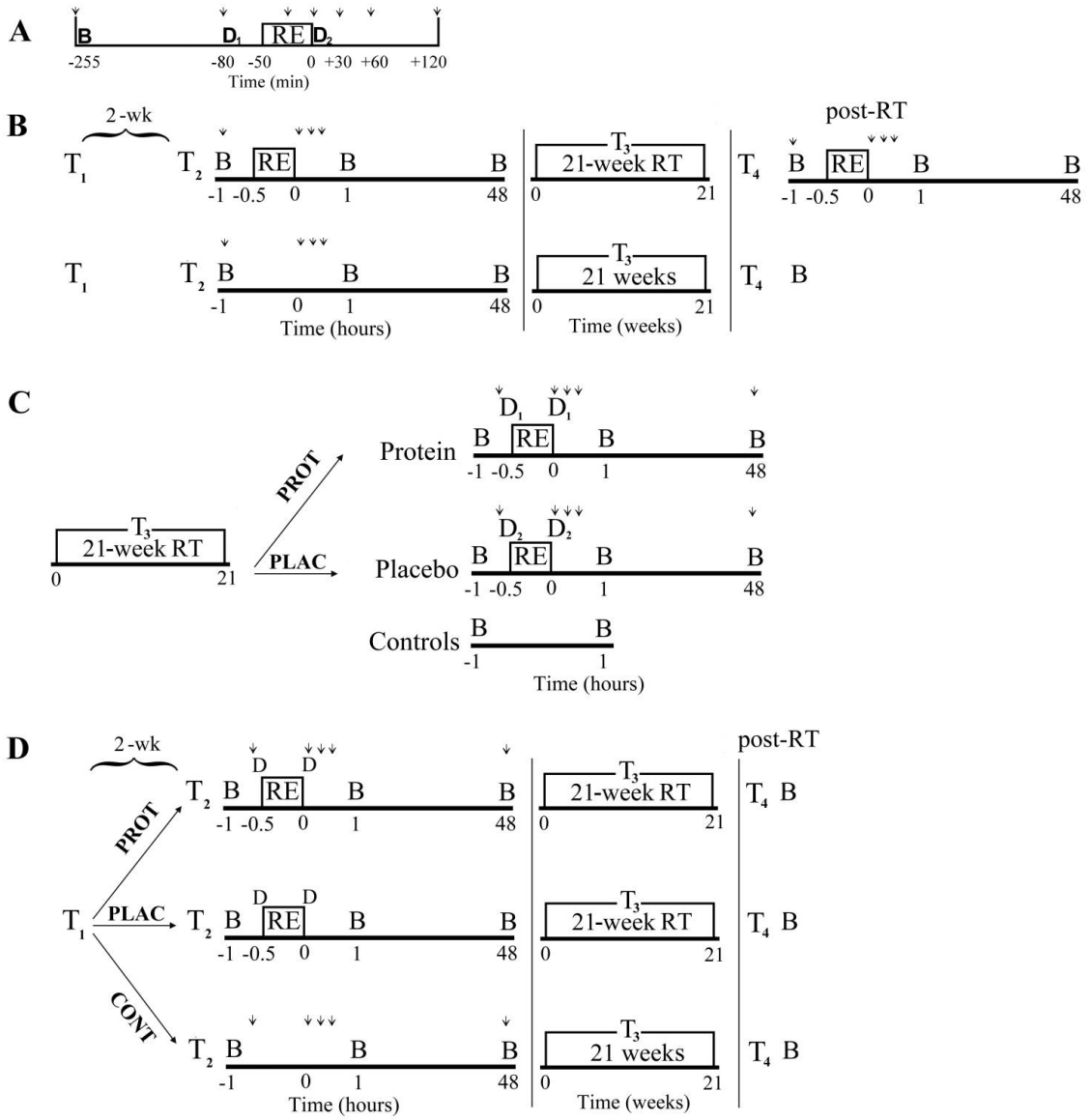


FIGURE 7 Experimental design. A) Cross-over design with either placebo or protein (25 g) ingested before the RE bout ($D_1$) (study I). $D_2$ was in both protein and placebo conditions 25 protein and 50 g carbohydrates. B) Design investigating the effects of the training state on acute RE-induced responses in older (II) and young and older men (VII). In strength trained men the acute RE bout (not depicted) was only once and biopsies were not taken at post 48h. C) After 21 weeks of training some subjects in a larger study design were matched to protein or placebo groups (III, IV). D) Long-term study investigating the effects of whey protein ngestion in the young men with acute RE bout before the 21-week training period (V, VI). B = biopsy, D = protein (15 g) or placebo drink, arrow = blood sample, T = muscle force or anthropometry testing at baseline ($T_{1/2}$) or after 10,5 or 21 weeks of the experimental time period ($T_{3/4}$).


FIGURE 8 Experimental resistance exercise bout with David 210 system. Concentric leg-extension 1RM was tested with this device.

### 4.2.2 Experimental resistance training period (Papers II, V-VII)

During the 21-week RT period, total-body heavy RE workouts were carried out twice a week. A minimum of two days of rest was required between the two sessions each week. All training sessions were supervised by experienced trainers making sure that proper techniques and progression was used in each exercise. The training program followed the recommendations of the ACSM position stand (Kraemer et al. 2002). The training program was especially focused on knee extensors since the analysis of muscle cross-sectional area and muscle biopsies were obtained from a knee extensor muscle (i.e.,vastus lateralis). The following exercises were used in each training session: two exercises for the knee extensor muscles, bilateral leg press and bilateral knee extension and one exercise for the leg flexors, bilateral knee flexion. The RT program also included exercises for the other main muscle groups of the body: chest and shoulders (bench press), upper back, trunk extensors and flexors, upper arms, ankle extensors, and hip abductors and adductors.

The first two exercises in each workout were always the leg press and bench press. Recovery between the sets was 2-3 minutes. RT was performed with progressive training loads of 40 to 85% of the subject's 1RM in a periodized training program. The number of sets of each exercise during RE workout increased (from 2-3 to 3-5) and the number of repetitions in each set decreased (15-20 to 5-6) during the 21-week RT period. The loads were individually determined throughout the RT period. More specifically, RT

consisted of three specific seven-week training periods aiming: 1) to familiarize to REs and to especially improve muscle strength endurance, 2) to concentrate on increasing muscle size and 3) to optimize gains in maximal strength while still increasing muscular hypertrophy. The last exercise workout was performed with 10% lighter loads because of the following post-RT measurements.

### 4.2.3 Protein supplements and supplementation

*Nutritional supplementation during resistance training* (V, VI). The subjects ingested immediately before *and* after each RE workout in the gym either 15 grams of whey isolate protein with minimal lactose and fat (Protarmor 907 LSI, Armor Proteins, Brittany, France) dissolved in 250 ml of water or an equivalent volume of non-energetic placebo. The reason for selection of a non-energetic placebo drink instead of iso-caloric carbohydrate drink was because the carbohydrate *per se* has also many effects on many of the studied variables. The drinks were provided for the subjects in a double-blind fashion. The drinks were made in our own laboratory by the personnel who coded the drinks for the training supervisors. The whey protein drink (15 grams) consisted of following essential amino acids: histidine (0.2 g), isoleucine (1.0 g), leucine (1.7 g), lycine (1.4 g), methionine (0.4 g), phenylalanine (0.5 g), threonine (1.0 g), tryptophan (0.2 g) and valine (0.8 g). These amino acids are important in activating the muscle protein synthesis (Borsheim et al. 2002). The similar supplement has been previously shown to rapidly absorb and increase blood amino amino acid concentrations in humans (Boirie et al. 1997; Dangin et al. 2001; Dangin et al. 2002), and also with a comparable speed to amino acids supplemented as a free form (Boirie et al. 1997; Mero et al. 2008; Mero et al. 2009).

Drinks contained exotic fruit, trinatriumsitrate, acesulfame-K, xanthane gum and betacarotene for flavor, viscosity and color, and tasted as identical as possible. In investigating the long-term effects of the protein (V, VI), the subjects in either protein or placebo group did not eat anything 1h before and 0.5h after experimental exercise workouts during RT period.

*Nutritional supplementation before and after a single bout of RE* (I-II-VI). In studies II-VII, the subjects fasted for three hours before the first biopsy. Either a 250 ml whey protein isolate containing 15 g of whey protein or an equivalent volume of placebo was ingested immediately before and after the bout of RE. Details of the drinks were explained above. For study I, the subjects ate a standardized breakfast (500 kcal: 21% protein, 66% carbohydrate and 13% fat) in the morning after the fasting blood samples. Thirty minutes before the RE bout, either a 500 ml whey-caseinate supplement (Härmä Food Ltd) or an equivalent volume of identical looking and tasting placebo was ingested in a randomized cross-over design separated by at least 7 days. The placebo provided only minimal calories, but the protein supplement was composed of 5.0 grams of whey protein hydrolysate, 12.5 grams of whey protein isolate and 7.5 grams of calcium-caseinate. In both conditions a protein-carbohydrate drink (the same 25 g of protein as the pre-drink and also 50 g carbohydrates: 35 g

maltodextrin and 15 g saccarose) was ingested immediately after the post-RE blood sample to mimic the common practice and recommendations for resistance-trained young men (Kerksick et al. 2008).

### 4.2.4 Measurements

For more details of the methods, such as coefficients of the variation and correlation coefficients for the reliability analysis of the methods, the original articles (I-VII) should be consulted.

#### 4.2.4.1 Muscle biopsy (II-VII)

Muscle biopsies were obtained 0.5h before and 1h and 48h after the RE session as well as in the training studies 4-7 days after the last RE workout from the 21 weeks of RT period. Studies II and VII also included a post-training RE bout and its 1h and 48h post-biopsies (Figure 7).

The post 1h biopsy time-point was selected to represent fast responses of the RE bout and the post 48h time-point the more delayed responses. The effects of the last exercise workout and the protein ingestion on the post-RT biopsy were minimized by obtaining the biopsy after RT 4-6 days after the last exercise workout. Biopsies were taken from the VL muscle with a 5-mm Bergström biopsy needle technique (Bergström and Hultman 1966) after local subcutaneous anaesthetics (lidocaine-adrenalin), midway between the patella and greater trochanter (Figure 9). The pre-RE biopsy and the 48h post-RE and post 21-week biopsies were taken from the same leg. The 48h biopsy was taken 2 cm above the previous biopsy location to minimize the effects of the pre-biopsy. The muscle sample was cleaned of any visible connective and adipose tissue as well as blood and thereafter frozen immediately in liquid nitrogen (-180°C) and stored at -80°C. The pre 21-week and post 21-week samples for immunohistochemical analysis were obtained with another needle and they were immediately mounted on a cork, and frozen rapidly in isopentane cooled to -160°C in liquid nitrogen and thereafter stored at -80°C.

#### 4.2.4.2 Blood sampling (I, III-IV, VI-VII)

Blood samples were drawn from an antecubital vein using 21-gauge disposable needles. For study I, blood was obtained before, during (after squats) and after the bout of RE. For the others, blood was obtained before and after the RE bout (Figure 7).

Blood was centrifuged (3500 RPM and 4°C for 10 min). Blood samples were kept frozen at -20°C until analysed the same day, except samples for hormones which were stored at -80°C and analysed at a later date.

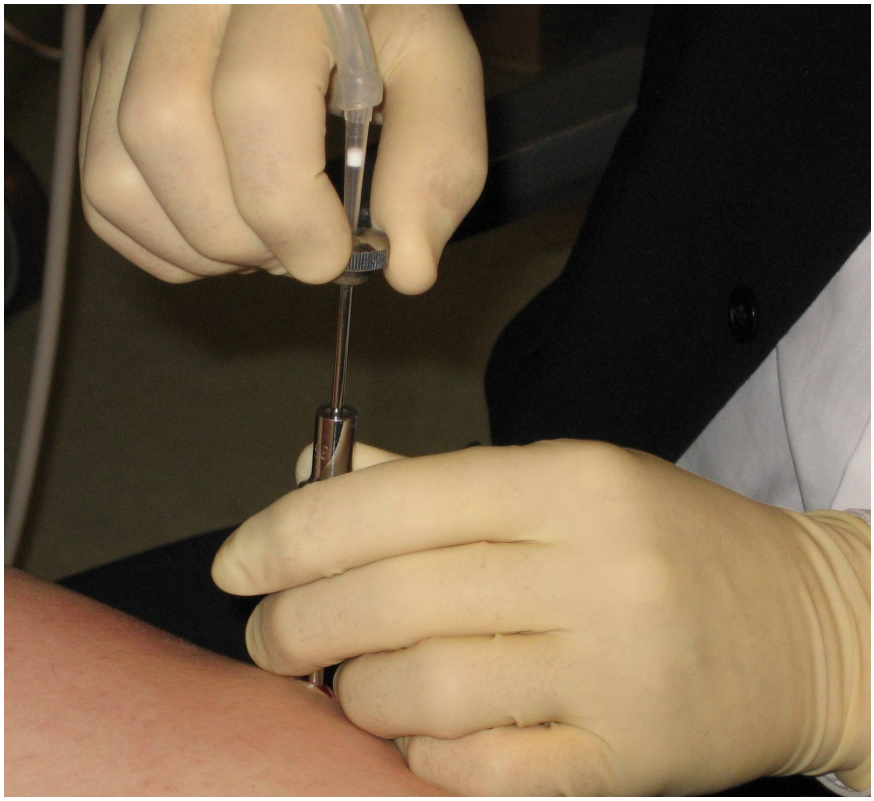

FIGURE 9 Muscle biopsy. Biopsies were taken from the vastus lateralis muscle with a 5-mm Bergström biopsy needle technique (Bergström and Hultman 1966) after local subcutaneous anaesthetics (lidocaine-adrenaline).

#### 4.2.4.3 Anthropometry and muscle cross-sectional area

*Anthropometry.* After overnight fastinging, body mass (kg) and fat percentage were measured. Body fat was measured with skinfolds (biceps and triceps brachii, subscapular and iliac crest) (Durnin and Womersley 1974).

*Bioimpedance (II) and muscle thickness (II, VI, VII).* After an overnight fast, the body fat percentage and amount of total body muscle mass (kg) was measured by bioelectrical impedance using an Inbody720 machine with a multifrequency current (Seoul, Korea). The subjects were advised to drink normally, but abstain from anything that has a dehydrating effect (e.g., sauna, physical work, or alcohol) during the day preceding the bioimpedance measurement. The thickness of the vastus lateralis and intermedius muscles were measured with an ultrasound device in a standardized supine position (Aloka SSD-2000, Aloka Co, Tokyo, Japan). The ultrasound measurement site (at the middle of the vastus lateralis muscle) was tattooed to ensure that the same site was used both before and after training.

*Muscle cross-sectional area (V).* The muscle cross-sectional area (CSA) of the quadriceps femoris muscle was determined before and after the 21-wk period from both RT and control young male subjects using magnetic resonance imaging (MRI) (GE Signa Exite HD 1.5 T) at a local MRI-center (Keski-Suomen Magneettikuvaus, Jyväskylä), the method shown to be high in quality (Reeves et al. 2004). During the measurement, the legs of the subjects were kept parallel

and strapped with a belt and a special cast designed to standardize the measurement as well as possible. Four axial-plane MRI scans were taken; the first image was 4 cm above the midway between the patella and greater trochanter ($image_1$) and, thereafter, the next three scans were taken at 2, 4 and 6 cm towards the patella ($images_{2-4}$). The MRI images were analysed with OsiriX (version 2.7.5) software.

#### 4.2.4.4 Muscle strength (II-VII)

Maximal isometric force of the bilateral leg extensor muscles was measured using an electromechanical dynamometer with a knee angle of 107° and hip angle of 110° (Häkkinen et al. 2001b). Unilateral isometric knee extension and flexion as well as bilateral bench press tests were measured with a David 200 system (David Fitness and Medical, Finland) (Häkkinen et al. 1998b) with 90° knee and elbow angles, respectively. A minimum of three trials were completed for each subject, and the best performance trial was used in the subsequent statistical analysis. The force signal of the isometric measurements was recorded and analyzed with a Signal software version 2.15 (Cambridge Electronic Design Ltd., Cambridge, UK). A David 210 system (David Fitness and Medical, Finland) was used to measure maximal bilateral concentric force production for leg extensors (hip and knee extensors). Separate trials were performed for concentric 1 repetition maximum (RM) testing. After each repetition, the load was increased until the subject was unable to extend his legs from ~60° to the full-extended ~180° knee angle position. The highest successfull load was determined as the 1 RM. The subjects were carefully familiarized with the test procedures and had several warm-up contractions in all devices before the actual testing.

### 4.2.5 Dietary intake

Dietary intake of the subjects was registered with dietary diaries for three (II-VII) to four days (I) before the day of the acute RE bout day and in the studies with biopsies also on the biopsy day, and the day thereafter. In the training studies, dietary intake was registered also after 10.5 weeks (4 days) and again before the 21$^{st}$ week biopsy (post 21 weeks, three days before, and on the biopsy day) and in II and VII also one day after post-biopsy. The diaries were analysed using the Micro Nutrica nutrient-analysis software version 3.11 (The Social Insurance Institution of Finland).

## 4.3 Biochemical analysis

### 4.3.1 Muscle biopsy

#### 4.3.1.1 Analysis of muscle messenger RNA (II-V, VII)

*Total RNA isolation, reverse transcription and cDNA synthesis.* Homogenization of the muscle samples was done with FastPrep (Bio101 Systems, USA) tubes containing Lysing Matrix D (Q-Biogene, USA) and total RNA was extracted using the Trizol-reagent (Invitrogen, Carlsbad, CA, USA). An optical density (OD) ratio ($OD_{260}/OD_{280}$) of 1.8 to 2.0 and gel electrophoresis showed that our extraction yielded DNA-free and un-degraded RNA, respectively. Three to five µg of total RNA were reverse transcribed to synthesize cDNA according to the manufacturer's instructions using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, Ca, USA).

*Real-time RT-PCR.* The mRNA expression was quantified with a real-time reverse transcriptase-PCR (RT-PCR) assay using Abi 7300 and 7700 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The probes and primers used were mostly pre-designed transcripts validated by Applied Biosystems bioinformatics design pipelines. The gene bank accession numbers and Applied Biosystems assay IDs, respectively, were: NM 005259 and Hs00193363_m1 (myostatin), NM 001106 and Hs00609603_m1 (activin receptor IIb), NM 005860 and Hs00610505_m1 (follistatin related gene protein: FLRG), NM 002478 and Hs00159528_m1 (MyoD), NM 002479 and Hs00231167_m1 (myogenin), NM_078467.1 and Hs00355782_m1 (p21), NM_052827.1 and Hs00608082_m1 (cdk2), NM_148177.1 and NM_058229.2 and Hs00369714_m1 (Muscle Atrophy F-Box: MAFbx / atrogin-1), NM 004064 and Hs00153277_m1 (p27), NM002046, Hs99999905_m1 (GAPDH), and X03205.1 and Hs99999901_s1 (18sRNA). PCR for AR, IGF-IEa and MGF was performed with SYBR green mix (QuantiTect, Qiagen, Crawley, UK). The primers used were designed by Oligo Explorer and Analyzer softwares (Gene Link, Inc) and these were synthesised by Oligomer Ltd. (Helsinki, Finland). The sequences of the primers for each target gene used are given in table 3 (Marcell et al. 2001; Hameed et al. 2003). A single peak on the DNA melting temperature curve was seen at the end of each reaction.

TABLE 3 SYBR green primers used in real time PCR (Marcell et al. 2001; Hameed et al. 2003)

| Primer name | Sequence (5′ to 3′) | Product size (bp) |
|---|---|---|
| IGF-IEa forward | ATCTAAGGAGGCTGGAGATGTATTGC | 114 |
| IGF-IEa reverse | TCAAATGTACTTCCTTCTGGGTCTTG | |
| MGF forward | CGAAGTCTCAGAGAAGGAAAGG | 150 |
| MGF reverse | ACAGGTAACTCGTGCAGAGC | |
| AR forward | TTGTCCACCGTGTGTCTTCTTCTGC | 225 |
| AR reverse | TGCACTTCCATCCTTGAGCTTGGC | |

Each sample was analyzed in triplicates. PCR cycle parameters used for all genes were: 50°C for 2 min, + 95°C for 10 min, 37-45 (depending on the mRNA analysed) cycles at 95°C for 15 s, and 60°C for 1 min. GAPDH mRNA was used as an endogenous control because it had been used successfully previously in exercise studies in our laboratory and in the studies II and III it was shown to be stable and more suitable to use in this design as a housekeeping gene than 18sRNA. Gene transcript results were calculated according to the mathematical model by Liu and Saint (Liu and Saint 2002). SigmaPlot (version 9.0, Systat Software inc., Richmond, Ca, USA) was used as a curve fitting software needed in the method. Only exception was study VII, in which the mRNA was measured according to the corresponding gene-specific standard curve created by serial dilutions of pooled samples.

#### 4.3.1.2 Analysis of proteins with western blotting (IV, VI, VII)

*Western blotting for the analysis of phosphoproteins and myostatin (VI)*. Muscle biopsy specimens were hand-homogenized in ice-cold buffer (20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 2 mM DTT, 1% Triton X-100, 0.2% sodium deoxycholate, 30 µg/ml leupeptin, 30 µg/ml aprotinin, 60 µg/ml PMSF, and 1% phosphatase inhibitor cocktail (P 2850; Sigma, St. Louis, USA) at a dilution of 15 µl/mg of wet weight muscle. Homogenates were rotated for 30 min at 4°C, centrifuged at 10 000 *g* for 10 min at 4°C to remove cell debris, and stored at –80°C. Total protein was determined using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, USA).

For 4E-BP1, but not others, homogenates were first heated 10 minutes at 95°C, spun 7000 g for 30 mins, +4°C (Dreyer et al. 2006b) and then continued with the Laemmli buffer and heating 95°C as was the case with the other proteins. Samples containing 30 µg of total protein were separated by SDS-PAGE for 60 to 90 min at 200 V using 4–20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). All four samples from each subject were run on the same gel. Proteins were transferred to PVDF membrane at 300 mA constant current for 3h on ice at 4°C. The uniformity of protein loading was checked always by staining the membrane with Ponceau S. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 1h and then incubated overnight at 4°C with commercially available rabbit polyclonal primary phosphospecific antibodies. Antibodies recognized phosphorylated Akt on $Ser^{473}$, mTOR on $Ser^{2448}$, $p70^{S6K}$ on $Thr^{389}$, rpS6 on $Ser^{235/236}$, 4E-BP1 on $Thr^{37/46}$ and eEF2 on $Thr^{56}$ (Cell Signaling Technology, Beverly, MA) and C-terminal myostatin protein (Chemicon/Millipore AB 3239, CA, USA) (Mendler et al. 2007). The rabbit polyclonal antibody used was raised against a peptide residing in the C-terminal of myostatin corresponding to amino acids 349-364 of human myostatin and therefore, being a similar antibody to those used previously (Gonzalez-Cadavid et al. 1998; Taylor et al. 2001).

All the antibodies were diluted 1:2000 (except eEF2 on $Thr^{56}$ which was 1:3000) in TBS-T containing 2.5% nonfat dry milk. Membranes were then washed in TBS-T, incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Cell Signaling Technology, USA) diluted 1:5000 in TBS-T with 2.5% milk for 1h followed by washing in TBS-T. Phosphorylated proteins were visualized by enhanced chemiluminescence (ECL) reagents according to the manufacturer's protocol (SuperSignal west femto maximum sensitivity substrate, Pierce Biotechnology, USA). Quantification was carried out using a ChemiDoc XRS in combination with Quantity One software (Bio-Rad Laboratories, USA). The membranes described above were incubated in Restore Western blot stripping buffer (Pierce Biotechnology) for 30 min and reprobed with appropriate antibodies for detection of the total expression levels of Akt and rpS6 (rpS6 rabbit monoclonal) (Cell Signaling Technology, USA) and $p70^{S6K}$ (Santa Cruz Biotechnology, USA) by immunoblot analysis as described above.

*Western blotting for the analysis of androgen receptors (IV and VII).* Total muscle protein concentration and western blot was quantified as described previously (Carson et al. 1996) with small modifications. Each specimen (~20-30 mg) was homogenized in a 4% (w/v) solution in Mueller buffer (50 mM Hepes pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7 \cdot 10H_2O$, 100 mM β-glycerolphosphate, 25 mM NaF, 1 mM $Na_3VO_4$, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, and 0.3 µg/ml aprotinin). The homogenate was centrifuged for 15 min at 10 000 g at 4°C. The protein concentration was determined using the Lowry based method (Bio-Rad, Hercules, CA). A sample volume containing 40 µg of total protein was incubated for 15 min at 65°C in sample buffer and then fractionated on an 8% gel and transferred on to a PVDF membrane overnight at 4°C. After blocking with 1% BSA for 1h, the membrane was probed with the polyclonal rabbit antibody against AR (Ab3510, Abcam, UK) overnight at 4°C. The membrane was then incubated for 1h with secondary antibody (goat anti-rabbit, G21234, Molecular Probes), and with ECL (ECL Plus, Amersham BioSciences, New Jersey, USA) after which it was exposed to an X-ray film for visualization. Quantification of AR was performed using densitometry scanning (Personal Densitometer SI, Molecular Dynamics). The 110 kDa size AR band analyzed was confirmed with the positive control of AR (T47D Cell Lysate, Santa Cruz BioTechnology).

#### 4.3.1.3 Immunohistochemistry (IV, VI, VII)

*Muscle fiber cross-sectional area.* Serial 8-µm-thick transverse sections were cut on a cryomicrotome (Leica CM 3000) at –24°C. Fiber type was classified by staining using myofibrillar ATPase method according to the earlier studies in our laboratory (Kovanen and Suominen 1987; Häkkinen et al. 2001b; Korhonen et al. 2006). Fiber sarcolemma was visualized with an antibody against dystrophin (DYS2, Novocastra Laboratories, UK) using avidin-biotin peroxidase kit (Vectastain PK-4002, Vector Laboratories, USA) with diaminobentzidine

(Abbott Laboratories, USA) as a chromogen (Kovanen and Suominen 1987; Häkkinen et al. 2001b). The measurements of fiber cross-sectional area comprised an average of 125 ± 57 type I and 129 ± 61 type II muscle fibers. Stained cross-sections were analyzed by Tema Image-Analysis System (Scan Beam) using a microscope (Olympus BX 50) and color video camera (Sanyo High Resolution CCD). Mean fiber areas of fiber types I and II were calculated.

*Immunohistochemical staining of androgen receptors* (IV)*, mTOR and rpS6* (VI). For the AR, mTOR and rpS6 localisation in human VL muscle biopsies, eight µm cross-sections, cut with cryomicrotome, were air-dried and fixed 15 minutes with 4% PFA-PBS. Sections were permeabilized with 0.2% Triton-X for 10 minutes and blocked 30 minutes with 3% BSA-PBS and thereafter incubated with primary antibodies overnight at +4°C. Double immunolabeling was performed using monoclonal antibody against rpS6 (rabbit monoclonal) or rabbit polyclonal antibodies against phospho-rpS6 on $Ser^{235/236}$ or phospho-mTOR on $Ser^{2448}$ Cell Signaling Technology, Beverly, 1:40 in 1% BSA-PBS) or polyclonal antibody against AR (rabbit polyclonal, N-20, sc-816; Santa Cruz Biotechnology, 1:100 in 1% BSA-PBS) (Sinha-Hikim et al. 2004) with either mouse monoclonal antibody against human slow myosin heavy chain I (MyHC I) (Developmental Studies Hybridoma Bank (DSHB), Iowa city: A4.951), or human fast MyHC II [DSHB: A4.74] developed by Dr. H.M. Blau (Hughes et al. 1993), or mouse monoclonal antibody against caveolin-3 (1:100) (BD Transduction laboratories, USA) to visualize muscle sarcolemma. Nuclei were stained by Hoechst 33258 (Sigma, St. Louis). Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 or 546, and goat anti-mouse Alexa Fluor 546 or 488 (Molecular Probes, Eugene, USA). Slides were mounted with mowiol-DABCO. Negative controls were done by omitting the primary or secondary antibodies. Moreover, for AR specificity, the preabsorbtion of the primary antibody was used overnight at 4°C with a fivefold excess of the blocking peptide (sc-816 P; Santa Cruz Biotechnology). This treatment completely eliminated the nuclear AR staining, providing evidence that the positive results are specific for AR.

An Olympus BX-50F light microscope (Olympus Optical, Tokyo, Japan) with Olympus colour CCD camera (Colorview III, Olympus Optical, Tokyo, Japan) and Analysis® software (version 5.0, Soft-Imaging System GmbH, Munster, Germany) were used for the imaging and analysis. For the confocal microscope in (VI), two samples from young men were also further analyzed. An Olympus IX81 microscope with confocal imaging system and software (Olympus Fluoview ver. 1.6a) were used for these analyses (Kivelä et al. 2007).

### 4.3.2 Blood analysis (I, III-IV, VI, VII)

Blood lactate concentration was determined using Nova Stat Profile PhOx Plus L autoanalyzer (Nova Biomedical, Waltham USA). A KX-21N autoanalyzer (Sysmex, Japan) was used to measure hematocrit and hemoglobin for correcting the plasma volume changes (Dill and Costill 1974). Serum insulin was analysed by the ADVIA Centaur insulin assay (I), which is a two-site sandwich

immunoassay using direct chemiluminescent technology (Bayer Ltd, USA), or with an immunometric chemiluminescence method with an Immulite® 1000 (DPC, Los Angeles, USA) (III, VI, VII). Serum testosterone was analysed either by enzyme immunoassay, ELISA (IBL, Hamburg, Germany) (I) or with Immulite® 1000 (DPC, Los Angeles, USA) (IV, VI, VII). Serum sex hormone-binding globulin (SHBG) was analysed with an Immulite® 1000. Free testosterone was calculated from total testosterone and immunoassayed SHBG concentrations (method validated by Vermeulen et al. 1999). To avoid inter-assay variation, all samples for each subject were assayed in the same assay run.

## 4.4 Statistical methods

All data are expressed as means ± SD, except where designated. The data were analysed by a one or two-factor repeated measures General Linear Model ANOVA. Any violations of the assumptions of sphericity were explored and, if needed, corrected with a Greenhouse-Geisser or Huynh-Feldt estimator. Shapiro-Wilk test revealed that mRNA and western blot data was not normally distributed and therefore for the statistical tests, those values were log-transformed. Holm-Bonferroni post hoc tests were performed to localize the effects. Exception to the statistical analysis was study I in which cross-over setting was used. In that study, the data from the RE session were analyzed by an analysis of variance according to a linear model for a two-period repeated measurements cross-over design (Wallenstein and Fisher 1977). All the analyses of the RE session in that study were performed by means of SAS (8.0.2.) system using the MIXED procedure. In the other studies, SPSS versions 11.0-15.0 were used for statistical analyses (SPSS, Inc., Chicago, IL). The level of significance was set at $p \leq 0.05$.

# 5 RESULTS

The main findings of the present series of studies are presented below. For more details the original articles (I-VII) should be consulted.

## 5.1 Nutrient intake

There were no differences in total energy or macronutrient consumption between the protein and placebo conditions during four days before the RE bout (I) or during three days before RE bout, during RE day and the day thereafter (5 days in total, III-VI) (Table 4), or before training and after training (II, VII). In the training studies comparing protein and placebo groups (V, VI), the nutrient or energy intake did not differ between the groups at week 0, 10.5, or 21, or in the averaged values of those three time-points.

TABLE 4 Dietary intake in studies comparing protein and placebo. It does not include the protein supplement in the protein group. Studies I and III/IV are acute settings with dietary diary collected during four days before the RE bout (I) or during three days before RE bout, during RE day and the day thereafter (5 days in total, III-VI). In studies V and VI, an averaged energy and macronutrient intake is shown in the protein and placebo groups throughout the 21-week training period (week 0, week 10.5 and week 21). g/kg bw = g per kg body mass. There were no significant differences between the groups in total energy or any macronutrient consumption

| Study | Variable | Protein | Placebo |
|---|---|---|---|
| I | Energy (1000 kJ) | 10.9 ± 1.3 | 10.3 ± 1.3 |
| | Protein (g/kg bw) | 1.9 ± 0.5 | 1.8 ± 0.5 |
| III/IV | Energy (1000 kJ) | 8.0 ± 2.2 | 7.8 ± 1.9 |
| | Protein (g/kg bw) | 1.1 ± 0.3 | 1.1 ± 0.3 |
| V/VI | Energy (1000kJ) | 10.5 ± 1.5 | 10.2 ± 3.0 |
| | Protein (g/kg bw) | 1.5 ± 0.3 | 1.4 ± 0.4 |

## 5.2 Acute responses after resistance exercise session with or without protein ingestion (I-VII)

### 5.2.1 Resistance exercise loading

RE loadings were 5x10 repetition leg press (II-VII) or 5x1 RM and 3x10 RM squat and 4x10 RM leg press (I). Total work of the RE bouts (loads x sets x repetitions) was in overall in (I): protein: 14003 ± 1726 kg and in placebo: 14035 ± 1613 kg, in (II): before training: 6095 ± 926 kg, after 21 weeks 7578 ± 1046 kg (difference $p<0.001$), and in (VII) in the older before training: 6225 ± 926 kg and after 21 weeks 7800 ± 1131 kg, and in the young before training 7363 ± 477 kg and after 21 weeks 8863 ± 336 kg (difference between the pre and post loadings, $p<0.001$). In (III-IV) with older men (after 21 weeks of RT), the total work was 8094 ± 1378 in the protein group and 7572 ± 1105 kg in the placebo group. In (V-VI) in previously untrained men, the total work of the RE bout was 6722 ± 1210 kg in the placebo group and 6994 ± 1327 kg in the protein group. There were no differences between the protein and placebo conditions in the total volume of the loadings in any of the studies.

### 5.2.2 Isometric force and blood lactate after the exercise session

The RE bouts led to a significant transient decrease in muscle isometric force (27-38%, $p<0.01$) (II-VII) or explosive force measured by vertical jump (I) (36-41%, $p<0.001$). Protein ingestion (I, III-VI) or training state of the muscle (II, VII) did not affect the magnitude of this decrease. Increases in blood lactate (up to 9.8-13.5 mmol/l) occurred also, showing the glycolytic nature of the exercises.

### 5.2.3 Blood hormones

Serum **testosterone** significantly increased in both protein and placebo conditions during the RE bout in trained young men ($p<0.01$) being higher in the placebo compared to the protein immediately post-RE ($p=0.05$) (I) (Figure 10). In previously untrained young men, serum testosterone increased significantly compared to the control group during the bout of RE only in the placebo group ($p<0.05$). In agreement with that result, in the older, increased serum total and free testosterone at post 0 min occurred only in the placebo group ($p<0.05$).

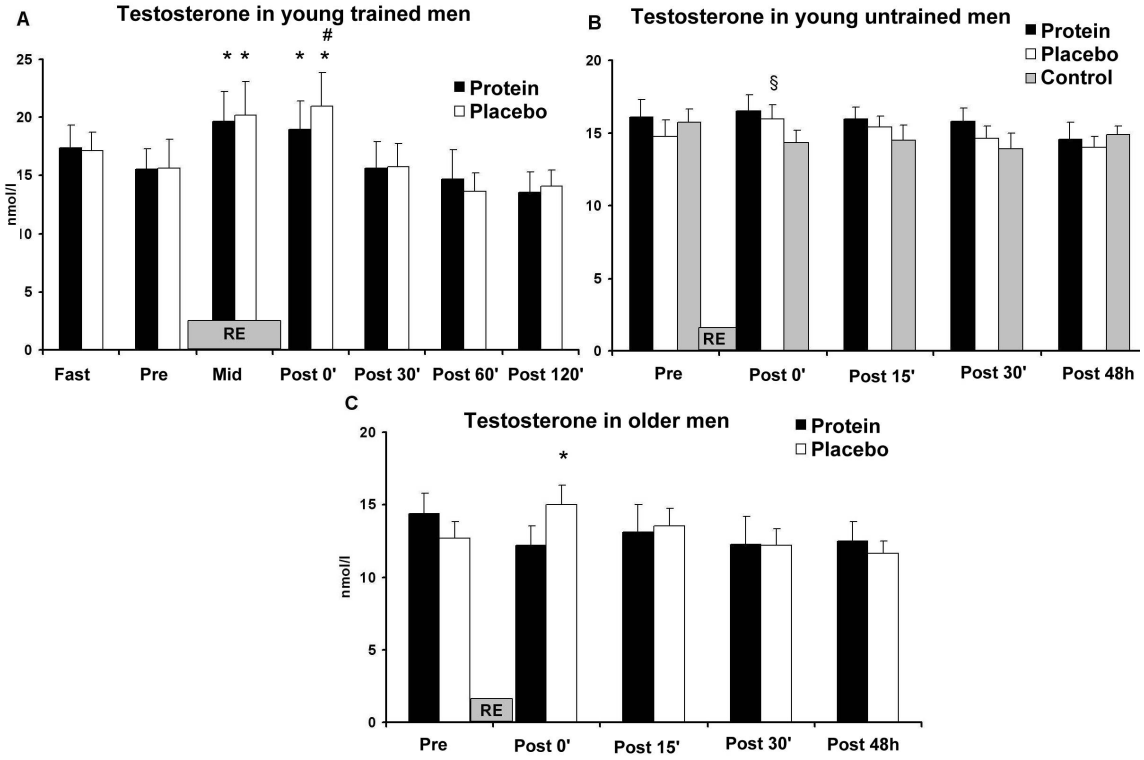


FIGURE 10 Resistance exercise induced changes in serum total testosterone concentration in young trained men (A), young previously untrained men (B) and older men after 21 weeks of RT period (C). The results for serum total testosterone are analysed and shown as uncorrected to the changes in plasma volume. All the values are means ± SE. Mid = blood sample immediately after the squat sets, * = $p<0.05$ difference to the pre-value in either protein or placebo condition, # = $p=0.05$ difference between the protein and placebo conditions at post, and § = $p<0.05$ increase in the placebo group compared to controls (I, IV, VI).

When investigating the effects of a squat and leg press exercise in trained men (I), serum **insulin** was significantly increased after the squat sets only in the protein condition ($p=0.01$). The area under the curve of the insulin concentrations during the RE bout (calculated from pre-RE before the protein drink to post-RE) was significantly higher in the protein condition compared to the placebo (51.6%, $p<0.001$). In the older men and previously untrained young men, serum insulin did not change significantly after RE bout in any group.

### 5.2.4 Muscle total RNA

In the older men (II), the total RNA concentration (µg RNA extracted per mg of muscle wet weight) was increased following the RE bout in the untrained condition at 48h post-RE (18%, $p<0.05$) but not anymore after the 21-week RT period. In the young men, the same increase in the pre-RT state was not seen.

### 5.2.5 Muscle mRNA and protein content of myostatin and AR

#### 5.2.5.1 Effect of resistance exercise with protein ingestion

There were no significant changes in any of the measured mRNA values in the control group (who did not exercise) at any time point when compared to the pre-situation (in the older controls pre vs. post 1h and pre vs. post 21 weeks, and in the young, in addition to those, also pre vs. post 48h) (II-V, VII). Heavy RE bout led to a decrease in **myostatin** mRNA in the young placebo group at post 1h and in the older placebo group at post 48h ($p<0.05$) but not when protein was ingested (Figure 11). In the older, myostatin mRNA also tended to be decreased at post 1h ($p=0.06$). In the young, also the myostatin 26 kDa sized protein already decreased at post 1h in the placebo group ($p<0.05$) but not in the protein or control groups (Figure 16).

In the young, the receptor of myostatin, **activin receptor IIb** mRNA, decreased in both protein and placebo groups after the RE bout being significant at 48h after the RE bout ($p<0.05$) (Figure 11). In the older, no changes after the RE bout were found when comparing the protein and placebo groups (III). However, in the setting investigating the effects of the subject's training state (II), there was a decrease also in the older after the RE bout (see section: effect of training state, Figure 14).

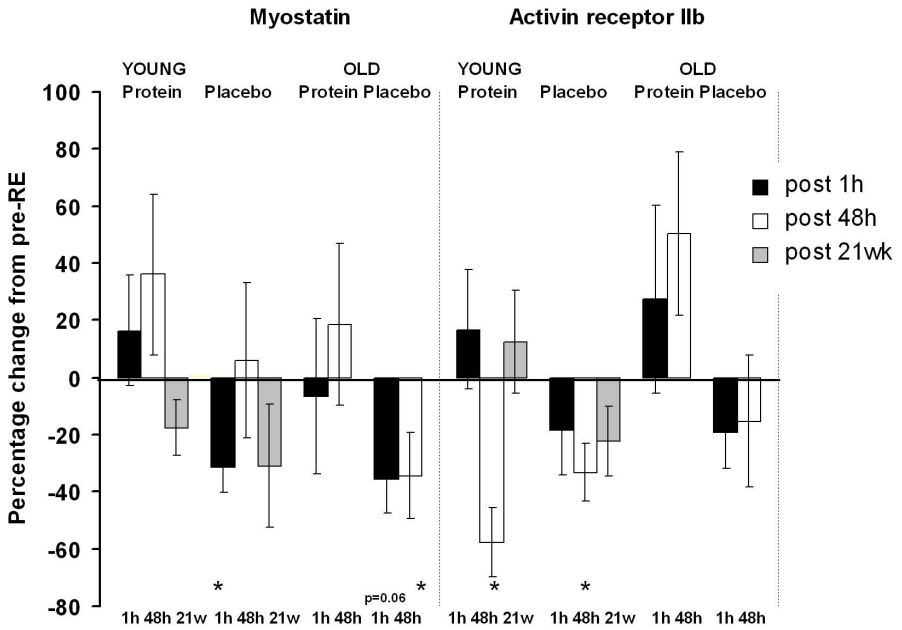


FIGURE 11 Real-time RT-PCR results for myostatin and activin receptor IIb mRNA expressions before and after single RE bout (black bar: 1h and white bar: 48h) as well as in young men also after 21 weeks of RT (grey bar: 21w) from vastus lateralis muscle in protein and placebo conditions. Results are normalized to GAPDH mRNA expression and changes are presented in relation to pre-RE levels. All the values are means ± SE. * = significant ($p<0.05$) difference compared to the pre-value in either protein or placebo condition. The data of the control groups is not shown because there were no changes in the control groups (III, V).

In the young protein group, a significant increase in cdk2 mRNA was observed at 1h post-RE (p=0.01) and a trend at 48h post-RE (p=0.08) (Figure 12). In the older, the increase in cd2 mRNA was significant in the protein group from at 48h post-RE (p=0.02).

In the young, **p21** mRNA increased in both protein and placebo groups after the RE bout, being significant in the placebo group from post 1 to 48h and in the protein group at post 1h. In the older, the average increase seemed to be smaller; p21 mRNA was significantly increased only in the placebo group at 1h post-RE (Figure 12).

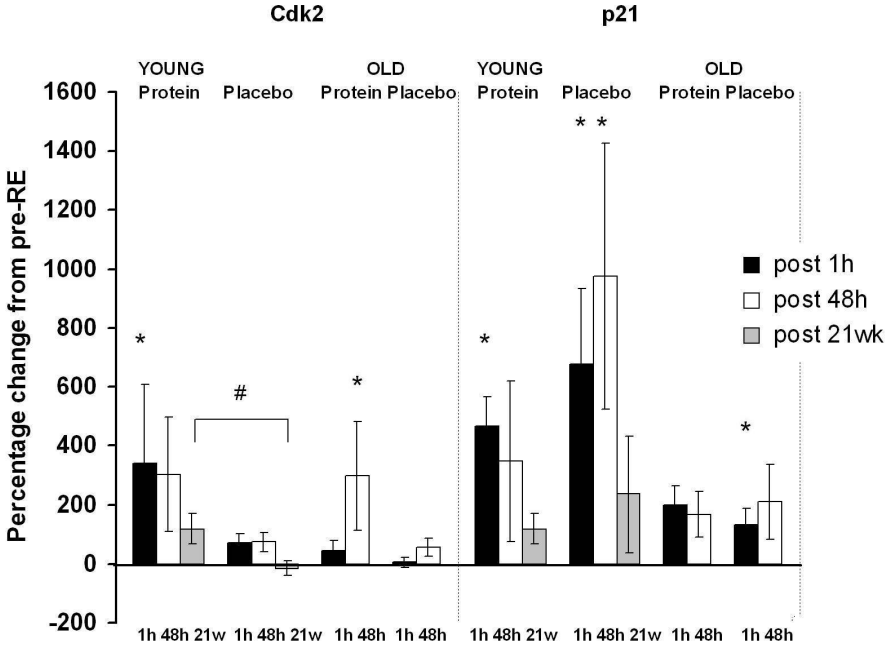


FIGURE 12 Real-time RT-PCR results for cdk2 and p21 mRNA expressions before and after single RE bout (black bar: 1h and white bar: 48h) as well as in young men also after 21 weeks of RT (grey bar: 21w) from vastus lateralis muscle in protein and placebo conditions. # = $p<0.05$ difference in the change between protein and placebo group See further explanations from the figure 11. The data of the control groups is not shown because there were no changes in the control groups (III, V).

In the young, **myogenin** mRNA was decreased at 1h after the RE bout in the placebo group (p=0.005) (Figure 13). At post 48h there was at least tendency for an increase in myogenin mRNA in all the RE-groups; however this was significant only in the young pooled (protein and placebo) data ($p<0.05$). In the young, no effect of the RE bout or protein was observed for follistatin related gene protein (**FLRG**) but in the older, it was increased in the protein group at 48h post-RE (p=0.02).

In the older, no significant changes due to the RE bout in either the protein or placebo condition were observed in **MGF** or **IGF-IEa** mRNA. In the older combined protein and placebo groups, the **androgen receptor** (AR) mRNA was increased at post 48h (p=0.01) with no differences between the protein and

placebo groups (Figure 13). In the older, no significant changes in the AR protein were seen in either group. **MyoD** mRNA did not change due to the RE bout in any group. **p27** mRNA which was measured in the older, and ubiquitin-ligase **MAFbx** mRNA in the young, also remained un-changed.

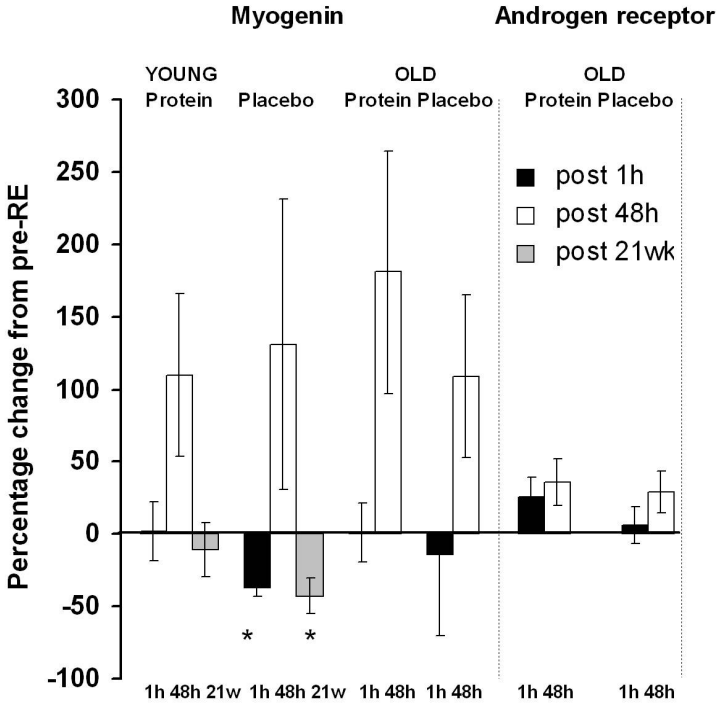


FIGURE 13 Real-time RT-PCR results for myogenin and androgen receptor mRNA expressions before and after single RE bout (black bar: 1h and white bar: 48h) as well as in young men also after 21 weeks of RT (grey bar: 21w) from vastus lateralis muscle in protein and placebo conditions. * = significant ($p<0.05$) difference compared to the pre-value. In the older, the pooled data of protein and placebo showed a RE loading effect for an increase ($p=0.05$). In the young pooled data, myogenin mRNA was increased at 48h after the RE bout ($p<0.05$). With pooled data, AR mRNA was increased at post 48h ($p=0.01$). The data of the control groups are not shown because there were no changes in the control groups when compared to the pre-situation (in the older controls pre vs. post 1h and pre vs. post 21 weeks, and in the young, in addition to those, also pre vs. post 48h), making the complex images easier to interpret (III-V).

### 5.2.5.2 Effect of the training state

Effects of the training state were investigated for the myostatin, FLRG, activin receptor IIb, myogenin, MyoD and p27 mRNA in the young (II) and for the AR, MGF and IGF-IEa mRNA and AR protein in both the young and the older (VII). Myostatin mRNA decreased following the RE bout but only after 21 weeks of RT being localized at 48h post-RE ($p<0.05$) (Figure 14). The post 1h mRNA of activin receptor IIb was significantly decreased compared to pre-RE levels in the before-RT state (Figure 14). An averaged (post 1 and 48h) MyoD mRNA expression after the RE bout was larger in the post-RT condition compared to

the pre-RT ($p<0.05$). Myogenin mRNA was significantly increased due to the RE bout at post 48h in the pre-RT state.

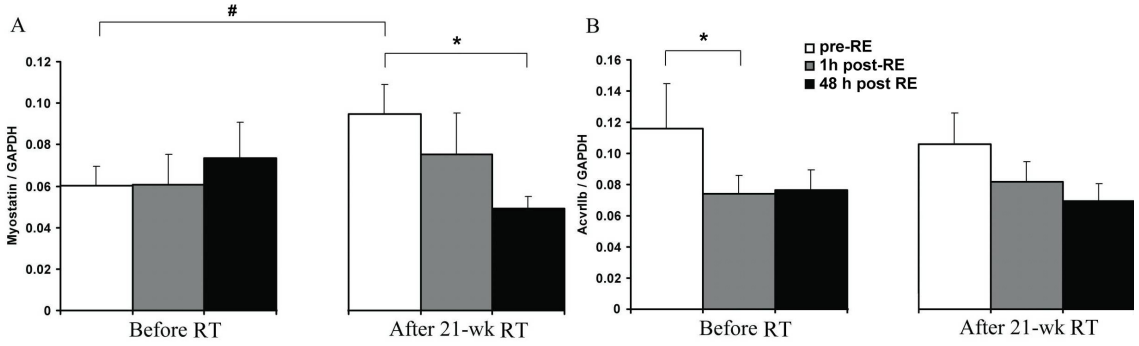


FIGURE 14 Real-time RT-PCR results for RE-induced a) myostatin and b) activin IIb mRNA expressions from vastus lateralis muscle. Results are shown both before and after 21 weeks of RT and expressed as normalized to GAPDH mRNA expression. Muscle biopsies were obtained before resistance exercise (pre-RE, white bars), and 1 (post-RE 1h, grey bars) and 48h (post-RE 48h, black bars) post-exercise. Values are means ± SE calculated according to Liu & Saint (2002) mathematical model (expressed as $10^3$). * = Significantly different from corresponding pre value in RE and # = difference between pre- and post-ST resting levels ($p<0.05$) (II).

IGF-IEa mRNA was increased only in young strength trained men at post 48h ($p<0.001$). MGF mRNA was increased at post 48h ($p<0.05$) in young strength trained men and previously untrained older men. No significant changes due to the RE bout in either the before- or after-RT state was observed also in FLRG, p27 and AR mRNA, and AR protein.

### 5.2.6 Muscle phosphoproteins

#### 5.2.6.1 Effect of resistance exercise with protein ingestion

Muscle phosphoproteins were studied in young men. No changes were seen in the control group. However, the phosphorylated **$p70^{S6K}$** and **rpS6** were increased 1h after the RE bout (Figure 15). The change in the phosphorylation of $p70^{S6K}$ was greater with the protein ingestion compared to the placebo ($p<0.001$), while also the phosphorylation of rpS6 tended to be larger in the protein vs. placebo group ($p=0.11$). Phosphorylated **mTOR** increased only in the protein group, and the increase persisted from post 1h to 48h ($p<0.05$) (Figure 15). In the placebo group, the increase of phosphorylated rpS6 persisted 48h after the RE bout.

There was a strong trend for decreased **4E-BP1** phosphorylation in the placebo group at post 1h ($p=0.06$). The decrease was significant when compared to the controls ($P<0.05$) (Figure 16). In the placebo group, 7/9 subjects showed a decrease (average 43%) and 2/9 an increase (8%) of phosphorylated 4E-BP1 from pre to post 1h, whereas in the protein group 6/9 showed an increase (112%) and 3/9 a decrease (32%) (between the group difference in the change $p<0.05$).

In the protein group a trend for an increase existed in the phosphorylation of **Akt** at post 1h (p=0.096) (Figure 15). No change was seen in the phosphorylation of **eEF2** (Figure 16) and total protein content of $p70^{S6K}$, Akt and rpS6.

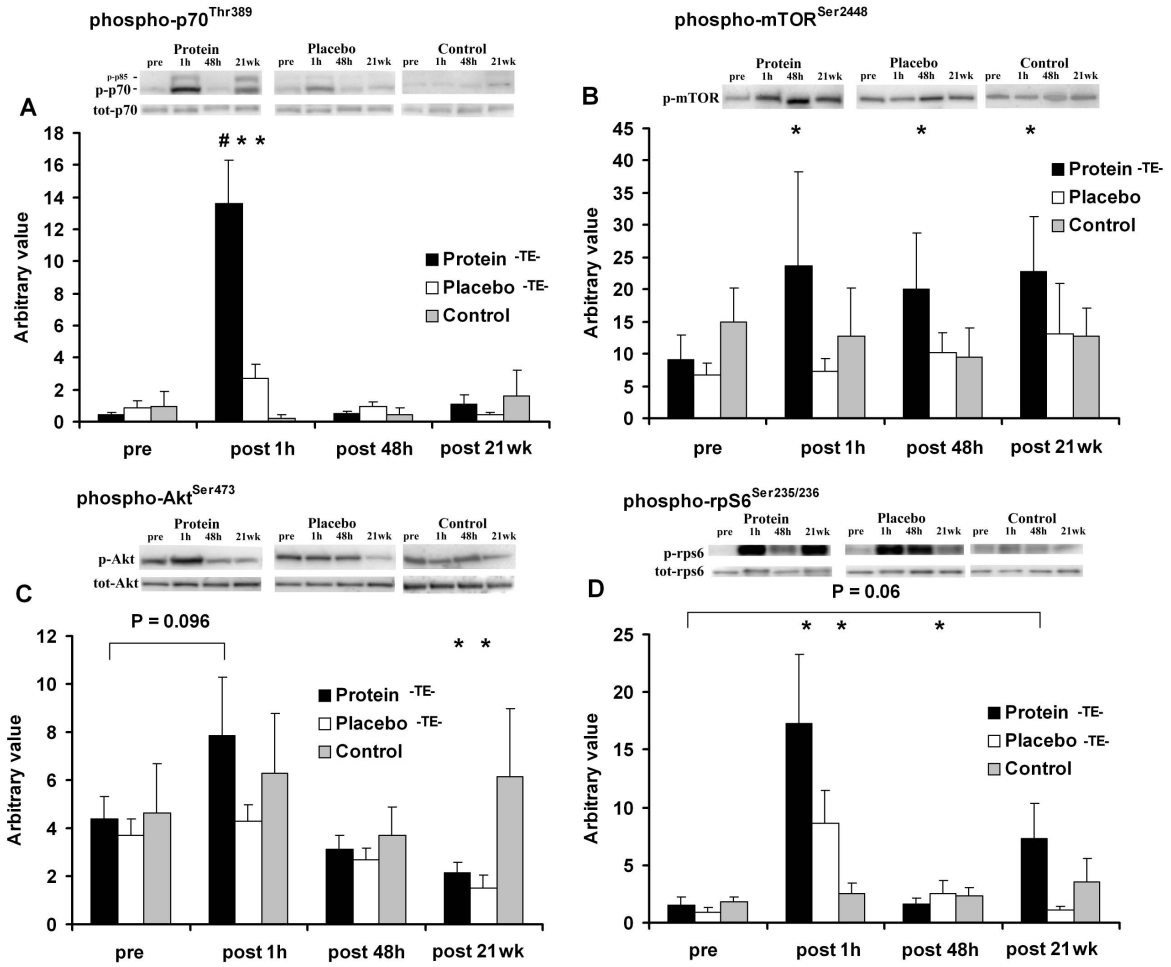


FIGURE 15 Phosphorylation of A) $p70^{S6K}$ at $Thr^{389}$, B) mTOR at $Ser^{2448}$, C) Akt at $Ser^{473}$, and D) rpS6 at $Ser^{235/236}$. Immunoblot of one individual is shown on top of each figure as well as total forms of $p70^{S6K}$, Akt and rpS6. $p70^{S6K}$ figure (blot) shows also that phosphorylation of other isoform of S6K1, $p85^{S6K}$ at $Thr^{412}$ followed the same trend as $p70^{S6K}$. Values are arbitrary units (means ± SE). *p<0.05 vs. pre, #p<0.05 difference between protein and placebo, and -TE- p<0.05 time-effect of ANOVA. For the protein and placebo groups n=9 in these figures and for the control group n=6 (VI).

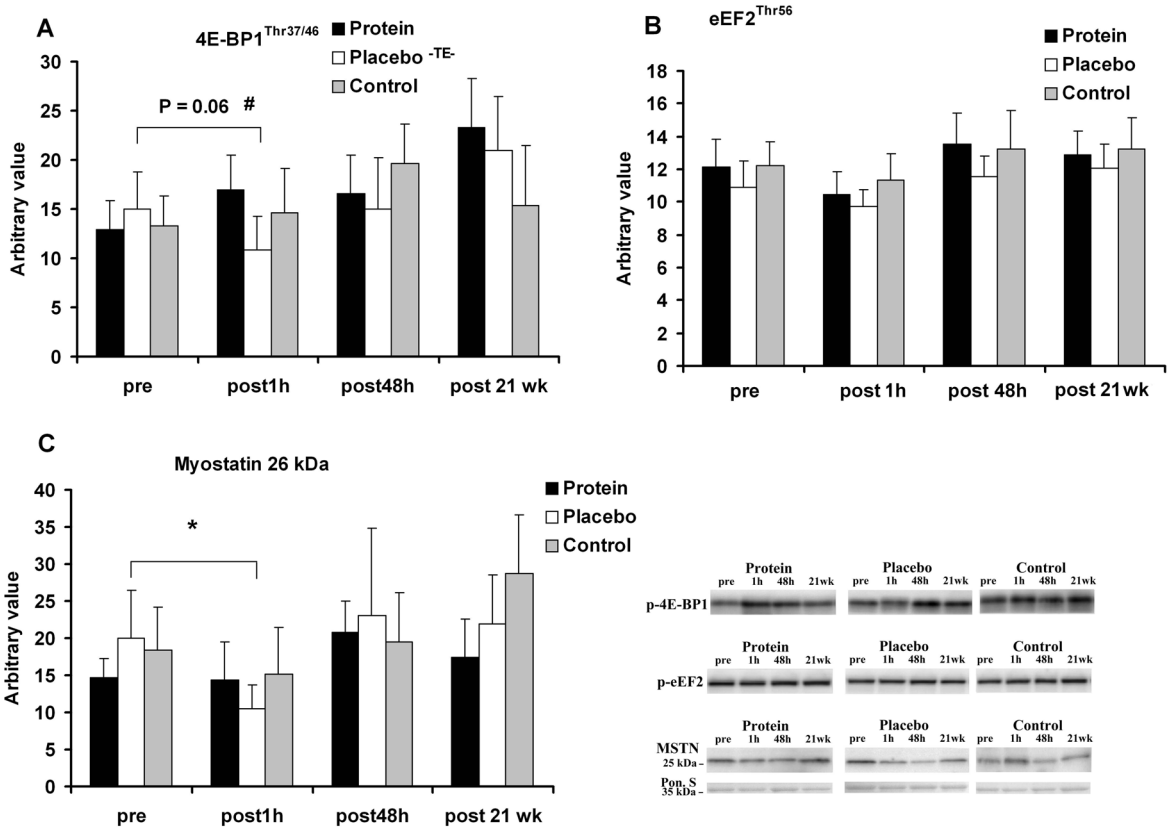


FIGURE 16 Phosphorylation of A) 4E-BP1 at Thr$^{37/46}$, B) eEF2 at Thr$^{56,}$ and C) myostatin 26 kDa C-terminal protein. Ponseau S staining shows equal protein loading. Immunoblot of one individual is shown in the right corner. See explanations in the text of figure 15. There was a between-group difference between the protein and placebo group and also between the placebo and control group in the change from pre to post 1h (# = $p<0.05$). For the protein and placebo groups n=9 in these figures and for the control group n=6 (VI).

## 5.3 Basal responses after resistance training period with or without protein ingestion (II, V-VII)

### 5.3.1 Muscle size and force

*Anthropometric measurements.* The body mass of young men increased significantly in the training groups after 21 weeks compared to the control group ($p<0.05$). In the protein group, the average increase was 3.5 ± 2.0 kg and in the placebo group 2.3 ± 2.1 kg. However, at 10.5 weeks, only the protein group had increased its body mass compared to the control group ($p=0.01$), not the placebo group. Fat percentage remained unchanged in every group.

*Muscle size.* In young previously untrained men, cross-sectional area (CSA) of the quadriceps femoris (QF) increased significantly after 21 weeks of RT in both protein and placebo groups ($p<0.01$) but not in the control group (V). The

change of the average QF CSA was larger (ns.) in the protein group (9.9±7.4%) compared to placebo (7.5±4.8%) (Figure 17). CSA of the VL muscle increased in all four axial-plane images in both protein and placebo groups ($p<0.001$) but not in the control group. The average increase in the VL muscle was significantly largest in the protein group ($p<0.05$) (Figure 17). The protein group also demonstrated significant increase in VL muscle thickness both after 10.5 (within-group $p<0.05$) and 21 weeks ($p<0.01$) of RT but placebo group only after 21 weeks of RT ($p<0.05$) (VI). Moreover, compared to the control group, only the protein group increased its muscle thickness significantly after both 10.5 and 21 weeks ($p<0.05$), whereas the placebo group only approached a trend after 21 week ($p=0.12$).

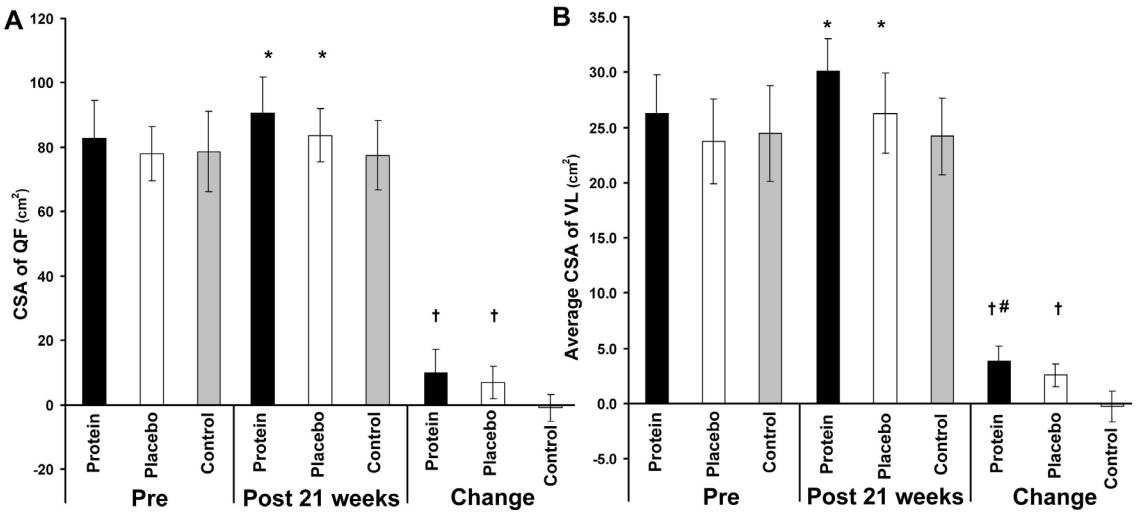


FIGURE 17 The average CSA of A) quadriceps femoris and B) vastus lateralis from all four MRI-images. Significantly ($p<0.05$) different compared to the Pre (*), to the Control (†) or to the Placebo (#). All the values are means ± SD (V).

*Muscle Force.* Maximal bilateral 1 RM leg extension, isometric bench press, and unilateral isometric knee extension and flexion increased during the 21-week training period in both the protein and placebo groups of young men ($p<0.05$) (V). Compared to the control group (8.0 ± 9.5%), isometric leg extension increased significantly only in the protein group (a relative increase of 24.3 ± 12.3%, difference to the placebo $p=0.02$), whereas the increase was not significant in the placebo group (19.3 ± 15.5%, $p=0.23$).

When comparing the changes between the young and the older subjects (VII), the isometric force and dynamic 1RM of the leg extensors increased in both young (18 ± 15%, 19 ± 11%, respectively) and older men (17 ± 14%, 21 ± 8%) during the 21-week RT period ($p<0.05$).

### 5.3.2 Specific muscle mRNAs

A significant increase in myostatin mRNA levels was observed in response to RT in the older ($p<0.05$) (II) but not young men (V) (Figures 11 and 14). There was no change in muscle myostatin 26 kDa protein in the young men (Figure

16). In the young, a trend for increased cdk2 mRNA was observed after 21 weeks of RT ($p=0.08$), whereas a decrease was seen in the placebo group ($p<0.05$) (Figure 12). This response differed between the protein and placebo groups ($p<0.05$). In the young, a significant decrease in myogenin mRNA was observed at 21 weeks of RT in the placebo group but not in the protein group. In the older, no effects of long-term protein ingestion was studied but a significant increase in myogenin mRNA level was observed in response to RT without additional protein supplementation ($p<0.001$) (II).

### 5.3.3 Phosphorylation of muscle protein kinases

The phosphorylation of mTOR was increased only in the protein group at 21 weeks ($p<0.05$) (Figure 15). The phosphorylation of Akt decreased after 21 weeks of RT in both RT groups ($p<0.05$) (Figure 15). The phosphorylation of eEF2, $p70^{S6K}$, rpS6 and total protein levels of $p70^{S6K}$, Akt and rpS6 were unaffected by the RT period (Figures 15 and 16).

### 5.3.4 Correlations between the biological responses to the RE bout and the change in the training-induced muscle size

In the young placebo group, individual changes in $VL_1$, $VL_2$ as well as $QF_1$ and $QF_2$ (all from approximately the muscle biopsy location) correlated negatively with the 21-week change in myostatin mRNA ($r= -0.76$ to $r= -0.86$, $p=0.006$ to $p=0.01$). There was also a negative correlation in VL change with myostatin mRNA change at post 48h in the placebo group being significant in $VL_4$ ($r= -0.71$, $p=0.05$). Therefore the larger the delayed or more chronic decrease in the myostatin transcript level, the greater the VL muscle CSA increase during RT. In agreement with this, in the older men at the pre-RT situation, the RE-induced 48h change from pre-RE in myostatin mRNA levels correlated inversely with the increase in total body muscle mass (Figure 18) and muscle thickness of the sum of vastus lateralis and intermedius during the RT period ($r= -0.82$, $p=0.002$ and $r= -0.52$, $p=0.12$, respectively). However, in the young, the similar relationships were not seen with the increase in fiber size. Surprisingly, in the young placebo group, the fast 1h change in myostatin mRNA was positively correlated to muscle average VL CSA increase during RT (average VL: $r=0.85$, $p=0.02$).

Relative changes in AR protein concentration during 21 weeks of RT correlated with the relative changes in average type I and II muscle fiber CSA ($r = 0.62$, $p<0.001$), type I fiber CSA ($r = 0.72$, $p<0.01$), lean body mass ($r = 0.55$, $p<0.05$) and vastus lateralis thickness ($r = 0.47$, ns. $p=0.058$) in the combined group of young and older men (Figure 18).

There were no significant correlations between the RE- or RT-induced change in phosphorylated protein kinases or in the myostatin protein with corresponding RT-induced changes in the VL fiber size or the muscle thickess measured by ultrasonography or the VL cross-sectional area measured by MRI.

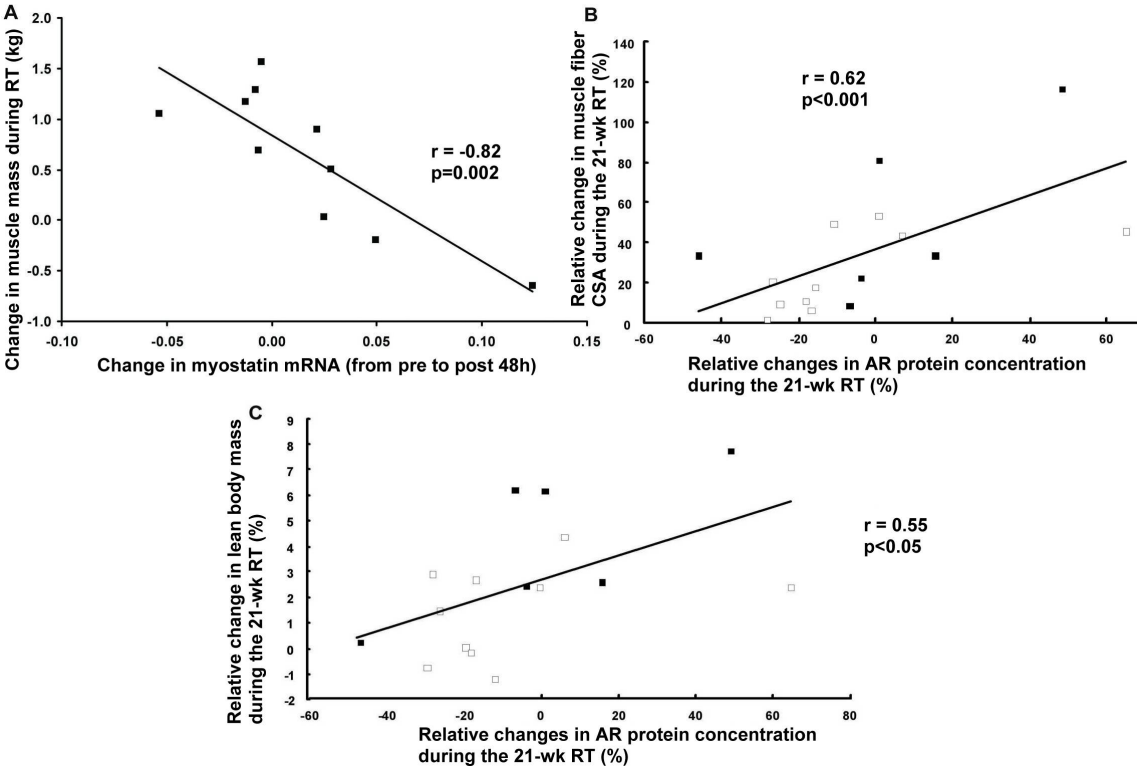


FIGURE 18 The correlation between muscle mass change and muscle A) RE-induced 48h change in myostatin mRNA, and B) and C) 21-week RT-induced change in the AR protein concentration. The muscle mass/size method was in A) bioimpedance, in B) average type I+II VL muscle fiber size and C) lean body mass by weight scale and by scalpels (body fat). In B and C, black squares = YT, young men training group, white squares = OT, older men training group.

### 5.3.5 Immunohistochemistry

*Muscle fiber CSA.* In (VI), VL CSA of type I and II fibers increased significantly after 21 weeks of RT in both young protein (mean ± SE, 42.2 ± 11.1% and 54.5 ± 11.4%, respectively) and placebo groups (44.0 ± 11.4% and 55.6 ± 14.9%) ($p<0.01$) and also significantly ($p<0.05$) compared to the control group (3.5 ± 2.3% and 3.1 ± 6.0%, $p>0.2$). There was no significant difference in the change between protein and placebo groups. In (VII), fiber size increased in young (49 ± 41%) and older males (31 ± 21%) during the 21-week RT period ($p<0.05$), but the change in older men in comparison with the control group did not reach statistical significance.

*Localization of ARs, mTOR and rpS6.* The most intensive staining of ARs was clearly in the nuclei in both fiber types I and II (Figure 19). There may be more ARs at or close to the cell borders of the type I fibers but this was not quantified. Both phosphorylated mTOR at $Ser^{2448}$ and rpS6 at $Ser^{235/236}$, as well as total rpS6, were localized primarily close to nuclei and sarcolemma, outside an area where contractile proteins are located (Figure 20. A large part of the signal for these proteins emanated from inside the muscle fibers but also to some extent outside the sarcolemma.

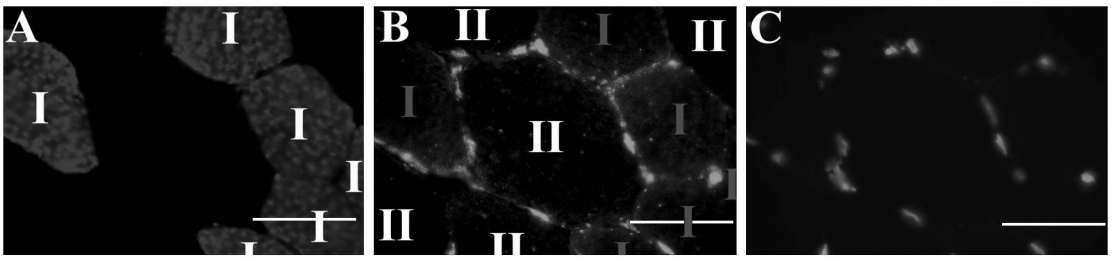


FIGURE 19 Immunofluorescence staining of VL muscle cross-sections with MyHC I (A), androgen receptors (B), and nuclei with Hoechst (C). High magnification microscopic images show that AR staining within muscle fibers is highest in the nuclei while some less intensive staining is found near the nuclei in both type I and type II muscle cells. Control staining (see methods) that the specific AR staining was mostly nucleolar. Scale bars are 50µm.

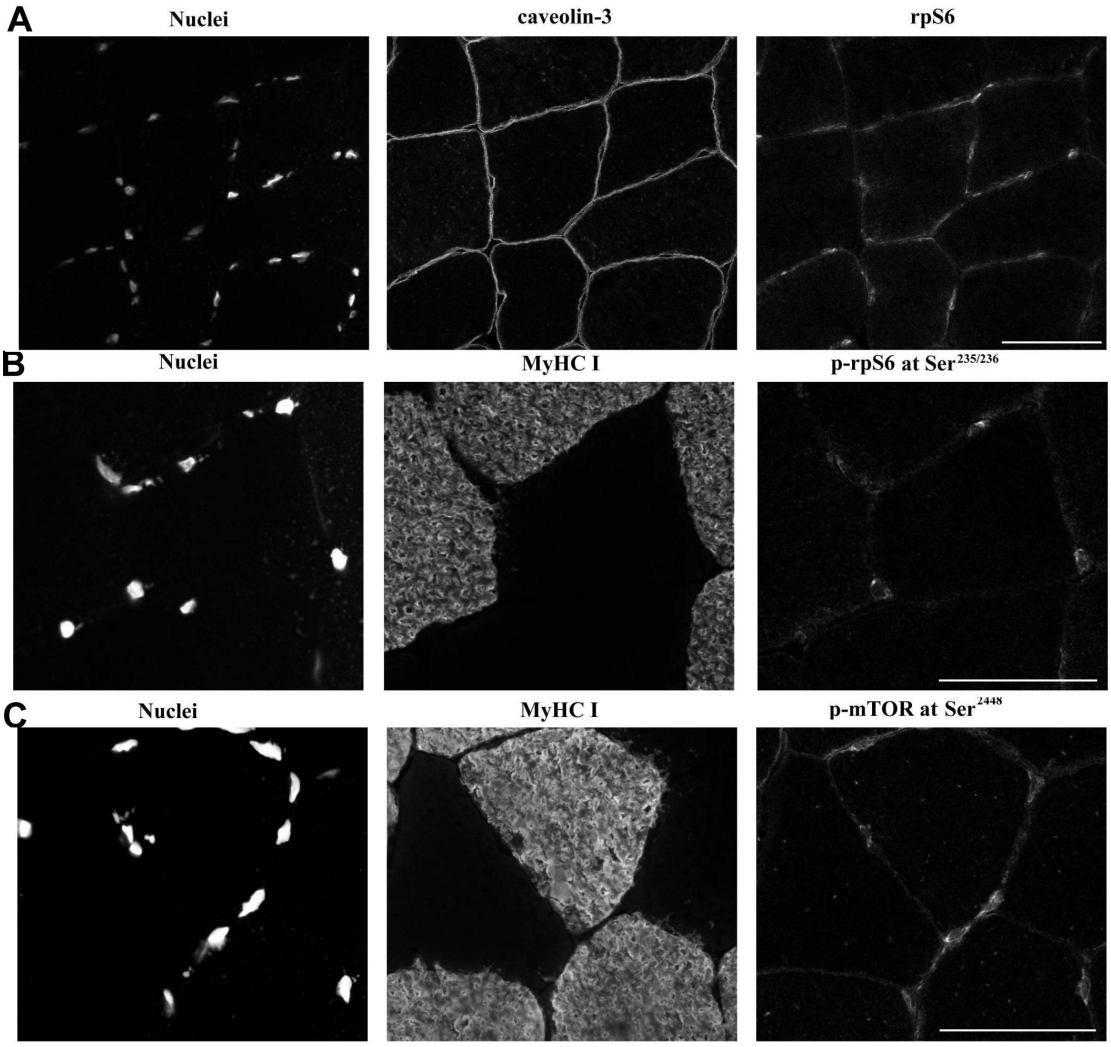


FIGURE 20 Confocal microscopy images of total (A) and phosphorylated rpS6 (B) and phopshorylated mTOR (C) in VL muscle cross-sections. Nuclei were stained with Hoechst, sarcolemma with anti-caveolin-3 and myofibrillar area with anti-MyHC I. Phosphorylated mTOR at $Ser^{2448}$, rpS6 at $Ser^{235/236}$, and total rpS6 were primarily localized close to the nuclei and sarcolemma (caveolin-3), outside the area where contractile proteins are located (MyHC). The images were obtained with the settings in which the secondary antibody (not shown) only gave minimal signal. Scale bars are 50µm.

## 5.4 Summary of the results

In this thesis, the effect of a single resistance exercise bout or long-term resistance training period with or without protein ingestion, were investigated. Table 5 summarizes the main quantified results of the study.

TABLE 5 Summary of the main results. ↑ = upregulated, ↓ = downregulated, ↔ = no change. US = ultrasonography, BI = bioimpedance. Bolded results are the main findings

| **Variable** | **Effect of the RE bout** | **Effect of protein on RE bout responses** | **Effect of the RT period** | **Effect of protein during RT (young)** |
|---|---|---|---|---|
| **Muscle size and strength** | | | | |
| MRI: CSA | | | ↑ | ↑ |
| Muscle fiber size | | | ↑ young > older | ↔ |
| Muscle thickness (US) | | | ↑ | ↑ ↔ |
| Muscle strength: dynamic and isometric | | | ↑ young ≈ older | ↔ |
| **Muscle mRNA** | | | | |
| Myostatin | **↓ (↔ untrained older)** | **prevented ↓** | ↑ (older) ↓ ↔ (young) | ↔ |
| AcvrIIb | ↓ | ↔ | ↔ | ↔ |
| FLRG | ↔ | ↑ ↔ (older) ↔ (young) | ↔ | ↔ |
| Myogenin | ↑ (at 48h) | ↔ / prevented ↓ (young) ↔ (older) | ↑ (older) ↓ ↔ (young) | ↔ / prevented ↓ |
| MyoD | ↔ | ↔ | ↔ | ↔ |
| Cdk2 | ↔ | ↑ | ↔ | ↑ |
| p21 | ↑ | ↔ | ↔ | ↔ |
| MAFbx | ↔ | ↔ | ↔ | ↔ |
| MGF | ↔↑ | ↔ | ↔ | |
| IGF-IEa | ↔↑ | ↔ | ↔ | |
| AR | ↔↑ | ↔ | ↔ | |
| **Muscle protein** | | | | |
| AR | ↔ | **↔** | ↔ | |
| Myostatin | ↓ | **Prevented ↓** | ↔ | ↔ |
| $p70^{S6K}$ | ↔ | ↔ | ↔ | ↔ |
| Akt | ↔ | ↔ | ↔ | ↔ |
| rpS6 | ↔ | ↔ | ↔ | ↔ |
| **Phosphorylated muscle phosphoproteins** | | | | |
| $p70^{S6K}$ at $Thr^{389}$ | ↑ | ↑ | ↔ | ↔ |
| mTOR at $Ser^{2448}$ | ↔ | ↑ | **↔** | ↑ |

| | | | | |
|---|---|---|---|---|
| Akt at $Ser^{473}$ | ↔ | ↔↑ | ↓ | ↔ |
| rpS6 at $Ser^{235/236}$ | ↑ | ↔↑ | ↔ | ↔↑ |
| 4E-BP1 at $Thr^{37/46}$ | ↓ | **Prevented ↓** | ↔ | ↔ |
| eEF2 at $Thr^{56}$ | ↔ | ↔ | ↔ | ↔ |
| **Serum hormones** | | | | |
| Testosterone | ↑ | **Prevented ↑** | | |
| Insulin | ↔ | ↔↑ | | |

The effects of the RE bout or the RT period on protein or phosphoprotein levels of myostatin, $p70^{S6K}$, Akt, rpS6, 4E-BP1, mTOR and eEF2 and mRNA of MAFbx were investigated only in the young. Also, the effects of the RT period on p21 and cdk2 mRNA were also measured only in the young. The effect of protein ingestion on AR mRNA and protein and on IGF-IEa and MGF mRNA was investigated only in the older men

# 6 DISCUSSION

This study investigated the effects of heavy resistance exercise with or without protein ingestion. This type of exercise has previously been shown to increase muscle hypertrophy. It was found that protein ingestion in the context of a resistance exercise workout can affect resistance exercise-induced responses at the levels of blood hormones, muscle gene expression both at the mRNA and protein level, phosphorylation of muscle protein kinases, and finally also whole muscle size.

## 6.1 Acute responses after a resistance exercise session

Skeletal muscle is a tissue with high capacity to increase its size and strength after mechanical loading such as resistance training (Morpurgo 1879; Goldberg 1967; Goldberg et al. 1975; Kraemer et al. 2002; Kraemer and Ratamess 2004; Wernbom et al. 2007). Training adaptations of this kind are thought to result from the cumulative effects of repeated bouts of resistance exercise on post-exercise molecular responses (Haddad and Adams 2002). Since these acute post-exercise responses are still poorly understood, they were investigated in the present thesis along with longer term adaptations.

### 6.1.1 Post-exercise responses with or without protein ingestion

Ingestion of nutrition containing amino acids, such as protein, in the context of a resistance exercise (RE) workout has gained large popularity not only in the gym but also in research during the last few years. This is partly because without ingestion of amino acids or protein soon before or after a RE bout the muscle protein net balance (synthesis - degradation) remains negative (Biolo et al. 1997; Tipton et al. 1999; Tipton et al. 2007) and some studies have suggested that with protein ingestion the muscle hypertrophy response to a longer term training period may be substantially larger (Esmarck et al. 2001; Cribb and Hayes 2006). However, the mechanisms underlying and the responses to post-

and/or pre-RE protein nutrition are unclear. Therefore, the effects of the ingestion of high quality protein were investigated in the present study in humans. Many novel results emerged from the study, the major ones of which are discussed below.

**Testosterone and androgen receptors.** An acute bout of hypertrophic-type heavy RE with multiple sets and repetitions transiently elevated blood testosterone concentrations as has been found earlier (Häkkinen and Pakarinen 1993; Chandler et al. 1994; Kraemer et al. 1998; Bloomer et al. 2000; Ahtiainen et al. 2003; Ahtiainen et al. 2005; Kraemer et al. 2006). Heavy training of this kind will eventually lead to muscle hypertrophy (Häkkinen et al. 2001b; Campos et al. 2002; Kraemer et al. 2002; Hubal et al. 2005; Wernbom et al. 2007). It could therefore be speculated that this transient increase in blood testosterone has some importance in muscle adaptation to training, since testosterone is clearly positive for muscle size and regulation (Bhasin et al. 1996; Ferrando et al. 1998; Kadi et al. 1999; Sinha-Hikim et al. 2003; Eriksson et al. 2005; Kvorning et al. 2006; Sinha-Hikim et al. 2006). Surprisingly, such an acute increase in blood testosterone has been shown to be attenuated by post- or pre- and post-exercise feeding in young men (Chandler et al. 1994; Kraemer et al. 1998; Bloomer et al. 2000; Kraemer et al. 2006). The results of the present study extends these findings, showing that ingestion of milk proteins or whey alone before the RE bout can attenuate the increase in serum testosterone during the bout in different groups of individuals; i.e. in moderately trained older men, and in untrained and trained young men. The results of the present study and others (Hoffman et al. 2006; Kalman et al. 2007) have shown that the effect of protein ingestion on blood testosterone does not seem to be long-lasting.

Mechanism underlying the transient decrease in testosterone when protein is ingested is unknown, but it may be related to an increased content of the testosterone receptors and, therefore, possibly also to the enhanced uptake of testosterone to muscle fibers as suggested recently (Kraemer et al. 2006). However, in the older men in the present study, no positive effect of protein feeding on **androgen receptor** content was found at the protein level and there was only a trend for an increase at mRNA level. Therefore, other explanations for the attenuation of the testosterone response to protein ingestion have to be taken into account. Pre- and post-exercise nutrition can re-distribute part of the blood flow away from active muscles to the gastrointestinal tract (McKirnan et al. 1991). This could transiently lower the uptake of hormones to muscles during a bout of RE. Nutrition may also possibly acutely decrease the testicular circulation and therefore transiently decrease testosterone release into circulation (Wang et al. 1983). Clearly, the mechanisms underlying the decrease in blood testosterone following protein ingestion remain to be studied in the future.

It was also found, however not quantified, that at or near the cell borders of type I muscle fibers of the present subjects there seemed to be more intensive androgen receptor-spesific staining compared to type II fibers, at least in the VL muscle. If the fiber type difference really exists, it may be explained by the findings that type I fibers may have more myonuclei (Tseng et al. 1994) and

capillaries (Andersen 1975) surrounding them as well as possibly satellite cells (Schmalbruch and Hellhammer 1977) compared to type II fibers because myonuclei, vascular endothelial and smooth muscle cells, mast cells and satellite cells all express androgen receptors (Sinha-Hikim et al. 2004). Interestingly, many studies suggest that exogenous androgen administration-induced increase in muscle size can be larger in type I muscle fibers compared to type II fibers (Hartgens et al. 1996; Kadi et al. 1999; Sinha-Hikim et al. 2003; Ustunel et al. 2003; Eriksson et al. 2005; Sinha-Hikim et al. 2006).

**Myostatin.** The gene expression of myostatin decreased after the RE bout in the placebo groups of both young and older men. The acute decrease in myostatin mRNA following a RE bout is supported by large number of studies (Kim et al. 2005a; Coffey et al. 2006a; Raue et al. 2006; Kim et al. 2007; Kvorning et al. 2007; Louis et al. 2007; Mascher et al. 2008). The decrease in myostatin expression after an exercise bout is theoretically advantageous for muscle hypertrophy since myostatin inhibits muscle protein synthesis and growth (McPherron et al. 1997; Gonzalez-Cadavid et al. 1998; Schuelke et al. 2004; Welle et al. 2006).

The fact that the decrease in myostatin protein was also observed in the young suggests trascriptional regulation because the myostatin mRNA and protein level changes from pre to post 1h also correlated positively ($r=0.66$, $p=0.007$). It has been assumed that the detected ~26 kDa myostatin is a glycosylated tightly bound dimer of a 110 amino acid carboxy-terminal peptide of myostatin and/or that the monomer of myostatin is strongly bound by some other protein (Gonzalez-Cadavid et al. 1998; Taylor et al. 2001). In a recent human study by Kim et al. (2007) a myostatin propeptide of size 28 kDa and myostatin protein complex of size 50 kDa remained un-changed 24 hours after a bout of RE, but the latter seemed to increase after 16 weeks of RT. The present study is the first to show in humans that the myostatin peptide, thought to be the active form of myostatin, can follow RE-induced decreased myostatin transcript levels. Uncertainty exists, however, about the myostatin protein and its function in humans, and therefore at the moment, myostatin protein results in humans need to be interpreted with caution.

Another novel finding was the effect of protein ingestion on acutely preventing the decrease in myostatin expression after the RE bout. The effect of protein has also previously been shown to be important for this response in rats (Nakazato et al. 2006). However, the results on different animal species and in different study settings on the effects of various nutritional intakes on the expression of myostatin are conflicting (Carlson et al. 1999; Chauvigne et al. 2003; Jeanplong et al. 2003; Guernec et al. 2004; Nakazato et al. 2006; Terova et al. 2006; Smith et al. 2008). The only published study on humans showed recently that in a resting state without exercise, myostatin mRNA decreased in old men and women after feeding a mixed meal (Smith et al. 2008). Our results suggest that it is possible that nutrition has a different effect in the context of exercise. However, the effect of protein (whey) alone may also be different from that of a mixed meal. It also has to be admitted that the different timing of the biopsies may also have resulted in a different finding, and therefore definite

conclusions on the theoretically negative effect of protein ingestion on myostatin expression should not be drawn. The observed larger increase in mTOR signaling, and cdk2 mRNA, similar or larger myogenin mRNA and similar responses in p21, p27 and MyoD mRNA after the RE session in the protein compared to the placebo group indicate the possibility that protein ingestion did not increase myostatin signaling as these molecules are downstream of myostatin (Rios et al. 2002; McCroskery et al. 2003; Amirouche et al. 2009).

**Activin receptor IIb.** The gene expression of the myostatin receptor, activin receptor IIb, decreased after the RE bout, which if it also led to decrease in the amount of its protein content, may lower myostatin signaling in muscle. This is because myostatin mediates its signals mainly through this receptor (Lee and McPherron 2001). This response may, therefore, be advantageous for muscle hypertrophy. Recent study, however, reported no change for activin receptor IIb 24 hours after a RE bout or after 16 weeks of RT (Kim et al. 2007). As far it is known, in healthy humans no other exercise or training studies examining this receptor exists. Protein supplementation in the present study was not found to affect the RE-induced gene expression of activin receptor IIb in either the young or older men. Further studies need to be conducted to confirm the findings observed in this thesis, also in the level of protein content of this receptor.

**Cdk2.** Gene expression of cyclin-dependent kinase 2 (cdk2) increased significantly after the RE bout, but only in the protein group, in both older and young men. In the protein group in the young men it was also found to be elevated after 21 weeks of RT when compared to the placebo group. Some studies suggest that increased cdk2 could be theoretically positive for muscle hypertrophy. Cdks are important in cell proliferation (Malumbres et al. 2000; Berthet et al. 2003) and cdk2 knockout mice are slightly smaller than wild-type mice (Berthet et al. 2003). The expression as well as activity of cdk2 are increased in proliferating myoblasts (Ohkubo et al. 1994; Hlaing et al. 2002; McCroskery et al. 2003). It is, therefore, possible that whey protein ingestion before and/or after a RE workout may increase satellite cell proliferation. When repeated during long-term training, this may lead to an increased number of satellite cells, a speculation supported by a recent study (Olsen et al. 2006). An *in vitro* study in turkey cells also supports this hypothesis: nutrition feed increased DNA synthesis and satellite cell number when compared to a food-deprived state, and this increase was also accompanied by upregulating the expression of myogenic transcription factor, myogenin (Halevy et al. 2003). Also, in the present study protein ingestion in young men seemed to prevent the small but rapid decrease in **myogenin** gene expression after the single RE bout and also the decrease after the 21 weeks of resistance training, both decreases being observed only in the placebo group. Myogenin is an important regulator for muscle satellite cell differentiation (Rios et al. 2002; Charge and Rudnicki 2004) but it is also expressed inside the muscle fibers (Ishido et al. 2004; Kadi et al. 2004a) and thus probably makes a significant contribution to the adaptive processes of the muscle fiber itself.

Some of the larger cdk2 gene expression responses observed with protein ingestion may also reflect other proliferating cells than satellite cells (or other muscle myogenic cells), such as fibroblasts, immune cells and endothelial cells (Kovanen 2002; Sinha-Hikim et al. 2004). Recent study in humans showed that one week of low protein intake *decreased* several transcript levels in muscle that relate positively to cell proliferation and, vice versa, *increased* transcript levels that negatively regulate cell proliferation (Thalacker-Mercer et al. 2007). Some studies have shown increased recovery from heavy exercise and decreased muscle damage or soreness when nutrition containing amino acids is ingested in the context of exercise (Nosaka et al. 2006; Shimomura et al. 2006; Cockburn et al. 2008). Neither this phenomenon nor the number of satellite cells or other proliferating cells was directly investigated in the present thesis but it is tempting to hypothesize that the possible protein-induced larger cell proliferating capacity may have something to do with this possible enhanced muscle recovery after a heavy exercise workout. More in-depth studies on protein level are clearly needed.

Cdks are inhibited by cdk inhibitors such as **p21** and **p27** (Malumbres et al. 2000). In the present thesis, the gene expression of p21 increased after the RE bout especially in young men but p27, which was measured only in the older men, remained stable. In previous studies, gene expression of p21 increased in humans 24 hours after two bouts of electromyostimulation-induced loading (Bickel et al. 2003) and during compensatory hypertrophy (Adams et al. 1999) and resistance training (Kadi et al. 2004b); however, no changes after a bout of RE or RT were reported for the gene expression of p21 and p27 (Kim et al. 2007) but a decrease in p27 mRNA and protein content after a bout of RE in some subject groups (Bamman et al. 2004; Kim et al. 2005a). The averagely increased gene transcript levels of myogenin and p21 at 48h after the RE bout in the present thesis may suggest ongoing muscle satellite cell differentiation (Rios et al. 2002), while the increased gene expression of cdk2 in the protein group may indicate that some of the cells capable proliferating continue to do so especially when protein is ingested (McCroskery et al. 2003). The cdk2 and p21 responses appeared to be more pronounced in the young men, which may be speculated to reflect the possible age-related decline in responsiveness of muscle satellite cells, an assumption supported by a recent study (Dreyer et al. 2006a).

**mTOR signaling.** S6 protein kinase of size 70 kDa (**p70$^{S6K}$**) is an enzyme downstream of the TORC1 complex that contains mTOR and its regulatory proteins (Pullen and Thomas 1997; Kimball and Jefferson 2006). Whey protein intake increased the phosphorylation of p70$^{S6K}$ on Thr$^{389}$ 1h after RE bout. It can be speculated that this phosphorylation increased its activation because phosphorylation from this site is the chief event in the activation of p70$^{S6K}$ (Pullen and Thomas 1997) and since there was also a tendency for larger phosphorylation of one of its downstream target, **rpS6**. The results agree with those of a recent study showing that protein intake together with carbohydrate before, during and 1h after a RE bout increased phosphorylation of p70$^{S6K}$ at post 0 to 4h compared to carbohydrate only (Koopman et al. 2007). Interestingly, in the present study the phosphorylation of the second isoform of S6K1, **p85$^{S6K}$**,

clearly followed the same pattern as that of $p70^{S6K}$. This result is interesting since S6K1/$p70^{S6K}$ has been shown in animal and cell models to be important in muscle hypertrophy (Shima et al. 1998; Baar and Esser 1999; Ohanna et al. 2005).

The protein intake prevented a decrease in the phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein (**4E-BP1**), which was observed in the placebo group supporting a recent finding in humans using a slightly different time-scale and nutrients (Koopman et al. 2007). A RE bout itself without supplementary feeding has also been shown previously to decrease 4E-BP1 phosphorylation shortly after the RE bout (Dreyer et al. 2006b; Koopman et al. 2007; Deldicque et al. 2008; Mascher et al. 2008). It can be speculated that prevention of the dephosphorylation of 4E-BP1 after a bout of RE by ingestion of whey proteins probably prevents association of the 4E-BP1 with eIF4E (Prod'homme et al. 2005). This would allow a larger increase in protein synthesis due to the importance of eIF4E in the translation initiation process (Mader et al. 1995; Gautsch et al. 1998; Prod'homme et al. 2005; Kimball and Jefferson 2006). This could be one route through which whey proteins effectively increase muscle protein synthesis compared to a state of no nutritional supplementation (Tipton et al. 2004; Tipton et al. 2007; Katsanos et al. 2008).

**Whey protein and mTOR cascade.** It can be speculated that the consumption of whey proteins alone can activate the TORC1 complex of mTOR and its regulatory proteins. This sequence of events phosphorylates and activates $p70^{S6K}$ and hinders the dephosphorylation of 4E-BP1, the latter possibly also through a mechanism independent of TORC1 (Jacinto et al. 2004; Wang et al. 2005; Vary et al. 2007). The present results suggest that these effects occurred independently of blood insulin or phosphorylation of Akt at $Ser^{473}$. The increased phosphorylation and activation of $p70^{S6K}$ may be the reason for observed increased phosphorylation of **mTOR** itself at $Ser^{2448}$ (Chiang and Abraham 2005). Possibly a large increase in $p70^{S6K}$ phosphorylation is needed for this effect, since the smaller increase in the placebo group did not lead to increased phosphorylation of mTOR at this site in our study design. The phosphorylation of mTOR at $Ser^{2448}$ may be positive for the mTOR signaling and muscle hypertrophy, since an increase in phosphorylated mTOR from $Ser^{2448}$ has been shown to occur in parallel with the increase in muscle protein synthesis after the ingestion of amino acids and carbohydrates in human muscle (Dreyer et al. 2008). Furthermore, in rats, an increase in phosphorylation at this site has occurred along with a loading-induced muscle hypertrophy (Reynolds et al. 2002).

The phosphorylated mTOR was localized mainly close to the sarcolemmal membrane supporting the studies in rodents (Parkington et al. 2003; Hornberger et al. 2006). Most of the rpS6, phosphorylated or not, was located very close to nuclei supporting the earlier finding of phosphorylated rpS6 in humans (Koopman et al. 2007). Close proximity of this ribosomal protein to the nuclei is theoretically optimal for efficient protein synthesis.

In summary, the supplementation of whey proteins alone can enhance the mTOR signaling response to resistance exercise session, which theoretically can strengthen the adaption of muscle to resistance training.

**MAFbx.** In addition to the pathways of protein synthesis and cell cycle signaling, the regulation of protein breakdown signaling was also of interest in the present study since protein ingestion may have a minor acute effect on decreasing endogenous muscle protein degradation during heavy exercise (Nagasawa et al. 1998; Tipton and Wolfe 2001; Beelen et al. 2008a). The present results suggest that protein ingestion close to a RE bout does not have an acute effect on ubiquitin-ligase MAFbx (also called atrogin-1) transcript levels, one of the factors important in muscle protein degradation and atrophy (Bodine et al. 2001a). Therefore, if protein ingestion affects RE-induced proteolysis it is probably not through the transcript levels of MAFbx. It is possible that the whey protein fraction of milk, as compared to casein, as has been shown in the resting state by others (Boirie et al. 1997; Dangin et al. 2002), has also only a minor effect on protein degradation during a bout of RE. This is obviously an area calling for future study.

***The possible mechanisms*** underlying the effect of protein ingestion on muscle myostatin, cdk2, and mTOR signaling responses are unclear. Whey peptides or more probably particular amino acids (Ha and Zemel 2003) could affect gene expression and the phosphorylation of protein kinases in skeletal muscle when the muscle metabolism is at its most active. For the mTOR signaling, probably the most important ingredient in whey is its large content of branched chain amino acids, previously shown to elicit a similar $p70^{S6K}$ response in the context of a bout of RE as observed in the present study (Karlsson et al. 2004). It has been recently shown that the upstream mechanism activating mTOR signaling for some amino acids may operate through an increase in intracellular $Ca^{2+}$ which can in turn activate kinase hVps34 (human vacuolar protein sorting 34) (Gulati et al. 2008). Another possible pathway may be a mechanism utilizing Rag GTPases (Kim et al. 2008; Sancak et al. 2008). These and possible other upstream mechanisms remain to be studied in humans.

In summary, the resistance exercise bout transiently decreased myostatin gene expression in both young and older men, and the protein level study with young men showed that the decrease may also manifest as a decrease in myostatin protein concentration. However, whey protein ingestion before and after resistance exercise bout, seemed to prevent the decrease in myostatin expression without parallel changes in myostatin signalling pathway molecules. Protein ingestion increased cdk2 gene expression and mTOR signaling after the resistance exercise bout, a response which may positively relate to muscle recovery and hypertrophy. This also proves that controlling nutrition intake is important when studying molecular responses to an exercise session. Future research should chemically manipulate individual signaling pathways (by e.g. rapamycin that inhibits mTOR signalling) to investigate whether some or all of these signaling responses are sufficient or important independently for muscle hypertrophy to occur after months of training in humans.

### 6.1.2 Effects of the training state of muscle

The present studies did not show training state to have a dramatic effect on an acute RE-induced gene expression. Some differences, however, existed. Myostatin mRNA decreased following the RE bout in older men, but only after 21 weeks of training. In young men the effects of the training period on myostatin was not investigated, but a decrease in myostatin was already seen in the previously untrained subjects, suggesting a possible effect of aging. The average post-RE mRNA of **MyoD** was larger after the training period, which can be speculated to reflect enhanced satellite cell differentiation after exercise in the trained muscle; however, this hypothesis needs to be studied further. **IGF-IEa** mRNA was higher 48 hours after the RE bout only in the strength-trained young men, suggesting that for IGF-IEa, years of training or larger exercise volume may enhance its delayed up-regulation after a training session. Gene expression of IGF-IEa in humans following a bout of RE have been inconsistent; either an increase (Kim et al. 2005b; Greig et al. 2006) or no change has been found (Hameed et al. 2003; Psilander et al. 2003). The present results suggest that for some proteins thought to regulate muscle size the gene expression response to an exercise session may vary depending on the training state of the muscle. This possible effect of training state on gene expression tends to agree with the results of a recent cross-sectional study (Coffey et al. 2006a). Some studies also suggest that the same may, at least in part, be also true with mTOR signaling (Coffey et al. 2006b; Wilkinson et al. 2008). RT period may also attenuate and shorten the duration of the total muscle protein synthesis response to an acute bout of RE (Phillips et al. 1999; Phillips et al. 2002; Tang et al. 2008; Wilkinson et al. 2008) and may concentrate the response more in the myofibrillar proteins (Wilkinson et al. 2008). The molecular events behind these changes are unclear.

In summary, these results show that the expression of some but not all gene transcripts can vary depending on the training state of the individual. Nevertheless, controlling the training state is important when studying gene expression responses to exercise.

## 6.2 Muscle adaptation to long-term resistance training (II, V, VI, VII)

### 6.2.1 Muscle size and strength

**Effects of protein ingestion.** Ingestion of protein or essential amino acids during RT has increased muscle fiber cross-sectional area or lean body mass in most (Burke et al. 2001; Andersen et al. 2005; Bird et al. 2006; Candow et al. 2006a; Holm et al. 2006; Kerksick et al. 2006; Cribb et al. 2007; Cribb et al. 2007; Hartman et al. 2007; Hartman et al. 2007; Willoughby et al. 2007; Willoughby et al. 2007) but not in all studies (Antonio et al. 2000; Godard et al. 2002; Candow

et al. 2006b; Olsen et al. 2006; Verdijk et al. 2009). The present study investigated the effects of timed ingestion of protein alone close a RE workout on training-induced whole muscle hypertrophy in young men, using high-quality magnetic resonance imaging (MRI). The subjects who ingested 15 grams of whey proteins both immediately before and after each RE workout, two times a week for 21 weeks, had larger vastus lateralis (VL) muscle hypertrophy than the placebo group. This result, in agreement with the finding of earlier studies (Andersen et al. 2005; Cribb and Hayes 2006), seems to further suggest the importance of consuming high quality protein-containing nutrition soon after and possibly also before or during each heavy RE workout. It may be speculated that the reason for the significant difference observed, however, only in the VL muscle of the quadriceps femoris muscle group, may be that our selection of exercises was designed to specifically load and increase the size of the VL muscle. On average, VL had the greatest training-induced hypertrophy of the quadriceps femoris muscles which supports earlier results utilizing a similar training program (Häkkinen et al. 2001b).

Also somewhat faster increase in muscle thickness and body mass with protein ingestion was found. However, muscle fiber sizes were not observed to be larger after the full 21-week RT period. It can be speculated that protein ingestion may have shown bigger effects in subjects with a smaller habitual ingestion of protein than ~1.4-1.5 g / body weight observed, as can be suggested based on some earlier studies (Tarnopolsky et al. 1992; Hoffman et al. 2006). Alternatively, using subjects with a high level of training background could have resulted in more robust effects. In untrained subjects the variability of an adaptation in muscle size to even a short period of RT is enormous (Hubal et al. 2005), and this may override the effects of protein, especially in a measure with a high variation such as muscle fiber size. The training volumes (loads x sets x repetitions) for the leg extensor muscles (leg press and knee extension) between the protein and placebo groups were similar between the protein and placebo groups throughout the training period.

Various muscle strength variables were also studied. Of those, protein ingestion had possible effect only on the isometric leg strength, where the increase was significant compared to controls only in the protein group. The finding that protein did not have consistent effect on maximal muscle force is in agreement with some (Burke et al. 2001; Andersen et al. 2005; Candow et al. 2006b; Kerksick et al. 2006; Verdijk et al. 2009), but not all previous studies (Candow et al. 2006a; Holm et al. 2006; Cribb et al. 2007). The small effect of protein ingestion on muscle force in relatively short-term 6-21 week training studies conducted usually with previously untrained subjects may be due to neural mechanisms, which may in turn explain most of the force production enhancement instead of the increase in muscle size during the first weeks of RT (Häkkinen et al. 2001b). Neural mechanisms may not be affected significantly by protein ingestion.

A recent study (Verdijk et al. 2009) examined the effect of 10 grams of casein proteins ingested immediately before and after each RE workout during a 12-week RT period in older men and found no larger muscle hypertrophy or

strength increases than in the placebo group with a daily average protein intake of ~1.1 g / kg body weight in both groups. Therefore, both that study and the present thesis shows that while many short-term positive effects of protein or amino acid ingestion are often clear when studied acutely after each exercise bout, the long-term positive effects are less evident, especially if daily normal nutrition is unrestricted. This is especially true if the training session is conducted in a fasting state, as is too often the case. Clearly, more evidence gathered using different protein sources, different subject groups and different training protocols are needed with subjects in the normal nutritional state instead of fasting before an exercise bout. The effects of gender, in addition to age also need to be studied in more detail in future.

**Effects of age.** There was a tendency for larger muscle hypertrophy in the young compared to older subjects during the 21-week RT period. Some previous studies have shown a smaller relative muscle hypertrophy response to RT in older compared to the young subjects (Moritani and deVries 1980; Welle et al. 1996; Kosek et al. 2006), while some studies have not found any such difference (Häkkinen et al. 1998a; Roth et al. 2001). The possible existence of an age-difference may depend on the training programs used (e.g., frequency and volume of training), ages being compared or even gender (Ivey et al. 2000; Häkkinen et al. 2001a). It is probable that the optimal training program will differ between young and older individuals. The present twice weekly heavy whole body training, even when highly periodized, may have been too demanding for some individuals, especially those in the older group. The reason for this may also be the relatively low energy and protein intake in the older men compared to the young men, as energy intake of the young men was significantly larger during the first week of the study. However, muscle strength adaptation to training was not affected by age.

To summarize, protein ingestion before and after each resistance exercise workout enhanced the increase in whole muscle size during the resistance training period when measured by MRI. Protein ingestion also seemed to accelerate the increase in body mass and muscle thickness. However, this larger increase with protein ingestion at the whole muscle level was not accompanied by enhanced muscle fiber hypertrophy or muscle strength in the present previously untrained young men. This may be explained by the rather large habitual ingestion of protein-containing nutrition in these subjects even without the supplementary protein. Muscle hypertrophy, but not strength, tended to increase more after the resistance training period in the young compared to the older men.

### 6.2.2 mTOR pathway signaling and gene expression of cdk2

The only long-lasting and consistent effect of protein ingestion was the increased phosphorylation of mTOR which remained increased in the protein group from 1 to 48h after the RE before the training and also after 21 weeks of RT. Surprisingly, the phosphorylation of Akt decreased ~0.5 fold in both training groups after 21 weeks of RT. In previous studies 8-10 weeks of RT

increased the phopshorylation of Akt at the same amino acid site ($Ser^{473}$) (Leger et al. 2006; Wilkinson et al. 2008). This different response may, owing to possible complexity of the temporal pattern of the Akt phosphorylation, depend on the timing of the biopsies or additionally on the length or type of the training period. Also different nutritional states in the present study compared to the other studies may have affected (Leger et al. 2006; Wilkinson et al. 2008). The phosphorylation of $p70^{S6K}$ and 4E-BP1 remained at resting levels after the RT period supporting previously published results on $p70^{S6K}$ (Leger et al. 2006; Wilkinson et al. 2008) and 4E-BP1 (Leger et al. 2006). A new finding was that also the phosphorylated elongation factor **eEF2** did not change due to the RT period. Therefore, the more chronic phosphorylation of mTOR and Akt which both showed change may be regulated differently or through other pathways than these other TORC1 and TORC1 pathway proteins which remained unchanged. In addition to these changes in the factors regulating translation, as was already mentioned in the cdk2 paragraph, transcript levels of cdk2 remained to be elevated after 21 weeks of RT in the protein group when compared to the placebo group. This suggests that protein ingestion combined with heavy training may also have rather long-lasting effects on gene expression in muscle.

The biopsies were taken 4-6 days after the last RE workout. This time-point represents roughly the time-point when the next RE session would have usually taken place in the gym making the pre and post 21-week biopsies comparable. The observed more "chronic" responses to the training period were probably due to a) a very long-lasting (4-6 days) effect from the last workout and/or b) a more permanent RT-induced change in the basal state of phosphorylation of these proteins. These changes may affect e.g. the level of the protein synthesis of the resting muscle (Wilkinson et al. 2008) and/or the strenght of the signal needed to activate the cascade through these pathways after e.g. mechanical loading or nutrition.

In summary, this thesis suggests that the phosphorylation state of at least some of the mTOR pathway protein kinases and transcript level changes of some muscle hypertrophy enhancing genes, such as cdk2, can show rather long-lasting and thus less transient responses following training with or without protein supplementation.

### 6.2.3 Possible associations between the molecular responses and muscle hypertrophy

*Myostatin.* In previously untrained young men, the rapid decrease in myostatin mRNA and protein content after the RE bout was prevented with whey protein ingestion. However, at the same time the protein supplemented group, in comparison to placebo group, showed larger whole muscle hypertrophy following the training period. This result may suggest that the acute decrease in myostatin after a RE bout may not be the commanding regulator of the increase in muscle size that occur during training. This would agree with a recent study showing similar expression of myostatin in responders and non-responders to

RT-induced hypertrophy (Bamman et al. 2007). In the present study, the young subjects, who increased their whole muscle CSA the *most,* had the *lowest* decrease in myostatin gene expression at 1h after the RE bout before the RT period. However, the delayed and long-term expression of myostatin appeared to manifest differently; the young and older placebo subjects who had the deepest decrease in myostatin transcript 48h after the RE bout or after 21 weeks of RT, also showed the highest increase in muscle hypertrophy. However, in the younger subjects, the change in RT-induced muscle fiber size was measured, and it was found that it did not correlate with the change in myostatin from pre to post 48h, which further complicates the speculations.

*$p70^{S6K}$*. An acute increase in the phosphorylation of $p70^{S6K}$ after resistance loading has previously been shown to correlate with a long-term increase in the skeletal muscle mass in rats (Baar and Esser 1999) and fiber size as well as fat-free mass in a small group of trained humans (Terzis et al. 2008), and recently also with an acute myofibrillar protein synthesis increase after a RE bout in humans (Kumar et al. 2009). However, in the present study there were no similar correlations in our previously untrained subjects. This would suggest that magnitude of the phosphorylation of $p70^{S6K}$ after a RE bout may not always predict hypertrophic response of the muscle occurring due to long-term training.

*Androgen receptors.* Interestingly, a possible relationship was found between the individual changes during 21 weeks of RT in muscle androgen receptor protein concentration and training-induced changes in muscle size. This suggests that the changes of the androgen receptor protein concentration in skeletal muscle during RT may have an impact on training-induced muscular adaptations. It can be speculated that the mechanism for this could be a larger uptake capacity of testosterone through its increased receptor amount in some previously untrained individuals compared to others. The result seemed to be explained by type I fibers.

# 7 PRIMARY FINDINGS AND CONCLUSIONS

The present study showed that protein supplementation before and after a resistance exercise workout can modify the responses to this type of exercise at various levels: blood hormones, gene expression, phosphorylation of proteins and whole muscle hypertrophy. These results could be applied in the planning of training and rehabilitation protocols and nutritional intake, with the aim to increasing or at least maintaining good muscle size and functional capacity. Specifically, the main findings and conclusions of the present study are as follows:

1) Heavy resistance training for 21 weeks led to muscle hypertrophy and increase in muscle dynamic and isometric strength in both young and older men (II, V-VII). The findings in the young men suggest that whey protein ingestion before and after each exercise workout can also increase whole muscle cross-sectional area and may accelerate the increase in muscle thickness as well as body mass (V, VI). However, no positive effect was found in muscle force and fiber size (V, VI).

2) Protein ingestion before the resistance exercise bout hindered the increase of serum testosterone during exercise in both young and older men (I, IV, VI). The study in the older men suggests that this response is not explained by a significant difference in the expression of androgen receptors between the protein and placebo groups (IV).

3) One single heavy resistance exercise bout in both young and older men led to decreased myostatin and its receptor gene expression (II, III, V) and in young men also to decreased myostatin protein concentration (VI). However, when protein was ingested before and after the exercise session, the decrease in myostatin did not occur (III, V, VI).

4) Protein ingestion possibly enhanced cell proliferation capacity by increasing cdk2 gene expression after a single resistance exercise bout in

both young and older men (III, V), and also after the 21-week resistance training in the young men (V). The result shows that protein ingestion can induce short and long-term effects on cell cycle regulatory mechanisms. Clearly, controlling nutrition intake is important when studying gene expression responses to exercise.

5) One bout of resistance exercise rapidly increased mTOR pathway signaling, and protein ingestion before and after the exercise strengthened this response. The only delayed or long-term effect of protein ingestion on mTOR signaling was the increased phosphorylation of mTOR itself, suggesting that at least some of the mTOR pathway protein kinases can show rather long-lasting phosphorylation responses due to protein supplementation during resistance training (VI).

6) Heavy resistance training for 21 weeks did not consistently change the acute gene transcript response to a single bout of resistance exercise in either young or older men (II, VII).

7) Decrease in myostatin gene expression 48 hours after an acute bout of resistance exercise was associated with larger muscle hypertrophy during the 21 week resistance training period (II, V). Also, individual changes in the androgen receptor concentration may affect the muscle hypertrophy response. The larger the increase in the individual androgen receptor protein concentration during the training period, the greater the hypertrophy response (VII).

Based on the present findings and recent literature, various positive and negative signaling pathways are activated or inactivated after a resistance exercise bout and whey protein ingestion can affect these responses. Of the specific responses, whey protein ingestion increased cdk2 gene expression and mTOR signaling after the resistance exercise bout, a response which may positively relate to muscle hypertrophy and recovery from exercise. The correlative data suggested that the changes in the androgen receptor protein concentration in skeletal muscle during resistance training, and myostatin changes after a resistance exercise bout, may have an impact on training-induced muscular adaptations. In future, it is important to investigate whether these, or other signaling responses, are sufficient for muscle hypertrophy to occur after some months of training with or without protein supplementation.

# YHTEENVETO (Finnish summary)

## Voimaharjoittelun fysiologiset ja molekyylibiologiset vaikutukset lihaskasvun säätelyssä lisäproteiinia nautittaessa tai ilman

Lihasten koon, voiman ja sitä kautta toimintakyvyn lasku ikääntymisen myötä on suuri yhteiskunnallinen ongelma väestön ikääntyessä. Toisaalta usein jo nuorilla lihasten kuormittamattomuus ja vääränlainen ravinto johtavat heikkoon toimintakykyyn. Tässä tutkimuksessa selvitettiin voimaharjoittelun mekanismeja ja vasteita lihasten kasvun säätelyssä nuorilla ja vanhoilla terveillä miehillä. Tärkeänä tutkimuskysymyksenä oli, vaikuttaako heraproteiinin nauttiminen yksittäisen voimaharjoituksen vasteeseen ja toisaalta myös pidempikestoisen voimaharjoittelujakson aikaansaamaan lihasten kehittymiseen. Hera on aminohappokoostumukseltaan korkealaatuisen maidon toinen proteiinifraktio kaseiinin lisäksi. Voimaharjoituksen vastetta tutkittiin lihasnäytteistä ja verinäytteistä kahden vuorokauden aikana harjoituksen jälkeen. Lihasten adaptoitumista tutkittiin 21 viikon voimaharjoittelun jälkeen sekä koko kehon että kudos- ja solutason muuttujien kautta. Tutkimus toteutettiin satunnaistettuna ja kontrolloituna kaksoissokkotutkimuksena.

Tutkimuksessa havaittiin, että voimaharjoittelu kaksi kertaa viikossa lisäsi lihasvoimaa aiemmin harjoittelemattomilla nuorilla ja vanhoilla miehillä, ja lihasten kokoa sekä kudos- että solutasolla mitattuna. Nuorilla miehillä tehdyssä tutkimuksessa 15 g heraproteiinia sisältävän juoman nauttiminen ennen ja jälkeen kunkin voimaharjoituksen lisäsi magneettikuvauksella mitattua ulomman reisilihaksen poikkipinta-alan kasvua sekä nopeutti kehon massan ja etureiden lihaksen paksuuden kasvua plasebo-juomaa nauttineisiin verrattuna. Tämä siitäkin huolimatta, että koehenkilöiden päivittäinen proteiininsaanti normaalista ruoasta oli suurta (n. 1,5 g /painokilo). Lihassolujen koon suurempaa kasvua ei tällä koehenkilöjoukolla havaittu.

Selittävänä tekijänä lisäproteiinin saannin aiheuttamalle lihasmassan lisääntymiselle ovat, ainakin osittain, todennäköisesti proteiinisynteesin säätelyreitillä havaitut positiiviset muutokset lähinnä p70S6 -kinaasin aktivoitumisessa ja 4E-BP1 -proteiinin inaktivoitumisessa fosforyloinnin avulla. Toinen selittävä tekijä voi olla lihassolujen ympärillä olevien jakautumiseen kykenevien satelliittisolujen jakautumiskapasiteetin lisääntyminen, mille antaa viitteitä solusyklin etenemiseen positiivisesti vaikuttavan proteiinin, sykliini-riippuvaisen kinaasi 2:n (cdk2) geenin ilmentymisen lisääntyminen proteiinia nautittaessa. Tämä mahdollisesti edesauttaa muun muassa aiemmissa tutkimuksissa havaittua nopeampaa harjoituksesta palautumista kun proteiinia on nautittu intensiivisen harjoituksen yhteydessä.

Yksittäinen raskas voimaharjoitus laski lihasten koon negatiivisen säätelijän, myostatiinin, ilmentymistä sekä lähetti-RNA- että proteiinitasolla. Jälkimmäinen havaittiin ensimmäistä kertaa ihmisillä. Tulos on, ainakin teoriassa, lihaskasvun kannalta suotuisa vaste. Tutkimuksessa havaittiin kuitenkin yllättäen, että myostatiinin ilmentyminen laski voimaharjoituksen jälkeen vain plase-

boryhmällä. Uusi havainto oli myös, että myostatiinin reseptorin, aktiviini IIb:n, geenin ilmentyminen vähentyi yksittäisen voimaharjoituksen jälkeen. Teoriassa tämä saattaa johtaa vähentyneeseen myostatiinin signalointiin lihassoluissa ollen edullinen vaste voimaharjoituksen jälkeen.

Proteiinin nauttiminen voimaharjoituksen yhteydessä vähensi jossain määrin myös voimaharjoituksen aiheuttamaa seerumin testosteronin nousua. Tässä ja useassa muussakin tutkimuksessa on osoitettu voimaharjoitusten yhteydessä nautitun korkealaatuisen proteiinin positiivinen vaikutus luurankolihasten adaptaatioihin. Proteiini kuitenkin näyttää siis vaikuttavan kuitenkin akuutisti yksittäisten voimaharjoitusten vasteisiin melko yllättävästi tiettyjen muuttujien osalta. Proteiinin aiheuttamien myostatiini- ja testosteronivasteiden fysiologisesta merkityksestä ei ole tietoa, mutta ne saattavat olla kehon homeostaattinen, tasapainottava reaktio. Proteiinin vähentävä vaikutus seerumin testosteroniin ei ollut selitettävissä androgeenireseptorien määrällä proteiiniryhmällä plaseboon verrattuna. Toisaalta proteiinin estävä vaikutus myostatiinin ilmentymisen laskuun ei vaikuttanut epäsuotuisasti myostatiinin alavirrassa olevaan signalointiin tämän tutkimuksen muuttujien tasolla. Lisätutkimuksia kuitenkin tarvitaan.

Lienee selvää, että voimaharjoitus ja proteiinin nauttiminen molemmat vaikuttavat lihasten kasvun säätelyyn pääasiassa yksittäisten ja nopeiden harjoituksen jälkeisten vasteiden kautta. Toisaalta pidempikestoisiakin vasteita näillä molekyylireiteillä havaittiin. Proteiini lisäsi mTOR-proteiinin fosforylaatiota ja cdk2 geenin ilmentymistä voimaharjoittelujakson jälkeen. Nämä vasteet saattavat tehostaa lihasten kehittymiskapasiteettia.

Tutkimuksessa selvitettiin myös nuorten ja vanhojen miesten eroja voimaharjoittelun vasteissa. Tämä tutkimus viittaa siihen, että lihasmassa kasvaa nuorilla vanhoja hieman enemmän voimaharjoittelun seurauksena. On selvää, että ikä kannattaa ottaa huomioon, kun laaditaan kullekin yksilölle optimaalista voimaharjoitteluohjelmaa. Mahdollisesti kaksi kertaa kunkin lihasryhmän kova harjoittaminen kuntosalilla viikossa useiden kuukausien ajan voi olla joillekin vanhemmille miehille suhteellisesti liian kova rasitus. Tämä on totta varsinkin jos proteiinin- ja energiansaanti on riittämätöntä.

Testosteronin reseptorien suhteen mielenkiintoinen havainto oli, että voimaharjoittelujakson aikainen androgeenireseptorin pitoisuuden muutos korreloi merkitsevästi ja positiivisesti lihasten koon kasvun kanssa (lihassolukoko, rasvaton kehonpaino ja tendenssi myös lihaksen paksuudessa). Täten testosteronin reseptorien pitoisuuden muutoksella voi olla merkitystä lihasten koon kasvussa harjoittelun yhteydessä.

Johtopäätöksenä voidaan todeta, että voimaharjoittelu vaikuttaa sekä akuutisti että pitkällä aikavälillä useisiin lihasten kasvun kannalta sekä positiivisiin että negatiivisiin säätelijöihin. Proteiinin nauttimisella välittömästi ennen ja jälkeen voimaharjoituksen pystytään jossain määrin tehostamaan lihasten koon kasvua vaikuttamalla fysiologisiin ja molekyylibiologisiin vasteisiin. Tulokset rohkaisevat voimaharjoittelua harrastavia nauttimaan korkealaatuista proteiiniravintoa voimaharjoituksen yhteydessä.

## REFERENCES

Aagaard P, Andersen JL, Dyhre-Poulsen P, Leffers AM, Wagner A, Magnusson SP, Halkjaer-Kristensen J, Simonsen EB (2001) A mechanism for increased contractile strength of human pennate muscle in response to strength training: Changes in muscle architecture. J Physiol 534:613-623

Adams GR, Caiozzo VJ, Haddad F, Baldwin KM (2002) Cellular and molecular responses to increased skeletal muscle loading after irradiation. Am J Physiol Cell Physiol 283:C1182-95

Adams GR, Haddad F, Baldwin KM (1999) Time course of changes in markers of myogenesis in overloaded rat skeletal muscles. J Appl Physiol 87:1705-1712

Adams GR & Haddad F (1996) The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy. J Appl Physiol 81:2509-2516

Ahtiainen JP, Pakarinen A, Alen M, Kraemer WJ, Häkkinen K (2005) Short vs. long rest period between the sets in hypertrophic resistance training: Influence on muscle strength, size, and hormonal adaptations in trained men. J Strength Cond Res 19:572-582

Ahtiainen JP, Pakarinen A, Alen M, Kraemer WJ, Häkkinen K (2003) Muscle hypertrophy, hormonal adaptations and strength development during strength training in strength-trained and untrained men. Eur J Appl Physiol 89:555-563

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase balpha. Curr Biol 7:261-269

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J 15:6541-6551

Allen DL, Roy RR, Edgerton VR (1999) Myonuclear domains in muscle adaptation and disease. Muscle Nerve 22:1350-1360

Amirouche A, Durieux AC, Banzet S, Koulmann N, Bonnefoy R, Mouret C, Bigard X, Peinnequin A, Freyssenet D (2009) Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. Endocrinology 150:286-294

Andersen LL, Tufekovic G, Zebis MK, Crameri RM, Verlaan G, Kjaer M, Suetta C, Magnusson P, Aagaard P (2005) The effect of resistance training combined with timed ingestion of protein on muscle fiber size and muscle strength. Metabolism 54:151-156

Andersen P (1975) Capillary density in skeletal muscle of man. Acta Physiol Scand 95:203-205

Antonio J, Sanders MS, Ehler LA, Uelmen J, Raether JB, Stout JR (2000) Effects of exercise training and amino-acid supplementation on body composition and physical performance in untrained women. Nutrition 16:1043-1046

Baar K, Nader G, Bodine S (2006) Resistance exercise, muscle loading/unloading and the control of muscle mass. Essays Biochem 42:61-74

Baar K & Esser K (1999) Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. Am J Physiol 276:C120-7

Bamman MM (2007) Take two NSAIDs and call on your satellite cells in the morning. J Appl Physiol 103:415-416

Bamman MM, Petrella JK, Kim JS, Mayhew DL, Cross JM (2007) Cluster analysis tests the importance of myogenic gene expression during myofiber hypertrophy in humans. J Appl Physiol 102:2232-2239

Bamman MM, Ragan RC, Kim JS, Cross JM, Hill VJ, Tuggle SC, Allman RM (2004) Myogenic protein expression before and after resistance loading in 26- and 64-yr-old men and women. J Appl Physiol 97:1329-1337

Bamman MM, Hill VJ, Adams GR, Haddad F, Wetzstein CJ, Gower BA, Ahmed A, Hunter GR (2003) Gender differences in resistance-training-induced myofiber hypertrophy among older adults. J Gerontol A Biol Sci Med Sci 58:108-116

Bamman MM, Shipp JR, Jiang J, Gower BA, Hunter GR, Goodman A, McLafferty CL,Jr, Urban RJ (2001) Mechanical load increases muscle IGF-I and androgen receptor mRNA concentrations in humans. Am J Physiol Endocrinol Metab 280:E383-90

Barton ER (2006) Viral expression of insulin-like growth factor-I isoforms promotes different responses in skeletal muscle. J Appl Physiol 100:1778-1784

Barton-Davis ER, Shoturma DI, Musaro A, Rosenthal N, Sweeney HL (1998) Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. Proc Natl Acad Sci U S A 95:15603-15607

Beelen M, Koopman R, Gijsen AP, Vandereyt H, Kies AK, Kuipers H, Saris WH, van Loon LJ (2008a) Protein coingestion stimulates muscle protein synthesis during resistance-type exercise. Am J Physiol Endocrinol Metab 295:E70-7

Beelen M, Tieland M, Gijsen AP, Vandereyt H, Kies AK, Kuipers H, Saris WH, Koopman R, van Loon LJ (2008b) Coingestion of carbohydrate and protein hydrolysate stimulates muscle protein synthesis during exercise in young men, with no further increase during subsequent overnight recovery. J Nutr 138:2198-2204

Bergström J & Hultman E (1966) The effect of exercise on muscle glycogen and electrolytes in normals. Scand J Clin Lab Invest 18:16-20

Berthet C, Aleem E, Coppola V, Tessarollo L, Kaldis P (2003) Cdk2 knockout mice are viable. Curr Biol 13:1775-1785

Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW (2001) Testosterone dose-response relationships in healthy young men. Am J Physiol Endocrinol Metab 281:E1172-81

Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, Casaburi R (1996) The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. N Engl J Med 335:1-7

Bickel CS, Slade J, Mahoney E, Haddad F, Dudley GA, Adams GR (2005) Time course of molecular responses of human skeletal muscle to acute bouts of resistance exercise. J Appl Physiol 98:482-488

Bickel CS, Slade JM, Haddad F, Adams GR, Dudley GA (2003) Acute molecular responses of skeletal muscle to resistance exercise in able-bodied and spinal cord-injured subjects. J Appl Physiol 94:2255-2262

Biolo G, Tipton KD, Klein S, Wolfe RR (1997) An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. Am J Physiol 273:E122-9

Biolo G, Declan Fleming RY, Wolfe RR (1995a) Physiologic hyperinsulinemia stimulates protein synthesis and enhances transport of selected amino acids in human skeletal muscle. J Clin Invest 95:811-819

Biolo G, Maggi SP, Williams BD, Tipton KD, Wolfe RR (1995b) Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. Am J Physiol 268:E514-20

Bird SP, Tarpenning KM, Marino FE (2006) Independent and combined effects of liquid carbohydrate/essential amino acid ingestion on hormonal and muscular adaptations following resistance training in untrained men. Eur J Appl Physiol 97:225-238

Blomstrand E, Eliasson J, Karlsson HK, Kohnke R (2006) Branched-chain amino acids activate key enzymes in protein synthesis after physical exercise. J Nutr 136:269S-73S

Bloomer RJ, Sforzo GA, Keller BA (2000) Effects of meal form and composition on plasma testosterone, cortisol, and insulin following resistance exercise. Int J Sport Nutr Exerc Metab 10:415-424

Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ (2001a) Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294:1704-1708

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

Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS (2002) Functional improvement of dystrophic muscle by myostatin blockade. Nature 420:418-421

Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL, Beaufrere B (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. Proc Natl Acad Sci U S A 94:14930-14935

Bolster DR, Kimball SR, Jefferson LS (2003) Translational control mechanisms modulate skeletal muscle gene expression during hypertrophy. Exerc Sport Sci Rev 31:111-116

Borsheim E, Tipton KD, Wolf SE, Wolfe RR (2002) Essential amino acids and muscle protein recovery from resistance exercise. Am J Physiol Endocrinol Metab 283:E648-57

Bradley L, Yaworsky PJ, Walsh FS (2008) Myostatin as a therapeutic target for musculoskeletal disease. Cell Mol Life Sci 65:2119-2124

Brown EC, DiSilvestro RA, Babaknia A, Devor ST (2004) Soy versus whey protein bars: Effects on exercise training impact on lean body mass and antioxidant status. Nutr J 3:22

Burke DG, Chilibeck PD, Davidson KS, Candow DG, Farthing J, Smith-Palmer T (2001) The effect of whey protein supplementation with and without creatine monohydrate combined with resistance training on lean tissue mass and muscle strength. Int J Sport Nutr Exerc Metab 11:349-364

Buse MG & Reid SS (1975) Leucine. A possible regulator of protein turnover in muscle. J Clin Invest 56:1250-1261

Campbell WW, Barton ML,Jr, Cyr-Campbell D, Davey SL, Beard JL, Parise G, Evans WJ (1999) Effects of an omnivorous diet compared with a lactoovovegetarian diet on resistance-training-induced changes in body composition and skeletal muscle in older men. Am J Clin Nutr 70:1032-1039

Campos GE, Luecke TJ, Wendeln HK, Toma K, Hagerman FC, Murray TF, Ragg KE, Ratamess NA, Kraemer WJ, Staron RS (2002) Muscular adaptations in response to three different resistance-training regimens: Specificity of repetition maximum training zones. Eur J Appl Physiol 88:50-60

Candow DG, Burke NC, Smith-Palmer T, Burke DG (2006a) Effect of whey and soy protein supplementation combined with resistance training in young adults. Int J Sport Nutr Exerc Metab 16:233-244

Candow DG, Chilibeck PD, Facci M, Abeysekara S, Zello GA (2006b) Protein supplementation before and after resistance training in older men. Eur J Appl Physiol 97:548-556

Carlberg U, Nilsson A, Nygard O (1990) Functional properties of phosphorylated elongation factor 2. Eur J Biochem 191:639-645

Carlson CJ, Booth FW, Gordon SE (1999) Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. Am J Physiol 277:R601-6

Carson JA, Schwartz RJ, Booth FW (1996) SRF and TEF-1 control of chicken skeletal alpha-actin gene during slow-muscle hypertrophy. Am J Physiol 270:C1624-33

Cass LA, Summers SA, Prendergast GV, Backer JM, Birnbaum MJ, Meinkoth JL (1999) Protein kinase A-dependent and -independent signaling pathways

contribute to cyclic AMP-stimulated proliferation. Mol Cell Biol 19:5882-5891

Chandler RM, Byrne HK, Patterson JG, Ivy JL (1994) Dietary supplements affect the anabolic hormones after weight-training exercise. J Appl Physiol 76:839-845

Charge SB & Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. Physiol Rev 84:209-238

Chauvigne F, Gabillard JC, Weil C, Rescan PY (2003) Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. Gen Comp Endocrinol 132:209-215

Chen YW, Nader GA, Baar KR, Fedele MJ, Hoffman EP, Esser KA (2002) Response of rat muscle to acute resistance exercise defined by transcriptional and translational profiling. J Physiol 545:27-41

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

Chiang GG & Abraham RT (2005) Phosphorylation of mammalian target of rapamycin (mTOR) at ser-2448 is mediated by p70S6 kinase. J Biol Chem 280:25485-25490

Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibe B, Bouix J, Caiment F, Elsen JM, Eychenne F, Larzul C, Laville E, Meish F, Milenkovic D, Tobin J, Charlier C, Georges M (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat Genet 38:813-818

Cockburn E, Hayes PR, French DN, Stevenson E, St Clair Gibson A (2008) Acute milk-based protein-CHO supplementation attenuates exercise-induced muscle damage. Appl Physiol Nutr Metab 33:775-783

Coffey VG, Shield A, Canny BJ, Carey KA, Cameron-Smith D, Hawley JA (2006a) Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. Am J Physiol Endocrinol Metab 290:E849-55

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

Costa A, Dalloul H, Hegyesi H, Apor P, Csende Z, Racz L, Vaczi M, Tihanyi J (2007) Impact of repeated bouts of eccentric exercise on myogenic gene expression. Eur J Appl Physiol

Costill DL, Coyle EF, Fink WF, Lesmes GR, Witzmann FA (1979) Adaptations in skeletal muscle following strength training. J Appl Physiol 46:96-99

Creer A, Gallagher P, Slivka D, Jemiolo B, Fink W, Trappe S (2005) Influence of muscle glycogen availability on ERK1/2 and akt signaling after resistance exercise in human skeletal muscle. J Appl Physiol 99:950-956

Cribb PJ, Williams AD, Stathis CG, Carey MF, Hayes A (2007) Effects of whey isolate, creatine, and resistance training on muscle hypertrophy. Med Sci Sports Exerc 39:298-307

Cribb PJ & Hayes A (2006) Effects of supplement timing and resistance exercise on skeletal muscle hypertrophy. Med Sci Sports Exerc 38:1918-1925

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378:785-789

Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, Wackerhage H, Taylor PM, Rennie MJ (2005) Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. FASEB J 19:422-424

Cuthbertson DJ, Babraj J, Smith K, Wilkes E, Fedele MJ, Esser K, Rennie M (2006) Anabolic signaling and protein synthesis in human skeletal muscle after dynamic shortening or lengthening exercise. Am J Physiol Endocrinol Metab 290:E731-8

Dangin M, Guillet C, Garcia-Rodenas C, Gachon P, Bouteloup-Demange C, Reiffers-Magnani K, Fauquant J, Ballevre O, Beaufrere B (2003) The rate of protein digestion affects protein gain differently during aging in humans. J Physiol 549:635-644

Dangin M, Boirie Y, Guillet C, Beaufrere B (2002) Influence of the protein digestion rate on protein turnover in young and elderly subjects. J Nutr 132:3228S-33S

Dangin M, Boirie Y, Garcia-Rodenas C, Gachon P, Fauquant J, Callier P, Ballevre O, Beaufrere B (2001) The digestion rate of protein is an independent regulating factor of postprandial protein retention. Am J Physiol Endocrinol Metab 280:E340-8

D'Antona G, Lanfranconi F, Pellegrino MA, Brocca L, Adami R, Rossi R, Moro G, Miotti D, Canepari M, Bottinelli R (2006) Skeletal muscle hypertrophy and structure and function of skeletal muscle fibres in male body builders. J Physiol 570:611-627

Deldicque L, Atherton P, Patel R, Theisen D, Nielens H, Rennie MJ, Francaux M (2008) Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise. Eur J Appl Physiol 104:57-65

Dill DB & Costill DL (1974) Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. J Appl Physiol 37:247-248

Dreyer HC, Drummond MJ, Pennings B, Fujita S, Glynn EL, Chinkes DL, Dhanani S, Volpi E, Rasmussen BB (2008) 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

Dreyer HC, Blanco CE, Sattler FR, Schroeder ET, Wiswell RA (2006a) Satellite cell numbers in young and older men 24 hours after eccentric exercise. Muscle Nerve 33:242-253

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

Drummond MJ, Bell JA, Fujita S, Dreyer HC, Glynn EL, Volpi E, Rasmussen BB (2008) Amino acids are necessary for the insulin-induced activation of

mTOR/S6K1 signaling and protein synthesis in healthy and insulin resistant human skeletal muscle. Clin Nutr 27:447-456

Durnin JV & Womersley J (1974) Body fat assessed from total body density and its estimation from skinfold thickness: Measurements on 481 men and women aged from 16 to 72 years. Br J Nutr 32:77-97

Eliasson J, Elfegoun T, Nilsson J, Kohnke R, Ekblom B, Blomstrand E (2006) Maximal lengthening contractions increase p70 S6 kinase phosphorylation in human skeletal muscle in the absence of nutritional supply. Am J Physiol Endocrinol Metab 291:E1197-205

Eriksson A, Kadi F, Malm C, Thornell LE (2005) Skeletal muscle morphology in power-lifters with and without anabolic steroids. Histochem Cell Biol 124:167-175

Esmarck B, Andersen JL, Olsen S, Richter EA, Mizuno M, Kjaer M (2001) Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. J Physiol 535:301-311

Estrada M, Espinosa A, Muller M, Jaimovich E (2003) Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. Endocrinology 144:3586-3597

Etzel MR (2004) Manufacture and use of dairy protein fractions. J Nutr 134:996S-1002S

Fang Y, Park IH, Wu AL, Du G, Huang P, Frohman MA, Walker SJ, Brown HA, Chen J (2003) PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr Biol 13:2037-2044

Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294:1942-1945

Ferrando AA, Sheffield-Moore M, Yeckel CW, Gilkison C, Jiang J, Achacosa A, Lieberman SA, Tipton K, Wolfe RR, Urban RJ (2002) Testosterone administration to older men improves muscle function: Molecular and physiological mechanisms. Am J Physiol Endocrinol Metab 282:E601-7

Ferrando AA, Tipton KD, Doyle D, Phillips SM, Cortiella J, Wolfe RR (1998) Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. Am J Physiol 275:E864-71

Fiatarone MA, O'Neill EF, Ryan ND, Clements KM, Solares GR, Nelson ME, Roberts SB, Kehayias JJ, Lipsitz LA, Evans WJ (1994) Exercise training and nutritional supplementation for physical frailty in very elderly people. N Engl J Med 330:1769-1775

Flotow H & Thomas G (1992) Substrate recognition determinants of the mitogen-activated 70K S6 kinase from rat liver. J Biol Chem 267:3074-3078

Frier BC & Locke M (2007) Heat stress inhibits skeletal muscle hypertrophy. Cell Stress Chaperones 12:132-141

Frost RA & Lang CH (2007) Protein kinase B/Akt: A nexus of growth factor and cytokine signaling in determining muscle mass. J Appl Physiol 103:378-387

Fry AC (2004) The role of resistance exercise intensity on muscle fibre adaptations. Sports Med 34:663-679

Fujita S, Dreyer HC, Drummond MJ, Glynn EL, Volpi E, Rasmussen BB (2008) Essential amino acid and carbohydrate ingestion prior to resistance exercise does not enhance post-exercise muscle protein synthesis. J Appl Physiol (June 5, 2008). doi:10.1152/japplphysiol.90395.2008

Ganong WF (2001) Review of Medical Physiology. McGraw-Hill, USA

Gautsch TA, Anthony JC, Kimball SR, Paul GL, Layman DK, Jefferson LS (1998) Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. Am J Physiol 274:C406-14

Georget V, Lobaccaro JM, Terouanne B, Mangeat P, Nicolas JC, Sultan C (1997) Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor. Mol Cell Endocrinol 129:17-26

Girgenrath S, Song K, Whittemore LA (2005) Loss of myostatin expression alters fiber-type distribution and expression of myosin heavy chain isoforms in slow- and fast-type skeletal muscle. Muscle Nerve 31:34-40

Glass DJ (2005) Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 37:1974-1984

Glover EI, Oates BR, Tang JE, Moore DR, Tarnopolsky MA, Phillips SM (2008) Resistance exercise decreases eIF2B{epsilon} phosphorylation and potentiates the feeding-induced stimulation of p70S6k1 and rpS6 in young men. Am J Physiol Regul Integr Comp Physiol 295:604-10

Godard MP, Williamson DL, Trappe SW (2002) Oral amino-acid provision does not affect muscle strength or size gains in older men. Med Sci Sports Exerc 34:1126-1131

Goldberg AL (2003) Protein degradation and protection against misfolded or damaged proteins. Nature 426:895-899

Goldberg AL, Etlinger JD, Goldspink DF, Jablecki C (1975) Mechanism of work-induced hypertrophy of skeletal muscle. Med Sci Sports 7:185-198

Goldberg AL & Goodman HM (1969) Amino acid transport during work-induced growth of skeletal muscle. Am J Physiol 216:1111-1115

Goldberg AL (1967) Work-induced growth of skeletal muscle in normal and hypophysectomized rats. Am J Physiol 213:1193-1198

Goldspink G & Howells KF (1974) Work-induced hypertrophy in exercised normal muscles of different ages and the reversibility of hypertrophy after cessation of exercise. J Physiol 239:179-193

Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S (1998) Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proc Natl Acad Sci U S A 95:14938-14943

Greenhaff PL, Karagounis LG, Peirce N, Simpson EJ, Hazell M, Layfield R, Wackerhage H, Smith K, Atherton P, Selby A, Rennie MJ (2008) Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. Am J Physiol Endocrinol Metab 295:E595-604

Greenlund LJ & Nair KS (2003) Sarcopenia--consequences, mechanisms, and potential therapies. Mech Ageing Dev 124:287-299

Greig CA, Hameed M, Young A, Goldspink G, Noble B (2006) Skeletal muscle IGF-I isoform expression in healthy women after isometric exercise. Growth Horm IGF Res 16:373-376

Guernec A, Chevalier B, Duclos MJ (2004) Nutrient supply enhances both IGF-I and MSTN mRNA levels in chicken skeletal muscle. Domest Anim Endocrinol 26:143-154

Gulati P, Gaspers LD, Dann SG, Joaquin M, Nobukuni T, Natt F, Kozma SC, Thomas AP, Thomas G (2008) Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. Cell Metab 7:456-465

Gulati P & Thomas G (2007) Nutrient sensing in the mTOR/S6K1 signalling pathway. Biochem Soc Trans 35:236-238

Ha E & Zemel MB (2003) Functional properties of whey, whey components, and essential amino acids: Mechanisms underlying health benefits for active people (review). J Nutr Biochem 14:251-258

Haddad F & Adams GR (2002) Selected contribution: Acute cellular and molecular responses to resistance exercise. J Appl Physiol 93:394-403

Häkkinen K, Kraemer WJ, Newton RU, Alen M (2001a) Changes in electromyographic activity, muscle fibre and force production characteristics during heavy resistance/power strength training in middle-aged and older men and women. Acta Physiol Scand 171:51-62

Häkkinen K, Pakarinen A, Kraemer WJ, Häkkinen A, Valkeinen H, Alen M (2001b) Selective muscle hypertrophy, changes in EMG and force, and serum hormones during strength training in older women. J Appl Physiol 91:569-580

Häkkinen K, Newton RU, Gordon SE, McCormick M, Volek JS, Nindl BC, Gotshalk LA, Campbell WW, Evans WJ, Häkkinen A, Humphries BJ, Kraemer WJ (1998a) Changes in muscle morphology, electromyographic activity, and force production characteristics during progressive strength training in young and older men. J Gerontol A Biol Sci Med Sci 53:B415-23

Häkkinen K, Pakarinen A, Newton RU, Kraemer WJ (1998b) Acute hormone responses to heavy resistance lower and upper extremity exercise in young versus old men. Eur J Appl Physiol Occup Physiol 77:312-319

Häkkinen K & Pakarinen A (1993) Acute hormonal responses to two different fatiguing heavy-resistance protocols in male athletes. J Appl Physiol 74:882-887

Hajduch E, Alessi DR, Hemmings BA, Hundal HS (1998) Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. Diabetes 47:1006-1013

Halevy O, Nadel Y, Barak M, Rozenboim I, Sklan D (2003) Early posthatch feeding stimulates satellite cell proliferation and skeletal muscle growth in turkey poults. J Nutr 133:1376-1382

Hameed M, Orrell RW, Cobbold M, Goldspink G, Harridge SD (2003) Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise. J Physiol 547:247-254

Harber MP, Schenk S, Barkan AL, Horowitz JF (2005) Effects of dietary carbohydrate restriction with high protein intake on protein metabolism and the somatotropic axis. J Clin Endocrinol Metab 90:5175-5181

Harridge SD (2007) Translational review: The plasticity of human skeletal muscle: From gene expression to in vivo function. Exp Physiol 92:783-97

Hartgens F, Kuipers H, Wijnen JA, Keizer HA (1996) Body composition, cardiovascular risk factors and liver function in long-term androgenic-anabolic steroids using bodybuilders three months after drug withdrawal. Int J Sports Med 17:429-433

Hartman JW, Tang JE, Wilkinson SB, Tarnopolsky MA, Lawrence RL, Fullerton AV, Phillips SM (2007) Consumption of fat-free fluid milk after resistance exercise promotes greater lean mass accretion than does consumption of soy or carbohydrate in young, novice, male weightlifters. Am J Clin Nutr 86:373-381

Haub MD, Wells AM, Tarnopolsky MA, Campbell WW (2002) Effect of protein source on resistive-training-induced changes in body composition and muscle size in older men. Am J Clin Nutr 76:511-517

Hill JJ, Qiu Y, Hewick RM, Wolfman NM (2003) Regulation of myostatin in vivo by growth and differentiation factor-associated serum protein-1: A novel protein with protease inhibitor and follistatin domains. Mol Endocrinol 17:1144-1154

Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y (2002) The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277:40735-40741

Hill M & Goldspink G (2003) Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. J Physiol 549:409-418

Hlaing M, Shen X, Dazin P, Bernstein HS (2002) The hypertrophic response in C2C12 myoblasts recruits the G1 cell cycle machinery. J Biol Chem 277:23794-23799

Hoffman JR, Ratamess NA, Kang J, Falvo MJ, Faigenbaum AD (2006) Effect of protein intake on strength, body composition and endocrine changes in strength/power athletes. J Int Soc Sports Nutr 3:12-18

Holm L, Esmarck B, Mizuno M, Hansen H, Suetta C, Holmich P, Krogsgaard M, Kjaer M (2006) The effect of protein and carbohydrate supplementation on strength training outcome of rehabilitation in ACL patients. J Orthop Res 24:2114-2123

Hornberger TA & Chien S (2006) Mechanical stimuli and nutrients regulate rapamycin-sensitive signaling through distinct mechanisms in skeletal muscle. J Cell Biochem 97:1207-1216

Hornberger TA, Chu WK, Mak YW, Hsiung JW, Huang SA, Chien S (2006) 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

Hornberger TA, Stuppard R, Conley KE, Fedele MJ, Fiorotto ML, Chin ER, Esser KA (2004) Mechanical stimuli regulate rapamycin-sensitive signalling by a phosphoinositide 3-kinase-, protein kinase B- and growth factor-independent mechanism. Biochem J 380:795-804

Hubal MJ, Gordish-Dressman H, Thompson PD, Price TB, Hoffman EP, Angelopoulos TJ, Gordon PM, Moyna NM, Pescatello LS, Visich PS, Zoeller RF, Seip RL, Clarkson PM (2005) Variability in muscle size and strength gain after unilateral resistance training. Med Sci Sports Exerc 37:964-972

Hughes SM, Cho M, Karsch-Mizrachi I, Travis M, Silberstein L, Leinwand LA, Blau HM (1993) Three slow myosin heavy chains sequentially expressed in developing mammalian skeletal muscle. Dev Biol 158:183-199

Iijima Y, Laser M, Shiraishi H, Willey CD, Sundaravadivel B, Xu L, McDermott PJ, Kuppuswamy D (2002) c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. J Biol Chem 277:23065-23075

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

Inoue K, Yamasaki S, Fushiki T, Okada Y, Sugimoto E (1994) Androgen receptor antagonist suppresses exercise-induced hypertrophy of skeletal muscle. Eur J Appl Physiol Occup Physiol 69:88-91

Ishido M, Kami K, Masuhara M (2004) Localization of MyoD, myogenin and cell cycle regulatory factors in hypertrophying rat skeletal muscles. Acta Physiol Scand 180:281-289

Ivey FM, Roth SM, Ferrell RE, Tracy BL, Lemmer JT, Hurlbut DE, Martel GF, Siegel EL, Fozard JL, Jeffrey Metter E, Fleg JL, Hurley BF (2000) Effects of age, gender, and myostatin genotype on the hypertrophic response to heavy resistance strength training. J Gerontol A Biol Sci Med Sci 55:M641-8

Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol 6:1122-1128

Jacquemin V, Furling D, Bigot A, Butler-Browne GS, Mouly V (2004) IGF-1 induces human myotube hypertrophy by increasing cell recruitment. Exp Cell Res 299:148-158

Jeanplong F, Bass JJ, Smith HK, Kirk SP, Kambadur R, Sharma M, Oldham JM (2003) Prolonged underfeeding of sheep increases myostatin and myogenic regulatory factor myf-5 in skeletal muscle while IGF-I and myogenin are repressed. J Endocrinol 176:425-437

Jenkins GM & Frohman MA (2005) Phospholipase D: A lipid centric review. Cell Mol Life Sci 62:2305-2316

Jensky NE, Sims JK, Rice JC, Dreyer HC, Schroeder ET (2007) The influence of eccentric exercise on mRNA expression of skeletal muscle regulators. Eur J Appl Physiol 101:473-80

Joulia D, Bernardi H, Garandel V, Rabenoelina F, Vernus B, Cabello G (2003) Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Exp Cell Res 286:263-275

Joulia-Ekaza D & Cabello G (2007) The myostatin gene: Physiology and pharmacological relevance. Curr Opin Pharmacol 7:310-315

Kadi F, Johansson F, Johansson R, Sjostrom M, Henriksson J (2004a) Effects of one bout of endurance exercise on the expression of myogenin in human quadriceps muscle. Histochem Cell Biol 121:329-334

Kadi F, Schjerling P, Andersen LL, Charifi N, Madsen JL, Christensen LR, Andersen JL (2004b) The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. J Physiol 558:1005-1012

Kadi F (2000) Adaptation of human skeletal muscle to training and anabolic steroids. Acta Physiol Scand Suppl 646:1-52

Kadi F, Bonnerud P, Eriksson A, Thornell LE (2000) The expression of androgen receptors in human neck and limb muscles: Effects of training and self-administration of androgenic-anabolic steroids. Histochem Cell Biol 113:25-29

Kadi F, Eriksson A, Holmner S, Thornell LE (1999) Effects of anabolic steroids on the muscle cells of strength-trained athletes. Med Sci Sports Exerc 31:1528-1534

Kalman D, Feldman S, Martinez M, Krieger DR, Tallon MJ (2007) Effect of protein source and resistance training on body composition and sex hormones. J Int Soc Sports Nutr 4:4

Kambadur R, Sharma M, Smith TP, Bass JJ (1997) Mutations in myostatin (GDF8) in double-muscled belgian blue and piedmontese cattle. Genome Res 7:910-916

Karlsson HK, Nilsson PA, Nilsson J, Chibalin AV, Zierath JR, Blomstrand E (2004) Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. Am J Physiol Endocrinol Metab 287:E1-7

Katsanos CS, Chinkes DL, Paddon-Jones D, Zhang XJ, Aarsland A, Wolfe RR (2008) Whey protein ingestion in elderly persons results in greater muscle protein accrual than ingestion of its constituent essential amino acid content. Nutr Res 28:651-658

Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR (2006) A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. Am J Physiol Endocrinol Metab 291:E381-7

Kerksick C, Harvey T, Stout J, Campbell B, Wilborn C, Kreider R, Kalman D, Ziegenfuss T, Lopez H, Landis J, Ivy JL, Antonio J (2008) International society of sports nutrition position stand: Nutrient timing. J Int Soc Sports Nutr 5:17

Kerksick CM, Rasmussen CJ, Lancaster SL, Magu B, Smith P, Melton C, Greenwood M, Almada AL, Earnest CP, Kreider RB (2006) The effects of protein and amino acid supplementation on performance and training

adaptations during ten weeks of resistance training. J Strength Cond Res 20:643-653

Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL (2008) Regulation of TORC1 by rag GTPases in nutrient response. Nat Cell Biol 10:935-945

Kim JS, Petrella JK, Cross JM, Bamman MM (2007) Load-mediated downregulation of myostatin mRNA is not sufficient to promote myofiber hypertrophy in humans: A cluster analysis. J Appl Physiol 103:1488-1495

Kim JS, Cross JM, Bamman MM (2005a) Impact of resistance loading on myostatin expression and cell cycle regulation in young and older men and women. Am J Physiol Endocrinol Metab 288:E1110-9

Kim JS, Kosek DJ, Petrella JK, Cross JM, Bamman MM (2005b) Resting and load-induced levels of myogenic gene transcripts differ between older adults with demonstrable sarcopenia and young men and women. J Appl Physiol 99:2149-2158

Kimball SR (2007) The role of nutrition in stimulating muscle protein accretion at the molecular level. Biochem Soc Trans 35:1298-1301

Kimball SR & Jefferson LS (2006) New functions for amino acids: Effects on gene transcription and translation. Am J Clin Nutr 83:500S-507S

Kimball SR, Shantz LM, Horetsky RL, Jefferson LS (1999) Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. J Biol Chem 274:11647-11652

Kivelä R, Havas E, Vihko V (2007) Localisation of lymphatic vessels and vascular endothelial growth factors-C and -D in human and mouse skeletal muscle with immunohistochemistry. Histochem Cell Biol 127:31-40

Koopman R, Pennings B, Zorenc AH, van Loon LJ (2007) Protein ingestion further augments S6K1 phosphorylation in skeletal muscle following resistance type exercise in males. J Nutr 137:1880-1886

Koopman R, Zorenc AH, Gransier RJ, Cameron-Smith D, van Loon LJ (2006) Increase in S6K1 phosphorylation in human skeletal muscle following resistance exercise occurs mainly in type II muscle fibers. Am J Physiol Endocrinol Metab 290:E1245-52

Koopman R, Wagenmakers AJ, Manders RJ, Zorenc AH, Senden JM, Gorselink M, Keizer HA, van Loon LJ (2005) Combined ingestion of protein and free leucine with carbohydrate increases postexercise muscle protein synthesis in vivo in male subjects. Am J Physiol Endocrinol Metab 288:E645-53

Korhonen MT, Cristea A, Alen M, Häkkinen K, Sipila S, Mero A, Viitasalo JT, Larsson L, Suominen H (2006) Aging, muscle fiber type, and contractile function in sprint-trained athletes. J Appl Physiol 101:906-917

Kosek DJ, Kim JS, Petrella JK, Cross JM, Bamman MM (2006) Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. J Appl Physiol 101:531-544

Kovanen V (2002) Intramuscular extracellular matrix: Complex environment of muscle cells. Exerc Sport Sci Rev 30:20-25

Kovanen V & Suominen H (1987) Effects of age and life-time physical training on fibre composition of slow and fast skeletal muscle in rats. Pflugers Arch 408:543-551

Kraemer WJ, Spiering BA, Volek JS, Ratamess NA, Sharman MJ, Rubin MR, French DN, Silvestre R, Hatfield DL, Van Heest JL, Vingren JL, Judelson DA, Deschenes MR, Maresh CM (2006) Androgenic responses to resistance exercise: Effects of feeding and L-carnitine. Med Sci Sports Exerc 38:1288-1296

Kraemer WJ & Ratamess NA (2004) Fundamentals of resistance training: Progression and exercise prescription. Med Sci Sports Exerc 36:674-688

Kraemer WJ, Adams K, Cafarelli E, Dudley GA, Dooly C, Feigenbaum MS, Fleck SJ, Franklin B, Fry AC, Hoffman JR, Newton RU, Potteiger J, Stone MH, Ratamess NA, Triplett-McBride T, American College of Sports Medicine (2002) American college of sports medicine position stand. progression models in resistance training for healthy adults. Med Sci Sports Exerc 34:364-380

Kraemer WJ, Volek JS, Bush JA, Putukian M, Sebastianelli WJ (1998) Hormonal responses to consecutive days of heavy-resistance exercise with or without nutritional supplementation. J Appl Physiol 85:1544-1555

Krissansen GW (2007) Emerging health properties of whey proteins and their clinical implications. J Am Coll Nutr 26:713S-23S

Krivickas LS, Walsh R, Amato AA (2009) Single muscle fiber contractile properties in adults with muscular dystrophy treated with MYO-029. Muscle Nerve 39:3-9

Kumar V, Selby A, Rankin D, Patel R, Atherton P, Hildebrandt W, Williams J, Smith K, Seynnes O, Hiscock N, Rennie MJ (2009) Age-related differences in dose response of muscle protein synthesis to resistance exercise in young and old men. J Physiol 15:211-7

Kvorning T, Andersen M, Brixen K, Schjerling P, Suetta C, Madsen K (2007) Suppression of testosterone does not blunt mRNA expression of myoD, myogenin, IGF, myostatin or androgen receptor post strength training in humans. J Physiol 578:579-593

Kvorning T, Andersen M, Brixen K, Madsen K (2006) Suppression of endogenous testosterone production attenuates the response to strength training: A randomized, placebo-controlled, and blinded intervention study. Am J Physiol Endocrinol Metab 291:E1325-32

Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R (2002) Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. J Biol Chem 277:49831-49840

Le Bacquer O, Petroulakis E, Paglialunga S, Poulin F, Richard D, Cianflone K, Sonenberg N (2007) Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. J Clin Invest 117:387-396

Lee SJ (2007) Quadrupling muscle mass in mice by targeting TGF-ss signaling pathways. PLoS ONE 2:e789

Lee SJ, Reed LA, Davies MV, Girgenrath S, Goad ME, Tomkinson KN, Wright JF, Barker C, Ehrmantraut G, Holmstrom J, Trowell B, Gertz B, Jiang MS, Sebald SM, Matzuk M, Li E, Liang LF, Quattlebaum E, Stotish RL, Wolfman NM (2005) Regulation of muscle growth by multiple ligands signaling through activin type II receptors. Proc Natl Acad Sci U S A 102:18117-18122

Lee SJ (2004) Regulation of muscle mass by myostatin. Annu Rev Cell Dev Biol 20:61-86

Lee SJ & McPherron AC (2001) Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci U S A 98:9306-9311

Leger B, Cartoni R, Praz M, Lamon S, Deriaz O, Crettenand A, Gobelet C, Rohmer P, Konzelmann M, Luthi F, Russell AP (2006) Akt signalling through GSK-3beta, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. J Physiol 576:923-933

Levenhagen DK, Gresham JD, Carlson MG, Maron DJ, Borel MJ, Flakoll PJ (2001) Postexercise nutrient intake timing in humans is critical to recovery of leg glucose and protein homeostasis. Am J Physiol Endocrinol Metab 280:E982-93

Liu W & Saint DA (2002) Validation of a quantitative method for real time PCR kinetics. Biochem Biophys Res Commun 294:347-353

Louis ES, Raue U, Yang Y, Jemiolo B, Trappe SW (2007) Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. J Appl Physiol 103:1744-51

Luthi JM, Howald H, Claassen H, Rosler K, Vock P, Hoppeler H (1986) Structural changes in skeletal muscle tissue with heavy-resistance exercise. Int J Sports Med 7:123-127

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

MacDougall JD, Sale DG, Alway SE, Sutton JR (1984) Muscle fiber number in biceps brachii in bodybuilders and control subjects. J Appl Physiol 57:1399-1403

MacDougall JD, Sale DG, Elder GC, Sutton JR (1982) Muscle ultrastructural characteristics of elite powerlifters and bodybuilders. Eur J Appl Physiol Occup Physiol 48:117-126

MacDougall JD, Elder GC, Sale DG, Moroz JR, Sutton JR (1980) Effects of strength training and immobilization on human muscle fibres. Eur J Appl Physiol Occup Physiol 43:25-34

Mader S, Lee H, Pause A, Sonenberg N (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol Cell Biol 15:4990-4997

Magee TR, Artaza JN, Ferrini MG, Vernet D, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, Gonzalez-Cadavid NF (2006) Myostatin short

interfering hairpin RNA gene transfer increases skeletal muscle mass. J Gene Med 8:1171-1181

Malumbres M, Ortega S, Barbacid M (2000) Genetic analysis of mammalian cyclin-dependent kinases and their inhibitors. Biol Chem 381:827-838

Marcell TJ, Harman SM, Urban RJ, Metz DD, Rodgers BD, Blackman MR (2001) Comparison of GH, IGF-I, and testosterone with mRNA of receptors and myostatin in skeletal muscle in older men. Am J Physiol Endocrinol Metab 281:E1159-64

Mascher H, Tannerstedt J, Brink-Elfegoun T, Ekblom B, Gustafsson T, Blomstrand E (2008) 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

Masure S, Haefner B, Wesselink JJ, Hoefnagel E, Mortier E, Verhasselt P, Tuytelaars A, Gordon R, Richardson A (1999) Molecular cloning, expression and characterization of the human serine/threonine kinase akt-3. Eur J Biochem 265:353-360

McCall GE, Byrnes WC, Dickinson A, Pattany PM, Fleck SJ (1996) Muscle fiber hypertrophy, hyperplasia, and capillary density in college men after resistance training. J Appl Physiol 81:2004-2012

McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R (2003) Myostatin negatively regulates satellite cell activation and self-renewal. J Cell Biol 162:1135-1147

McKirnan MD, Gray CG, White FC (1991) Effects of feeding on muscle blood flow during prolonged exercise in miniature swine. J Appl Physiol 70:1097-1104

McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387:83-90

McPherron AC & Lee SJ (1997) Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci U S A 94:12457-12461

Mendias CL, Bakhurin KI, Faulkner JA (2008) Tendons of myostatin-deficient mice are small, brittle, and hypocellular. Proc Natl Acad Sci U S A 105:388-393

Mendias CL, Marcin JE, Calerdon DR, Faulkner JA (2006) Contractile properties of EDL and soleus muscles of myostatin-deficient mice. J Appl Physiol 101:898-905

Mendler L, Baka Z, Kovacs-Simon A, Dux L (2007) Androgens negatively regulate myostatin expression in an androgen-dependent skeletal muscle. Biochem Biophys Res Commun 361:237-242

Mero A, Leikas A, Knuutinen J, Hulmi JJ, Kovanen V (2009) Effect of strength training session on plasma amino acid concentration following oral ingestion of leucine, BCAAs or glutamine in men. Eur J Appl Physiol 105:215-223

Mero A, Leikas A, Rinkinen N, Huhta P, Hulmi JJ, Pitkänen H, Knuutinen J (2008) Effect of strength training session on plasma amino acid concentration following oral ingestion of arginine or taurine in men. Amino Acids 35:99-106

Mero A (1999) Leucine supplementation and intensive training. Sports Med 27:347-358

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

Miyazaki M & Esser KA (2008) Cellular mechanisms regulating protein synthesis and skeletal muscle hypertrophy in animals. J Appl Physiol doi:10.1152/japplphysiol.91355.2008

Monks DA, Kopachik W, Breedlove SM, Jordan CL (2006) Anabolic responsiveness of skeletal muscles correlates with androgen receptor protein but not mRNA. Can J Physiol Pharmacol 84:273-277

Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM (2009) Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. Am J Clin Nutr 89:161-8

Moritani T & deVries HA (1980) Potential for gross muscle hypertrophy in older men. J Gerontol 35:672-682

Morpurgo B (1879) Ueber activitats-hypertrophie der willkuerlichen muskeln. Virchows Arch Path Anat 150:522-554

Mosher DS, Quignon P, Bustamante CD, Sutter NB, Mellersh CS, Parker HG, Ostrander EA (2007) A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. PLoS Genet 3:e79

Nader GA (2005) Molecular determinants of skeletal muscle mass: Getting the "AKT" together. Int J Biochem Cell Biol 37:1985-1996

Nagasawa T, Hirano J, Yoshizawa F, Nishizawa N (1998) Myofibrillar protein catabolism is rapidly suppressed following protein feeding. Biosci Biotechnol Biochem 62:1932-1937

Nakazato K, Hirose T, Song H (2006) Increased myostatin synthesis in rat gastrocnemius muscles under high-protein diet. Int J Sport Nutr Exerc Metab 16:153-165

Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR (1999) 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 Pt 2:427-431

Nelson DL & Cox MM (2000) Lehninger Principles of Biochemistry. Worth Publish, USA

Nosaka K, Sacco P, Mawatari K (2006) Effects of amino acid supplementation on muscle soreness and damage. Int J Sport Nutr Exerc Metab 16:620-635

Nygard O & Nilsson L (1990) Translational dynamics. interactions between the translational factors, tRNA and ribosomes during eukaryotic protein synthesis. Eur J Biochem 191:1-17

Ohanna M, Sobering AK, Lapointe T, Lorenzo L, Praud C, Petroulakis E, Sonenberg N, Kelly PA, Sotiropoulos A, Pende M (2005) Atrophy of

S6K1(-/-) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control. Nat Cell Biol 7:286-294

Ohkubo Y, Kishimoto T, Nakata T, Yasuda H, Endo T (1994) SV40 large T antigen reinduces the cell cycle in terminally differentiated myotubes through inducing Cdk2, Cdc2, and their partner cyclins. Exp Cell Res 214:270-278

Olsen S, Aagaard P, Kadi F, Tufekovic G, Verney J, Olesen JL, Suetta C, Kjaer M (2006) Creatine supplementation augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training. J Physiol 573:525-534

Osipova-Goldberg HI, Rogozkin VA, Feldkoren BI (2001) Properties of free and occupied androgen receptor in rat skeletal muscle cytosol: Effect of testosterone. J Steroid Biochem Mol Biol 78:481-492

Owino V, Yang SY, Goldspink G (2001) Age-related loss of skeletal muscle function and the inability to express the autocrine form of insulin-like growth factor-1 (MGF) in response to mechanical overload. FEBS Lett 505:259-263

Paddon-Jones D, Sheffield-Moore M, Aarsland A, Wolfe RR, Ferrando AA (2005) Exogenous amino acids stimulate human muscle anabolism without interfering with the response to mixed meal ingestion. Am J Physiol Endocrinol Metab 288:E761-7

Parkington JD, Siebert AP, LeBrasseur NK, Fielding RA (2003) Differential activation of mTOR signaling by contractile activity in skeletal muscle. Am J Physiol Regul Integr Comp Physiol 285:R1086-90

Pause A, Belsham GJ, Gingras AC, Donze O, Lin TA, Lawrence JC,Jr, Sonenberg N (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature 371:762-767

Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, Mueller M, Fumagalli S, Kozma SC, Thomas G (2004) S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol 24:3112-3124

Petrella JK, Kim JS, Cross JM, Kosek DJ, Bamman MM (2006) Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. Am J Physiol Endocrinol Metab 291:E937-46

Philip B, Lu Z, Gao Y (2005) Regulation of GDF-8 signaling by the p38 MAPK. Cell Signal 17:365-375

Philippou A, Stavropoulou A, Sourla A, Pissimissis N, Halapas A, Maridaki M, Koutsilieris M (2008) Characterization of a rabbit antihuman mechano growth factor (MGF) polyclonal antibody against the last 24 amino acids of the E domain. In Vivo 22:27-35

Phillips SM, Parise G, Roy BD, Tipton KD, Wolfe RR, Tamopolsky MA (2002) Resistance-training-induced adaptations in skeletal muscle protein turnover in the fed state. Can J Physiol Pharmacol 80:1045-1053

Phillips SM, Tipton KD, Ferrando AA, Wolfe RR (1999) Resistance training reduces the acute exercise-induced increase in muscle protein turnover. Am J Physiol 276:E118-24

Phillips SM, Tipton KD, Aarsland A, Wolf SE, Wolfe RR (1997) Mixed muscle protein synthesis and breakdown after resistance exercise in humans. Am J Physiol 273:E99-107

Pitkänen HT, Nykanen T, Knuutinen J, Lahti K, Keinanen O, Alen M, Komi PV, Mero AA (2003) Free amino acid pool and muscle protein balance after resistance exercise. Med Sci Sports Exerc 35:784-792

Prod'homme M, Balage M, Debras E, Farges MC, Kimball S, Jefferson L, Grizard J (2005) Differential effects of insulin and dietary amino acids on muscle protein synthesis in adult and old rats. J Physiol 563:235-248

Proud CG (2007) Signalling to translation: How signal transduction pathways control the protein synthetic machinery. Biochem J 403:217-234

Proud CG (2006) Regulation of protein synthesis by insulin. Biochem Soc Trans 34:213-216

Psilander N, Damsgaard R, Pilegaard H (2003) Resistance exercise alters MRF and IGF-I mRNA content in human skeletal muscle. J Appl Physiol 95:1038-1044

Pullen N & Thomas G (1997) The modular phosphorylation and activation of p70s6k. FEBS Lett 410:78-82

Rasmussen BB, Fujita S, Wolfe RR, Mittendorfer B, Roy M, Rowe VL, Volpi E (2006) Insulin resistance of muscle protein metabolism in aging. FASEB J 20:768-769

Rasmussen BB & Phillips SM (2003) Contractile and nutritional regulation of human muscle growth. Exerc Sport Sci Rev 31:127-131

Ratamess NA, Kraemer WJ, Volek JS, Maresh CM, Vanheest JL, Sharman MJ, Rubin MR, French DN, Vescovi JD, Silvestre R, Hatfield DL, Fleck SJ, Deschenes MR (2005) Androgen receptor content following heavy resistance exercise in men. J Steroid Biochem Mol Biol 93:35-42

Raue U, Slivka D, Jemiolo B, Hollon C, Trappe S (2006) Myogenic gene expression at rest and after a bout of resistance exercise in young (18-30 yr) and old (80-89 yr) women. J Appl Physiol 101:53-59

Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L (2003) Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. Mol Cell Biol 23:7230-7242

Reeves ND, Maganaris CN, Narici MV (2004) Ultrasonographic assessment of human skeletal muscle size. Eur J Appl Physiol 91:116-118

Rennie MJ, Bohe J, Smith K, Wackerhage H, Greenhaff P (2006) Branched-chain amino acids as fuels and anabolic signals in human muscle. J Nutr 136:264S-8S

Rennie MJ, Wackerhage H, Spangenburg EE, Booth FW (2004) Control of the size of the human muscle mass. Annu Rev Physiol 66:799-828

Reynolds TH,4th, Bodine SC, Lawrence JC,Jr (2002) Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. J Biol Chem 277:17657-17662

Rieu I, Balage M, Sornet C, Debras E, Ripes S, Rochon-Bonhomme C, Pouyet C, Grizard J, Dardevet D (2007) Increased availability of leucine with leucine-rich whey proteins improves postprandial muscle protein synthesis in aging rats. Nutrition 23:323-331

Rios R, Carneiro I, Arce VM, Devesa J (2002) Myostatin is an inhibitor of myogenic differentiation. Am J Physiol Cell Physiol 282:C993-9

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ (2001) 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

Rosenblatt JD & Woods RI (1992) Hypertrophy of rat extensor digitorum longus muscle injected with bupivacaine. A sequential histochemical, immunohistochemical, histological and morphometric study. J Anat 181:11-27

Roth SM, Martel GF, Ferrell RE, Metter EJ, Hurley BF, Rogers MA (2003) Myostatin gene expression is reduced in humans with heavy-resistance strength training: A brief communication. Exp Biol Med (Maywood) 228:706-709

Roth SM, Ivey FM, Martel GF, Lemmer JT, Hurlbut DE, Siegel EL, Metter EJ, Fleg JL, Fozard JL, Kostek MC, Wernick DM, Hurley BF (2001) Muscle size responses to strength training in young and older men and women. J Am Geriatr Soc 49:1428-1433

Rutherfurd SM & Moughan PJ (1998) The digestible amino acid composition of several milk proteins: Application of a new bioassay. J Dairy Sci 81:909-917

Ruvinsky I & Meyuhas O (2006) Ribosomal protein S6 phosphorylation: From protein synthesis to cell size. Trends Biochem Sci 31:342-348

Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P, Meyuhas O (2005) Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev 19:2199-2211

Ryazanov AG, Shestakova EA, Natapov PG (1988) Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation. Nature 334:170-173

Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM (2008) The rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320:1496-1501

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098-1101

Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14:1296-1302

Schmalbruch H & Hellhammer U (1977) The number of nuclei in adult rat muscles with special reference to satellite cells. Anat Rec 189:169-175

Schuelke M, Wagner KR, Stolz LE, Hubner C, Riebel T, Komen W, Braun T, Tobin JF, Lee SJ (2004) Myostatin mutation associated with gross muscle hypertrophy in a child. N Engl J Med 350:2682-2688

Schulte JN & Yarasheski KE (2001) Effects of resistance training on the rate of muscle protein synthesis in frail elderly people. Int J Sport Nutr Exerc Metab 11 Suppl:S111-8

Scott MP, Matsudaira PT, Lodish HF, Darnell JE, Zipursky L, Kaiser C, Berk A & Krieger M (2004) Molecular Cell Biology. W.H. Freeman and Co, USA

Sedliak M, Finni T, Cheng S, Kraemer WJ, Häkkinen K (2007) Effect of time-of-day-specific strength training on serum hormone concentrations and isometric strength in men. Chronobiol Int 24:1159-1177

Sefton BM & Shenolikar S (2001) Overview of protein phosphorylation. Curr Protoc Mol Biol Chapter 18:Unit 18.1

Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC (1998) Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J 17:6649-6659

Shimomura Y, Yamamoto Y, Bajotto G, Sato J, Murakami T, Shimomura N, Kobayashi H, Mawatari K (2006) Nutraceutical effects of branched-chain amino acids on skeletal muscle. J Nutr 136:529S-532S

Sinha-Hikim I, Cornford M, Gaytan H, Lee ML, Bhasin S (2006) Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. J Clin Endocrinol Metab 91:3024-3033

Sinha-Hikim I, Taylor WE, Gonzalez-Cadavid NF, Zheng W, Bhasin S (2004) Androgen receptor in human skeletal muscle and cultured muscle satellite cells: Up-regulation by androgen treatment. J Clin Endocrinol Metab 89:5245-5255

Sinha-Hikim I, Roth SM, Lee MI, Bhasin S (2003) Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. Am J Physiol Endocrinol Metab 285:E197-205

Smith EM, Finn SG, Tee AR, Browne GJ, Proud CG (2005) The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. J Biol Chem 280:18717-18727

Smith GI, Atherton P, Villareal DT, Frimel TN, Rankin D, Rennie MJ, Mittendorfer B (2008) Differences in muscle protein synthesis and anabolic signaling in the postabsorptive state and in response to food in 65-80 year old men and women. PLoS ONE 3:e1875

Smith K, Reynolds N, Downie S, Patel A, Rennie MJ (1998) Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. Am J Physiol 275:E73-8

Souza TA, Chen X, Guo Y, Sava P, Zhang J, Hill JJ, Yaworsky PJ, Qiu Y (2008) Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. Mol Endocrinol 22:2689-2702

Spangenburg EE, Le Roith D, Ward CW, Bodine SC (2008) A functional insulin-like growth factor receptor is not necessary for load-induced skeletal muscle hypertrophy. J Physiol 586:283-291

Stipanuk MH (2007) Leucine and protein synthesis: MTOR and beyond. Nutr Rev 65:122-129

Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ (2004) The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 14:395-403

Sun Y, Fang Y, Yoon MS, Zhang C, Roccio M, Zwartkruis FJ, Armstrong M, Brown HA, Chen J (2008) Phospholipase D1 is an effector of rheb in the mTOR pathway. Proc Natl Acad Sci U S A 105:8286-8291

Suzuki M, Doi T, Lee SJ, Okamura K, Shimizu S, Okano G, Sato Y, Shimomura Y, Fushiki T (1999) Effect of meal timing after resistance exercise on hindlimb muscle mass and fat accumulation in trained rats. J Nutr Sci Vitaminol (Tokyo) 45:401-409

Symons TB, Schutzler SE, Cocke TL, Chinkes DL, Wolfe RR, Paddon-Jones D (2007) Aging does not impair the anabolic response to a protein-rich meal. Am J Clin Nutr 86:451-456

Tang JE, Perco JG, Moore DR, Wilkinson SB, Phillips SM (2008) Resistance training alters the response of fed-state mixed muscle protein synthesis in young men. Am J Physiol Regul Integr Comp Physiol 294:R172-8

Tang JE, Manolakos JJ, Kujbida GW, Lysecki PJ, Moore DR, Phillips SM (2007) Minimal whey protein with carbohydrate stimulates muscle protein synthesis following resistance exercise in trained young men. Appl Physiol Nutr Metab 32:1132-1138

Tarnopolsky MA, Atkinson SA, MacDougall JD, Chesley A, Phillips S, Schwarcz HP (1992) Evaluation of protein requirements for trained strength athletes. J Appl Physiol 73:1986-1995

Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH,Jr, Kull FC,Jr, Gonzalez-Cadavid N (2001) Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. Am J Physiol Endocrinol Metab 280:E221-8

Terova G, Bernardini G, Binelli G, Gornati R, Saroglia M (2006) cDNA encoding sequences for myostatin and FGF6 in sea bass (dicentrarchus labrax, L.) and the effect of fasting and refeeding on their abundance levels. Domest Anim Endocrinol 30:304-319

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

Tesch PA, Colliander EB, Kaiser P (1986) Muscle metabolism during intense, heavy-resistance exercise. Eur J Appl Physiol Occup Physiol 55:362-366

Tesch PA & Larsson L (1982) Muscle hypertrophy in bodybuilders. Eur J Appl Physiol Occup Physiol 49:301-306

Thalacker-Mercer AE, Fleet JC, Craig BA, Carnell NS, Campbell WW (2007) Inadequate protein intake affects skeletal muscle transcript profiles in older humans. Am J Clin Nutr 85:1344-1352

Tipton KD, Elliott TA, Cree MG, Aarsland AA, Sanford AP, Wolfe RR (2007) Stimulation of net muscle protein synthesis by whey protein ingestion before and after exercise. Am J Physiol Endocrinol Metab 292:E71-6

Tipton KD & Witard OC (2007) Protein requirements and recommendations for athletes: Relevance of ivory tower arguments for practical recommendations. Clin Sports Med 26:17-36

Tipton KD, Elliott TA, Cree MG, Wolf SE, Sanford AP, Wolfe RR (2004) Ingestion of casein and whey proteins result in muscle anabolism after resistance exercise. Med Sci Sports Exerc 36:2073-2081

Tipton KD, Rasmussen BB, Miller SL, Wolf SE, Owens-Stovall SK, Petrini BE, Wolfe RR (2001) Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise. Am J Physiol Endocrinol Metab 281:E197-206

Tipton KD & Wolfe RR (2001) Exercise, protein metabolism, and muscle growth. Int J Sport Nutr Exerc Metab 11:109-132

Tipton KD, Ferrando AA, Phillips SM, Doyle D,Jr, Wolfe RR (1999) Postexercise net protein synthesis in human muscle from orally administered amino acids. Am J Physiol 276:E628-34

Tseng BS, Kasper CE, Edgerton VR (1994) Cytoplasm-to-myonucleus ratios and succinate dehydrogenase activities in adult rat slow and fast muscle fibers. Cell Tissue Res 275:39-49

Ustunel I, Akkoyunlu G, Demir R (2003) The effect of testosterone on gastrocnemius muscle fibres in growing and adult male and female rats: A histochemical, morphometric and ultrastructural study. Anat Histol Embryol 32:70-79

van Loon LJ, Saris WH, Verhagen H, Wagenmakers AJ (2000) Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate. Am J Clin Nutr 72:96-105

Vary TC, Anthony JC, Jefferson LS, Kimball SR, Lynch CJ (2007) Rapamycin blunts nutrient stimulation of eIF4G, but not PKCepsilon phosphorylation, in skeletal muscle. Am J Physiol Endocrinol Metab 293:E188-96

Vary TC & Lynch CJ (2007) Nutrient signaling components controlling protein synthesis in striated muscle. J Nutr 137:1835-1843

Verdijk LB, Jonkers RA, Gleeson BG, Beelen M, Meijer K, Savelberg HH, Wodzig WK, Dendale P, van Loon LJ (2009) Protein supplementation before and after exercise does not further augment skeletal muscle hypertrophy after resistance training in elderly men. Am J Clin Nutr 89:608-16

Vermeulen A, Verdonck L, Kaufman JM (1999) A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 84:3666-3672

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

Volpi E, Mittendorfer B, Rasmussen BB, Wolfe RR (2000) The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. J Clin Endocrinol Metab 85:4481-4490

Wackerhage H & Rennie MJ (2006) How nutrition and exercise maintain the human musculoskeletal mass. J Anat 208:451-458

Wagner KR, Fleckenstein JL, Amato AA, Barohn RJ, Bushby K, Escolar DM, Flanigan KM, Pestronk A, Tawil R, Wolfe GI, Eagle M, Florence JM, King WM, Pandya S, Straub V, Juneau P, Meyers K, Csimma C, Araujo T, Allen R, Parsons SA, Wozney JM, Lavallie ER, Mendell JR (2008) A phase I/IItrial of MYO-029 in adult subjects with muscular dystrophy. Ann Neurol 63:561-571

Wallenstein S & Fisher AC (1977) The analysis of the two-period repeated measurements crossover design with application to clinical trials. Biometrics 33:261-269

Wang J, Galil KA, Setchell BP (1983) Changes in testicular blood flow and testosterone production during aspermatogenesis after irradiation. J Endocrinol 98:35-46

Wang X, Beugnet A, Murakami M, Yamanaka S, Proud CG (2005) Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. Mol Cell Biol 25:2558-2572

Welle S, Bhatt K, Pinkert CA (2006) Myofibrillar protein synthesis in myostatin-deficient mice. Am J Physiol Endocrinol Metab 290:E409-15

Welle S, Bhatt K, Thornton CA (1999) Stimulation of myofibrillar synthesis by exercise is mediated by more efficient translation of mRNA. J Appl Physiol 86:1220-1225

Welle S, Totterman S, Thornton C (1996) Effect of age on muscle hypertrophy induced by resistance training. J Gerontol A Biol Sci Med Sci 51:M270-5

Wernbom M, Augustsson J, Thomee R (2007) The influence of frequency, intensity, volume and mode of strength training on whole muscle cross-sectional area in humans. Sports Med 37:225-264

Whittemore LA, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P, Knight A, Maylor R, O'Hara D, Pearson A, Quazi A, Ryerson S, Tan XY, Tomkinson KN, Veldman GM, Widom A, Wright JF, Wudyka S, Zhao L, Wolfman NM (2003) Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochem Biophys Res Commun 300:965-971

Wilkinson SB, Phillips SM, Atherton PJ, Patel R, Yarasheski KE, Tarnopolsky MA, Rennie MJ (2008) 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

Wilkinson SB, Tarnopolsky MA, Macdonald MJ, Macdonald JR, Armstrong D, Phillips SM (2007) Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption

of an isonitrogenous and isoenergetic soy-protein beverage. Am J Clin Nutr 85:1031-1040

Willard FS, Berven LA, Crouch MF (2001) Lysophosphatidic acid activates the 70-kDa S6 kinase via the lipoxygenase pathway. Biochem Biophys Res Commun 287:607-613

Willoughby DS, Stout JR, Wilborn CD (2007) Effects of resistance training and protein plus amino acid supplementation on muscle anabolism, mass, and strength. Amino Acids 32:467-477

Willoughby DS (2004) Effects of heavy resistance training on myostatin mRNA and protein expression. Med Sci Sports Exerc 36:574-582

Willoughby DS & Taylor L (2004) Effects of sequential bouts of resistance exercise on androgen receptor expression. Med Sci Sports Exerc 36:1499-1506

Willoughby DS & Rosene JM (2003) Effects of oral creatine and resistance training on myogenic regulatory factor expression. Med Sci Sports Exerc 35:923-929

Willoughby DS & Nelson MJ (2002) Myosin heavy-chain mRNA expression after a single session of heavy-resistance exercise. Med Sci Sports Exerc 34:1262-1269

Wolfman NM, McPherron AC, Pappano WN, Davies MV, Song K, Tomkinson KN, Wright JF, Zhao L, Sebald SM, Greenspan DS, Lee SJ (2003) Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. Proc Natl Acad Sci U S A 100:15842-15846

Wong TS & Booth FW (1990) Protein metabolism in rat gastrocnemius muscle after stimulated chronic concentric exercise. J Appl Physiol 69:1709-1717

Yang S, Alnaqeeb M, Simpson H, Goldspink G (1996) Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch. J Muscle Res Cell Motil 17:487-495

Yang W, Zhang Y, Li Y, Wu Z, Zhu D (2007) Myostatin induces cyclin D1 degradation to cause cell cycle arrest through a phosphatidylinositol 3-kinase/AKT/GSK-3 beta pathway and is antagonized by insulin-like growth factor 1. J Biol Chem 282:3799-3808

Yang W, Chen Y, Zhang Y, Wang X, Yang N, Zhu D (2006) Extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase pathway is involved in myostatin-regulated differentiation repression. Cancer Res 66:1320-1326

Yang Y, Creer A, Jemiolo B, Trappe S (2005) Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. J Appl Physiol 98:1745-1752

Yarasheski KE, Bhasin S, Sinha-Hikim I, Pak-Loduca J, Gonzalez-Cadavid NF (2002) Serum myostatin-immunoreactive protein is increased in 60-92 year old women and men with muscle wasting. J Nutr Health Aging 6:343-348