THE TEMPORAL CONTROL OF LOAD-DEPENDENT ANABOLIC AND CATABOLIC PATHWAYS IN HUMAN EXTENSOR MUSCLES

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ABSTRACT

Skeletal muscle displays a remarkable plasticity to loading and unloading. Such load-dependence is illustrated by the remodelling of metabolic and myofibrillar protein composition. To date the earliest documented molecular adjustments in unloaded human skeletal muscle consists of pre-translational alteration in the expression of factors regulating protein degradation and synthesis and mitochondrial metabolism. In project one I set out to investigate the changes in protein expression in the soleus and vastus lateralis muscle, muscles involved in posture and movement, after three days of unilateral limb suspension. My findings suggest that early unloading alters FAK-pY397 and metavinculin content, and deregulate the expression of factors that set the slow-oxidative phenotype. The underlying mechanisms governing disuse atrophy have not been characterized. Therefore, in the second project I examined muscle fibre morphology and potential factors associated with anabolic signalling as well as markers representative of the catabolic pathways in response to a chronic unloading period of 21 days in humans. Our findings support the proposition based on animal studies that mechanisms responsible for muscle atrophy involve a decrease in protein synthesis accompanied by an increase in protein degradation. It is well known that adjustments in muscle size are driven by changes in the content of myofibrils. However, how such myofibrillar alterations are integrated at the molecular and the architectural level in muscle fibres that undergo adaptive changes has not been resolved. Thus in project 3 I have examined changes in the content of costamere components with increased and reduced loading of human antigravity muscle in relation to changes in muscle size and molecular parameters of muscle size regulation. The findings suggest that load-dependent plasticity of

muscle size is integrated through costamere remodelling via a load-regulated process that involves level alterations of FAK and KAK-pY397 concentration.

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AUTHORS DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Manchester Metropolitan University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Manchester Metropolitan University and the Institute for Biomedical Research in Human Movement and Health.

This thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this Research Project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

SIGNED: ……. DATE: ……

DEDICATION

I would like to dedicate this thesis to my parents Peihong and Gaode and my sister Caroline for their love, support and belief in me throughout my life. Without them I would not be the person I am today. I would also like to deeply thank Professor Marco Narici and Professor Martin Flueck for their strong influence in my passion for a career in science. If I am half the scientist as they are, I will be very happy!

PUBLICATIONS AND PRESENTATIONS

Li R,Narici MV, Erskine RM, Seynnes OR, Rittweger J, Pisot R, Simunie B, Flueck M. (2013) Costamere remodeling with muscle loading and unloading in men. *Journal of Anatomy*, 223(5):525-36.

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CONTENTS

Abbreviations:

MRI	Magnetic resonance imaging
CT	Computerised tomography
IM	Immobilization
ULLS	Unilateral limb suspension
PI3K	Phosphoinositide 3-kinase
Akt /PKB	Protein Kinase B
PDK1	Phosphoinositide-dependent kinase-1
mTOR	Mammalian target of rapamycin
rpS6	Ribosomal protein S6
P70S6K/S6K1	P70 ribosomal protein S6 kinase/ribosomal protein S6 kinase beta-1

EIF4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1
eef2	Eukaryotic translation elongation factor2
Eif-2b	Eukaryotic initiation factor 2B
GSK3B	Glycogen synthase kinase 3beta
PtdIns(3,4)P2	Phosphatidylinositol (3,4)-bisphosphate
PtdIns(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
PP2A	Protein phosphatase 2
PTEN	Phosphatase and tensin homolog
SHIP2	SH2 domain containing inositol 5-phosphatase 2
FOXO	Forkhead box-O
ECM	Extracellular matrix
FAK	Focal adhesion kinase
S6K2	Serine/threonine protein kinase 2
5'TOP	5'terminal oligopyrimidine tract
mRNA	Messenger ribonucleic acid
eIF3	Eukaryotic initiation factor 3
UPS	Ubiquitin-proteasome system
ATP	Adenosine triphosphate
Ub	Ubiquitin
MAFbx	F-box protein 32
MuRF1	Muscle RING-finger protein-1
cDNA	Complementary deoxyribonucleic acid
RING	Really interesting new gene
SCF	Skp, Cullin, F-box
Skp1	S-phase kinase-associated protein 1
eIF3f	Eukaryotic translation initiation factor 3 subunit F
MyoD	Myogenic differentiation 1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IGF1	Insulin-like growth factor 1
PBS	Phosphate buffered saline
NaCl	Sodium chloride
KCl	Potassium chloride
Na_3PO_4	Sodium phosphate
KH_2PO_4	Potassium dihydrogen phosphate
dH_2O	Deionised hydrogen oxide
H_2O_2	Hydrogen peroxide
BSA	Bovine serum albumin
IgG	Insulin growth factor
FAB fragment	Fragment antigen-binding
AEC	3-amino-9-ethylcarbazole
DMF	Dimethylformamide
MHC	Myosin Heavy Chain
MHCII	Myosin heavy chain class II
MHC I	Myosin heavy chain class I
RIPA	Radioimmunoprecipitation assay
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
EDTA	Ethylenediaminetetraacetic acid
NP-40	Nonyl phenoxypolyethoxylethanol-40
Na_3VO_4	Sodium orthovanadate
PMSF	Phenylmethylsulfonyl fluoride
UV	Ultraviolet
APS	Adenosine 5'-phosphosulfate
TEMED	N,N,N',N'-Tetramethylethylenediamine
SDS	Sodium dodecyl sulfate
Tris base	Tris(hydroxymethyl)aminomethane
TBST	Tris buffered saline with Tween 20

ECL	Electrochemiluminescence
HRP	Horseradish peroxidase
RLT	RNA lysis buffer
RNase	Ribonuclease
DEPC	Diethylpyrocarbonate
RNA	Ribonucleic acid
DNase	Deoxyribonuclease
RW1	RNA wash buffer 1
RT	Reverse transcription
dNTP	Deoxyribonucleotide
PCR	Polymerase chain reaction
DNA	Deoxytibonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
$MgCl_2$	Magnesium chloride
KCl	Potassium chloride
cDNA	Complementary DNA
Taq	Thermus aquaticus
dUTP	Deoxyuridine triphosphate
AEC	3-amino-9-ethylcarbazole in dimethylformamide
DMSO	Dimethyl sulfoxide
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDHA	Succinate dehydrogenase complex, subunit A
ATP5A1	ATP synthase alpha subunit 1
UQCRC1	Ubiquinol-cytochrome c reductase core protein 1
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit, 9
NADH	Nicotinamide adenine dinucleotide hydride
FRNK	FAK-related nonkinase
VL	Vastus lateralis
SOL	Soleus
ANOVA	Analysis of variance
P	Probability value
MCSA	Mean cross-sectional area
CSA	Cross-sectional area
R	Correlation coefficient
vs.	Versus
Fig.	Figure
TBS	Tris buffered saline
Vmax	Maximum velocity
m.	Muscle
SE	Standard error
eIF-4E	Eukaryotic translation initiation factor 4E
3MH	3-mercaptohexanol

1. Review: The cellular and molecular basis of skeletal muscle disuse atrophy

1.1 Introduction: Disuse atrophy

Skeletal muscle atrophy can occur as a result of neural injuries, aging, starvation or lack of use and is a co-morbidity of many chronic diseases e.g. cancer. In particular, disuse happens to be a major cause of muscle atrophy in humans, typically occurring among patients, inactive elderly and astronauts. This form of muscle loss due to reduced loading demands imposed by inactivity has been regarded as an appropriate beneficial adaptation for reducing total muscular energy requirements (Murton *et al*., 2008). However, such muscle mass loss can be debilitating, significantly affecting the activities of daily living and decreasing quality of life of individuals as well as endangering the well-being of astronauts.

1.1.1 Functional and physiological changes with disuse atrophy

In response to inactivity, skeletal muscle undergoes structural and functional adaptations necessary to the reduced loading demands imposed in this new environment. The major unloading induceddeleterious alterations typically feature the loss of muscle mass and the associated decline in peak force and power, such that largest losses of function occur in the low-force-high-velocity region of the force-velocity relationship. Many of physiological changes of skeletal muscle associated to unloading can occur on the time scale of days or weeks (Booth and Thomason 1991, Flueck and Hoppeler 2003). The earliest documented molecular adjustments

involve pre-translational regulation of protein degradation and mitochondrial metabolism at just two days of disuse. The typical consequences underlying this load-dependent muscle loss become manifest after two weeks of unloading and consist of a distinctive muscle loss to a substantial degree, such that two weeks of leg-immobilization has shown an almost 5% reduction in quadriceps lean mass. A 20% reduction in type I muscle fibre cross sectional area and a 30% reduction in type II fibre area were observed following 60-days bed rest. Findings suggest that the extent of atrophy is muscle type specific with the greatest being observed in antigravity muscles, containing predominantly slow-twitch muscle fibres. Apart from the distinctive muscle fibre atrophy in reponse to immobilization, muscle fibres also undergo a fiber type transformation involving a shift from a slow-oxidative to an atrophic, fast-glycolytic phenotype, resulting in increased expression of fast myosin isoforms, which has been well documented in experiements on rodents (Desplanches *et al*., 1987, Fitts *et al*., 2000, Flueck and Hoppeler 2003), whereas there were few human studies in which a clear slow to fast shift in fibre-type distribution was observed following disuse (Hortobagyi *et al*., 2000, Borina *et al*., 2010). The mechanism underlying such fibre type switching represented by MHC-1-MHC-2A-MHC-2X shift in MHC isoform expression is not clear. However, a recent transgenic study demonstrated that focal adhesion kinase signaling promotes fibre type differentiation towards slow-oxidative muscle phenotype, whereby FAK overexpression in rat antigravitational muscle fibres upregulated transcript levels and protein content of MHC1 and components of mitochondrial respiration with a reduced expression of the major fast-type myosin heavy chain (MHC) isoform, MHC2A and such FAK-modulated muscle transcriptome was load-dependent (Durieux *et al*., 2009).

1.2 Models used in studying muscle unweighting

All data on disuse atrophy of human skeletal muscle are gathered from the utility of experimental designs that simulate the unloaded skeletal muscle conditions in humans. Due to extremely limited opportunities and sheer cost associated with space missions, several ground-based models of unweighting applied to study human disuse atrophy were first introduced as cost-effective and less time consuming alternatives to mimic the microgravity environment of space. These ground-based models are useful in the way to provide basic experimental design elements including control subjects as well as the ability to regulate variables like diet and exercise and they prove to be successful surrogates for providing valuable insights into disuse atrophy since the changes of musculoskeletal system appear similar to that in real situations. Below is a general overview of the effectiveness of the three different human models of unloading with particular consideration for the practicalaspects of each of the models based on their own unique characteristics.

1.2.1 Overview: Bed rest

Bed rest was established to be the principle ground-based model for mimicking the effects of microgravity during spaceflight on the musculoskeletal system. The particular version of the model involving head-down tilt successfully reproduces many of the associated cardiovascular and skeletal muscle-related aspects of the adaptation to microgravity (Adams *et al*., 2003). This model specifically induces unloading of the whole body hence effectively imposes inactivity on all postural body structures and tissues and generates a significant decrement in activity as well

as energy expenditure. Generally, results available to date from bed rest studies have compared favorably with those obtained from spaceflight. In the context of skeletal muscle physiology, both conditions share the same regulatory mechanisms for unweighting-induced adaptations, which concern the notion that much of the effect of altered loading stimulus on muscle is of a locally mediated response, initiated through mechanisms intrinsic to the muscle itself (Adams *et al*., 2003). The extent of the adaptive response is dependent on the degree of changes in loading experienced by particular muscle groups, i.e.postural muscles with antigravity functions will be primarily affected (LeBlanc *et al*., 1988, LeBlanc *et al*., 1995). Overall, the majority of the findings from literature suggest that the magnitude of alterations in size and performance in response to bed rest are consistent with those seen with space flight**.** A couple of potential cautionary issues in association with the intervention need to be addressed. Firstly, after relatively short periods of bed rest markers of increased bone resorption can be detected (Lueken *et al*., 1993, Smith *et al.,* 1998). Secondly, there has been evidence to indicate a certain degree of cardiovascular deconditioning following bed rest (Levine *et al*., 1997, Pawelczyk *et al*., 2001, Perhonen *et al*., 2001). This model imposes unweighting on all postural muscles as a result participants will require to follow a comprehensive programme of rehabilitation afterwards (Adams *et al*., 2003). Practically, bed rest studies involve a substantial amount of resources, such as specialized bed rest facilities that have showers to accommodate gurneys and the need for 24-hour nursing supervision (Widrick *et al*., 1997, Koryak 1998). Taking these above mentioned implications into consideration, it is questionable as to whether the use of this model is appropriate in situations where research interests just focus on the effects of unweighting on one or a few muscle groups. The fact that this intervention is particularly well suited for studying relatively complete whole body unweighting has restricted the application of this model to only

integrated musculoskeletal and cardiovascular investigations designed for studying multisystem effects of reduced activity (Adams *et al*., 2003).

1.2.2 Overview: Immobilization

Limb immobilization (IM) is another model that has been used to study disuse atrophy. This approach comprises lower limb casting, which involves the targeted limb being fixed with the knee joint in a flexed position. While carrying out ambulatory activities through the use of crutches the immobilized limb is floating above the ground. Data from strength measurements suggests that IM appears to induce a somewhat more rapid decline in maximum voluntary contraction performance compared to that during spaceflight and bed rest (Adams *et al*., 2003). Associated with this, IM findings indicates also a more rapid decline in muscle or muscle group size (cross-sectional area) (Adams *et al*., 2003). In terms of muscle fibre size measurements, there are only three reports in the literature for healthy IM subjects (Hespel *et al*., 2001, Hortobagyi *et al*., 2000, Veldhuizen *et al*., 1993). Amongst the existing data reported, already 14 days of IM resulted in muscle fibre CSA decrements of 8% for type I, 11% for type IIa and 9% for IIb.

Generally, mechanisms that underlie IM–induced muscle adaptations appear similar to those induced by spaceflight in which the postural antigravity muscles are to experience the greatest effect (Adams *et al*., 2003). The only deviation of this IM model from the other two models is the fixation of the knee joint. This added impact of the fixation might result in the more rapid change seen in IM in comparison to during spaceflight and bed rest. However, it is important to note that majority of IM data available to date are based on shorter durations and there are no

data after 28 days of IM. The fact of a lack of longer-period observations renders such conclusion of potential differences in response between IM, spaceflight and bed rest open to question. The immobilization model is specifically aimed at studying unloading-induced atrophy of skeletal muscle; the model induces inactivity to a certain extent, but mainly targets muscles in the suspended limb. This is advantageous for practical applications as participants can remain relatively active compared with e.g. bed rest models. Moreover, the resources in terms of facilities, equipment and personnel necessary to conduct IM studies are modest.

In contrast to that observed in space flight and bed rest IM limits adaptive response to only muscles in the suspended limb, avoiding problems associated with cephalad fluid shifts (Adams *et al*., 2003). Caution should be taken during the ambulatory period due to the possibility of falling associated with limb suspension and in cases involving improper use of crutches where axillary injuries may happen. There is a small risk of thrombosis (Bleeker *et al*., 2004), however, the overall risks associated with this intervention is low.

1.2.3 Overview: Unilateral limb suspension

Unilateral limb suspension (ULLS) is similar to the IM model in the way that it involves immobilization of the lower limb with unweighting of just one leg while the contralateral limb remains loaded. There happen to be two forms of the model.The older version employs the use of a support strap to suspend the target limb whereas the more recent one incorporates the use of a high-platform shoe for the loading leg (Berg *et al*., 1991, Ferretti *et al*., 2001). In each case, weight bearing is prevented on the targeted leg and individuals carry out ambulatory activity via the use of crutches. On the contrary to IM, ULLS does not involve constant fixation of the knee

joint. However, it should be noted that in the strap model, there is an element of knee flexion involved to certain extent, depending on the amount of time individuals spend in ambulatory activity, the associated changes in muscle strength is likely to simulate those postulated for IM due to the knee joint being in a flexed position. Interestingly, the actual findings from studies using the strap version of the ULLS model have demonstrated the observed changes in muscle strength to rather correspond to those data from space flight and bed rest. Therefore, the accelerated loss of strength as suggested previously in the IM section does not seem to take place after the periods of possible knee joint fixation involved in this model (Adams *et al*., 2003). The selective unloading of the treatment limb results in the adaptive responses to limit to affected muscles, such that the antigravity muscles of the leg experiences a greater reduction in muscle size compared to those of the thigh. The fact that the model has been developed relatively recent has lead to a very few number of muscle fibre size reports being available to date (Adams *et al*., 2003). Even though the existing fibre size alterations seems to be in accordance with those reported in space and bed rest, given the scarcity of results on this measurement factor it is not possible yet to determine whether the findings demonstrate a response that is comparable with the spaceflight or bed rest data. Furthermore, a recent report suggests that ULLS is not an appropriate model for spaceflight/bed rest induced alterations at the single-fibre level in skeletal muscle. However, such conclusions were reached based on comparisons made between fibres from a ULLS study with those from bed rest and spaceflight studies that included exercise countermeasures as part of the intervention, which raises questions as to whether such assessment forms a valid basis for evaluating the usefulness of the ULLS model (Baldwin *et al*., 1996, Riley *et al*., 1998, Riley *et al*., 2000, Widrick *et al*., 2002). Data indicate that the general mechanism governing ULLS induced changes conforms to the criteria of local effect rather than

effects of systemic phenomena, which are uniform to those observed in bed rest and spaceflight studies (Ferretti *et al*., 2001). For practical considerations, ULLS does prove to be a good model for studying local effects of unloading exclusively in skeletal muscle. Very little resources are required for conducting an ULLS study, in a way similar to IM, which is substantially less than those necessary for bed rest (Adams *et al*., 2003). The fact that ULLS model maintains lower limbs in normal anatomic positions for majority of the time allows participants to perform most occupational activities without compromising the study (Berg and Tesch 1996). In particular, the feature of unfixed knee joint in the platform shoe version enables participants to even achieve free range of limb motion in the targeted limb when performing ambulatory activities, as a result the direction of changes shown by the muscle size and functional data suggest responses to ULLS may more directly mimic spaceflight than those from the IM model (Dudley *et al*., 1992, Ploutz-Snyder *et al*., 1995, Ploutz-Snyder *et al*., 1996, Ferretti *et al*., 2001, Adams 2002). **S**uch periods of upright posture and the maintenance of relatively normal activity level majority of musculoskeletal system to remain loaded can successfully ameliorate the side effects imposed by relatively sedentary models including cardiovascular deconditioning (Adams *et al*., 2003). Moreover, the concern over falling during ambulatory activity with ULLS is considerably reduced in contrast to IM since there is the unloaded limb available to use in cases when balance is lost. A lone report has highlighted a risk of developing venous thrombosis in the calf following ULLS (Berg and Tesch 1996). This adverse event happened only in two of the subjects and appears to be a function of the attendant restriction of movement associating with the strap version of the model, through eliminating the strap element in the model have resolved this problem (Dudley *et al*., 1992, Ploutz-Snyder *et al*., 1995, Ploutz-Snyder *et al*., 1996, Ferretti *et al*., 2001, Adams 2002). Overall, like that of IM it is not sufficient for investigating

cardiovascular and whole body adaptation since it does not cause cephalad fluid shiftbut would rather represent an appropriate model to use for studying aspects of skeletal muscle specific unweighting. In our studies where ULLS was used, we have tried to limit the risk of thrombosis by keeping the knee joint in a slightly flexed position.

Figure 1.1 Model of unilateral limb suspension, with a left shoe with thick sole and the shoulder strap running around foot and ankle to unload the right foot

1.2.4 Evaluation of models

All of the three models discussed above can effectively bring aboutthe unloading-induced adaptations in skeletal muscle. Despite their impact on muscle fibres being of different degrees

of severity and/or rapidity, the overall response appears quite similar. The analysis of the data sets available on measured muscle physiological parameters including muscle size and strength performance do not favor the selection of one of the reviewed models over another. For selecting a specific model investigators should consider primarily the unique characteristics of each of the three models outlined in relation to the specific aims of a given study together with considerations for the more practical aspects of the models.

1.3 Proposed mechanisms governing disuse muscle atrophy

Skeletal muscle demonstrates a pronounced plasticity in response to loading status, which include remodeling of the contractile properties and metabolic capacities of individual muscle fibres (Booth 1991, Flueck and Hoppeler 2003). While the adaptations of skeletal muscle to changes in mechanical stimuli have been well documented, there is currently a lack of cellular and molecular understanding of such adjustments, especially regarding the underlying mechanisms. A very recent human study investigating the early events of unloading has reported the existence of differential gene expression patterns between muscle types, which are suggestive of regulatory mechanisms being possibly influenced by muscle phenotype and function (Gustafsson *et al*., 2010). In simple terms, the loss of muscle mass is the result of an imbalance between anabolic and catabolic regulations, favoring for an increased proteolysis and/or a decreased protein synthesis. Despite the fact that a variety of triggers and signaling proteins has been studied for a role in disuse atrophy, the cascade of signaling events that promotes the altered rate of protein turnover through anabolism or catabolism or both is still not clear. Based on animal unloading studies, protein degradation seems to be the major cause of muscle mass

loss, however, recent human isotope labeling experiments have lead to the proposition that the muscle atrophy in response to human disuse conditions is primarily the result of a decrease in muscle protein synthesis rather than an increase in protein degradation (de Boer *et al*., 2007a, Rennie *et al*., 2008). Reports of phosphorylation of specific translation factors at critical amino acids from unloading studies support the physiological regulation of protein synthesis as a control of muscle mass (Sandri 2008). At least modulated protein synthesis has been proposed by many to be the main mechanism underlying early regulations of load-dependent muscle protein turnover. In the review to follow, identified mechanisms are discussed with reference to key factors.

1.4 Anabolic signaling

1.4.1 The role of insulin-like growth factor 1 (IGF-1)/phophatidylinositol-3 kinase (PI3K)/AKT (Protein kinase B) pathway in disuse atrophy

The most well documented molecular pathway responsible for protein synthesis to date is the IGF-1/PI3K/Akt/ pathway. Insulin-like growth factor (IGF-1) activates the PI3K/Akt pathway necessary for the initiation of protein translation during mechanical loading of the muscle. The down-stream signalling cascade involves phosphoinositide-3-kinase (PI3K)-induced initial phosphorylation of protein kinase B (Akt) that in turn activates mammalian target of rapamycin (mTOR) and a number of its downstream effectors including p70 ribosomal protein S6 kinase (p70S6K) and translation initiation factors such as eIF4e-BP1 and eEF2 (Bodine *et al*., 2001b, Gingras *et al*., 2001, Pallafacchina *et al*., 2002, Deldicque *et al*., 2008, Sandri 2008). IGF-1 was

first suggested as an autocrine-paracrine anabolic factor produced by the muscle in a functional overload model, by Goldberg (1967). It was demonstrated that IGF-1 mRNA levels within the muscle increased in response to the load. This finding has been later confirmed by other investigators. Then, IGF-1 overexpression has been shown to increase muscle mass with concurrent activation of the Akt/mTOR signalling pathway (Barton 2006). Although animal studies indicate that the Akt/mTOR protein anabolic pathway seems to be down-regulated during unloading (Bodine *et al*., 2001b), its involvement in the human state of disuse atrophy has been questioned.

It has been generally agreed that mechanical stimuli can lead to increased protein synthesis via two initial triggers, increased growth factor concentration or direct mechanical tension. Akt/mTOR signaling integrates the growth factor-dependent control of muscle mass, whereas the latter form of stimulation has not been well characterized. At the moment there is strong evidence to support the presence of a load-dependent anabolic signaling pathway in skeletal muscle (Klossner *et al*., 2009). However, the signalling elements of this mechano-sensory pathway remain to be elucidated.

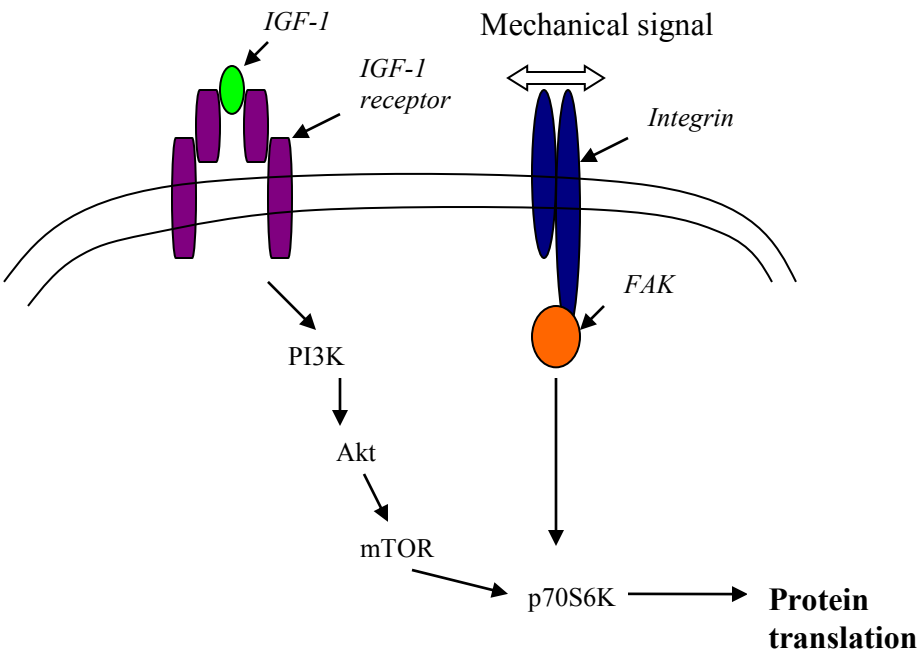


Figure 1.2 Model summarizing Insulin-like growth factor I (IGF-I)-mediated signalling pathways relevant to skeletal muscle hypertrophy and the Akt-independent FAK-mediated mechanical signalling towards enhanced protein translation via p70S6K (Klossner *et al*., 2009). *Abbreviations*: IGF-1, insulin-like growth factor 1; PI3K, phophatidylinositol-3 kinase; Akt, Protein kinase B; mTOR, mammalian target of rapamycin; FAK, focal adhesion kinase; p70S6K, p70 S6 kinase.

1.4.2 Costameres and its link to mechanotransduction

There is currently a limited understanding of the critical molecular events underlying the integration of mechanical signals toward downstream muscle protein synthesis responses. Attentions have been focused on mediators that are sensitive to mechanical stimuli, as these factors are most likely to contribute to signal transduction. In skeletal muscle, subsarcolemmal

focal adhesion assemblies known as costameres circumferentially align in register with the Z-disk of peripheral myofibrils, structurally link muscle fibres via the surrounding extracellular matrix to adjacent muscle fibres (Ervasti 2003). Consequently, given their location and associations costameres function to transmit contractile force laterally from the myofibrils across the sarcolemma to the extracellular matrix (ECM), serving to maintain the spatial organization of myofibrils as well as to insure the integrity of muscle fibres during contraction while physically couple force-generating sarcomeres with the sarcolemma. These costameres have been recognised as key structures for the myofascial transmission of forces. Indeed, it has been well known that costameric protein expression, stability and organization are closely regulated by mechanical tension (Quach and Rando 2006). Data currently supports the idea that costamere regulation plays a role in load-dependent muscle remodeling. Studies have demonstrated that costameric protein expression changes are associated with changes in muscle size in both animal and human loading and unloading models (Chopard *et al*., 2005, Durieux *et al*., 2007, Durieux *et al*., 2009). An in vitro skeletal myogenesis study of cultured differentiated skeletal muscle cells has shown that the formation and organization of aligned myofibrils is tightly coupled to costamerigenesis during skeletal myogenesis partly through contractile function and requires FAK signaling (Quach and Rando 2006). Furthermore, *in vivo* and *in vitro* data suggest that mechanosensitive protein complexes contained within costameres constitute potential sites for the conversion of contraction forces into mechano-sensitive signalling inside the myocellular compartments (Huijing *et al*., 1999, Bloch and Gonzalez-Serratos 2003**,** Samarel 2005, Quach and Rando 2006, Erskine *et al*., 2012). The suggestion that costameres are potential sites for mechanotransduction is supported in particular by load-dependent regulation of the amount and

activation status of the focal adhesion kinase (FAK) (Flueck *et al*., 1999, Flueck *et al*., 2002, Samarel 2005, Romer *et al*., 2006, Klossner *et al*., 2009).

1.4.3 Focal adhesion kinase (FAK) and mechano-dependent anabolic signaling

FAK is an integrin-bound cytoplasmic non-receptor tyrosine kinase that functions to transmit bi-directional signals at focal adhesions between ECM and the intracellular milieu. Its tyrosine phosphorylation at residue 397 represents the mechano-chemical coupling between mechanical stimulation of integrins and activation of intracellular signal transduction (Shyy and chien 1997, Flueck *et al*., 2002, Parsons 2003, Samarel *et al*., 2005, Ingber 2006, Romer *et al*., 2006). Briefly, FAK activation is primarily induced upon binding of integrins to ECM ligands. The resulting integrin clustering at focal adhesion complexes subsequently causes FAK auto-phosphorylation at Tyr-397 (Schlaepfer *et al*., 2004). This phosphorylation creates docking sites for proteins including Src-family kinases which further phosphorylate FAK at several other tyrosines such as Tyr 576/577, enabling FAK to achieve its full catalytic activity and to establish interactions with intracellular signaling cascades (Calalb *et al*., 1995, Parsons 2003). The mechanosensing property of FAK is especially illustrated by the observation of the immediate activation of FAK along with its downstream signaling components taking place within minutes after the deformation of integrins by mechanical tension.

The functional involvement of FAK in myogenesis and mechanosensing has been well documented in vivo and in vitro. Increased level of phosphorylated FAK was initially observed during chronic overload in rodents or synergist ablation in avian muscle. Conversely, a reduced level was reported with hindlimb suspension in rodents (Flueck *et al*., 1999, Gordon *et al*., 2001).

The phosphotransfer activity of FAK is stringently controlled by the magnitude of loading status. A 4-fold increase of FAK tyrosine phosphorylation has been reported in rat soleus muscle after 1 day of functional overload, whereas 7 days of muscle unloading through hindlimb suspension has induced a 10-fold reduction in phosphorylated FAK from the same muscle type. The distribution of sarcolemmal concentration of FAK is dependent on the degree of muscle fibre recruitment for contraction, with the highest level present in slow-oxidative muscles (Gordon *et al*., 2001, Flueck *et al*., 2002). In support of this idea, FAK has been recognized as a load-dependent regulator controlling the expression programme underlying the slow-oxidative phenotype in rat muscle (Durieux *et al*., 2009). Moreover, the altered level and phosphorylation of FAK have often been observed to associate with changes in myofibrillar content (Flueck *et al*., 1999, Carson and Wei 2000). In vitro, Rando and colleagues have found that FAK signaling is essential in directing myofibrillogenesis partly through contractile function during skeletal myogenesis in cultured differentiated skeletal muscle cells (Quach and Rando 2006). These observations suggest that FAK could be a load-dependent regulator of protein synthesis. p70S6K positioned downstream of FAK in cell culture is regarded as a potential effector of FAK activation since the phosphotransfer activity and phosphorylation status of both kinases correlate with protein synthesis and load-dependent increases in muscle mass, suggesting a possible functional coupling between the two kinases (Malik and Parsons 1996, Cary and Guan 1999, Flueck *et al*., 2002**,** Gan *et al*., 2006, Ingber 2006). Recently, a muscle-targeted transgenesis study in rat has identified FAK as an upstream regulator of p70s6k activation in a mechano-sensory anabolic pathway that is AKT-independent (Klossner *et al*., 2009).

Nevertheless, majority of the data on FAK signaling in muscle remodeling were obtained from cell lines and rodents. To date, few studies have examined the effect of mechanical loading state

on FAK in human skeletal muscle. Currently, only two human disuse studies have looked at FAK. De Boer and colleagues reported a 30% decrease in FAK phosphorylation at Tyr 576/577 and a 20% decrease in its content after 10 days of ULLS, with no apparent change in rest of the signaling proteins that they have measured (de Boer *et al*., 2007b). The other study has detected a reduced level of phosphorylated FAK (Tyr 576/577) as the most evident change after 14 days of unilateral knee immobilization. The author has suggested that much of the immobilization-induced muscle loss observed was the result of a decline in post–absorptive muscle protein synthesis (Glover *et al.,* 2008).

Overall, above findings point to FAK's function as an upstream regulatory molecule of a mechano-sensory pathway involved in anabolic signaling.

1.4.4 mTOR and p70S6K

One of the best-investigated downstream effectors of intracellular anabolic signaling in skeletal muscles is 70kDa ribosomal S6 kinase (p70S6K), a serine/threonine kinase with a well established role of promoting increased translational efficiency as well as translation capacity (Ruvinsky and Meyuhas 2006). Over the past 20 years, the cellular regulation and function of S6K as part of the anabolic signaling network has been widely studied. In particular, a great deal has learnt in regard to its association with the AKT/mTOR pathway.

In mammals, S6K represent a family composed of two distinct genes, S6K1 and S6K2, both of which encode two protein isoforms each generated by alternate ATG start site utilization. Among the isoforms, the 70kDA S6K1/p70S6K containing 502 amino acids has been the best characterized and most extensively studied protein, with a function in translational control. Its

activity is regulated by phosphorylation and dephosphorylation events. In its activated form, p70S6K phosphorylates the ribosomal protein S6 on five serine residues in the C-terminal tail, which then promotes the possible translation of a class of around 90 transcripts known as 5'TOP (terminal oligopyrimidine tract) mRNAs that are known to encode a variety of ribosomal proteins and translation factors. In addition, active S6K1 generated through dissociation from the multi-subunit scaffold eIF3 upon mTOR binding also phosphorylates a group of substrates involved in translation initiation or other steps for driving protein production (Ruvinsky and Meyuhas 2006, Zanchi and Lancha 2008, Fenton and Gout 2011, Magnuson *et al*., 2012).

The initial recognition of the critical nature of p70S6K in skeletal muscle is through the observation of significantly reduced cross sectional area of the individual muscle fibres in p70S6K-/- mice where S6K1 was genetically ablated compared to that of fibres from the wild-type animals (Ohanna *et al*., 2005). It has been widely accepted that the phosphorylaton of p70S6K at the 389 threonine site marks the increased protein anabolic response induced by mechanical stimulation (Baar and Esser 1999). Significantly elevated phosphorylation of p70S6K at Threonine 389 has been observed to correlate with increased muscle mass during loading protocols in rodents (Baar and Esser 1999, Nader and Esser 2001, Atherton *et al*., 2005**,** Burry *et al*., 2007). In line with the animal studies, elevations of activated p70S6K and its protein content have been reported to correlate with load-induced muscle mass increase as well as elevated protein synthesis from human studies of reloading (Hernandez *et al*., 2000, Cuthbertson *et al*., 2006, Dreyer *et al*., 2006, Eliasson *et al*., 2006, Fujita *et al*., 2007).

Among the upstream signaling pathways that regulates p70S6K, only one major pathway has been widely accepted, which is the phosphoinositide 3-kinase (PI3K)/Akt signalling cascade. This pathway is initiated by activation of PI3K, which then phosphorylates Akt and leads to the

activation of mTOR to enhance translational efficiency in part via inducing the downstream effector, S6K1. The involvement of this pathway in mechano-dependent anabolic signaling has been questioned. Firstly, S6K1 is capable to be activated by mechanical stimuli in the presence of an inhibitor of PI3K, Wortmannin (Hornberger and Chien 2006). In addition, recent studies have shown phosphorylation of S6K1 even in the absence of activated signaling components of PI3K/Akt/mTOR pathway (Eliasson *et al*., 2006, Fujita *et al*., 2007, Mascher *et al*., 2007, Deldicque *et al*., 2008, Terzis *et al*., 2008). Moreover, in an attempt to restore muscle mass PI3K-Akt activation was recovered but failed to attenuate the loss of muscle mass induced by unloading (Dupont *et al*., 2011). Mechanical stimuli happens to induce the phosphorylation event of mTOR at its 2448 serine residue which then leads to activations of several effectors including S6K1 (Reynolds *et al*., 2002). While mTOR/S6K1 signaling appears to be necessary for hypertrophy, the inhibition of this pathway by rapamycin does not induce significant muscle atrophy in adult animals. In fact, whether mTOR phosphorylation at Ser 2448 represents a positive, negative or inconsequential regulatory phosphorylation still remain unresolved. Recent studies with human subjects focusing on mechanical stimulation of the skeletal muscle have demonstrated that the activation of p70S6K (phosphorylation in threonine 389) is not always correlated with the activation of mTOR (phosphorylation in serine 2448) (Eliasson *et al*., 2006, Fujita *et al*., 2007). Three studies so far have assessed the effects of mechanical stimulation on another phosphorylation site, Ser 2481 of mTOR and no alterations were observed (Eliasson *et al*., 2006, Hornberger and Chien 2006, Mascher *et al*., 2007). The activation of mTOR seems not always a prerequisite for S6K1 phosphorylation and as a result studies have already begun to question the dependence of mTOR on p70S6K activation (Chiang and Abraham 2005, Holz and Blenis 2005, Mascher *et al*., 2007).

By taking these findings into consideration alternative mechano-sensitive pathway governing protein synthesis is present besides the Akt-mTOR pathway. Although mTOR is a regulator of S6K1 signaling, mediators of S6K1 activation in response to mechanical stimuli are not yet identified. However more recently a muscle-targeted transgenesis study in rat with a physiological model for hindlimb unloading and reloading has identified a mechano-sensory anabolic pathway that involves FAK as an upstream element to p70S6K (Klossner *et al*., 2009).

1.5 Catabolic pathways

In simplest terms, muscle mass loss is the result of decreased protein synthesis and/or increased protein degradation. Therefore to characterize the mechanisms underlying muscle atrophy an understanding of the implications of proteolytic systems is needed. The four main proteolytic pathways responsible for the cellular protein degradation seen in eukaryotic cells are cysteine-dependent aspartate specific proteases (caspases), cathepsins, calcium-dependent calpains, and the ubiquitin proteasome system (UPS), amongst which UPS appears to be the major proteolytic machinery involved in muscle protein breakdown, having its regulation being well documented in multiple types of muscle atrophy (Ventadour and Attaix 2006, Murton *et al*., 2008), yet evidences also suggest calpains, lysosomes and in some conditions even caspases to participate in muscle wasting (Berthon *et al*., 2007). The calcium-dependent calpains and caspase systems possibly play a role in initiating myofibrillar proteolysis, whereas the UPS is believed to degrade actin and MHC. Although all of the above mentioned proteolytic systems have been shown to be involved in the degradation of muscle protein during hindlimb unloading, a substantial amount of evidence from cell lines and animals to date supports the importance of UPS to unloading-

induced atrophy. However recent data are just beginning to shed light on its involvement in human muscle remodeling. Moreover, there is very limited data on the role of lysosomal proteolysis in muscle atrophy.

1.5.1 Ubiquitin-proteasome system (UPS)

In brief, the ATP-dependent ubiquitin-proteasome proteolytic process involves two distinct stages, namely the addition of ubiquitin (Ub) molecules to targeting proteins termed ubiquitination and the subsequent recognition of the ubiquitinylated substrates by proteasome for hydrolysis. The ubiquitination mechanism is coordinated by actions of three distinct groups of enzymes. The initial step of this cascade involves the binding of Ub to Ub-activating enzyme (E1) through a high-energy thioester bond. Then the formation of another thioester linkage between Ub and a cysteine residue of Ub-conjugating enzyme (E2) enables the successful transfer of Ub from E1 to E2. The third enzyme, Ub-ligase enzyme (E3) functions to catalyse the last step of ubiquitination whereby Ub is to establish an isopeptide bond with the target protein commonly between the ε-amino group of a lysine residue in the target protein and the carboxy-terminal glycine residue 76 in Ub with only a few exceptions (Passmore and Barford 2004, Hoeller *et al*., 2007, Li *et al*., 2007). These steps are repeated until the typical formation of a minimum of four Ub monomers having covalently attached via lysine residue 48 of Ub to the target protein is achieved as a prerequisite for the subsequent proteasomal hydrolysis (Thrower *et al*., 2000). This programme of ubiquitin transfer is an effective strategy for specifically targeting protein families to generate great substrate specificity. Upon recognition of the ubiquitinylated proteins by 26S proteasome for hydrolysis the protein substrate will unfold and enter the proteasome by means of

an ATP-dependent process. Structurally the 26S proteasome constitutes a 20S catalytic core and 19S regulatory caps, where the 20S core is formed by four heptameric rings consisting of alpha subunits to provide structural support and beta subunits in charge of the chymotrypsin-like, trypsin-like and caspase-like activities (Rivett *et al*., 1997, Attaix *et al*., 2003). The tagged proteins are cleaved into short oligonucleotides inside the proteasome. UPS functions to degrade non-intact myofibrils that are (partially) disintegrated through the action of calpains (Solomon and Goldberg 1996, Jagoe and Goldberg 2001).

While the literature largely explores the convergent role of the UPS components in a variety of atrophic conditions, its particular involvement in muscle disuse atrophy has been extensively investigated. Based on animal unloading data ubiquitin-proteasome system seems to be the mechanism responsible for the bulk of myofibrillar protein breakdown (Jackman and Kandarian 2004). Inhibition of proteasome function with available proteasome inhibitors since 1994 proved to significantly affect muscle proteolysis during disuse muscle atrophy. Signs indicative of UPS regulation have been recognized at various levels of the proteolytic cascade. A general upregulation of expression of mRNAs encoding distinct UPS constituents including many components of 26S proteasome and its subunits have been well documented in animal unloading models. A distinctive increase in the mRNA levels of E3 Ub-ligases, MAFbx and MuRF1was reported in particular (Murton *et al*., 2008). Other measured variables such as the ubiquitination rate and the level of ubiqutinated and polyubquitinated proteins have also been observed to increase. By examining the proteolytic activities of the 26S proteasome an elevated activity of chymotrypsin has been reportedly mentioned in response to several rat unloading regimes including hindlimb suspension, hindlimb casting and Achilles tendon laceration (tenotomy) (Berthon *et al*., 2007, Bialek *et al*., 2011). In vivo data revealed that activities of 26S proteasome

were differentially activated in slow and fast muscles in response to inhibition-induced proteolysis, it was shown that the activity of 26S proteasome was 50% increased in the gastronemius muscle during proteolysis, while, though not significantly so, the activity was decreased by 25% in the soleus muscle (Shi *et al*., 2009).

Overall, these findings indicate that UPS potentially accounts for the associated decrease in muscle mass often observed in conjunction with animal disuse studies. While other elements notably mRNA levels of 20S proteasome subunit alpha-4 (HC6) and amounts of ubiquitinated proteins appear elevated, to date most of human disuse studies have focused their attention generally on the transcriptional expressions of two ubiquitin ligases, MAFbx and MuRF1 (Murton *et al*., 2008).

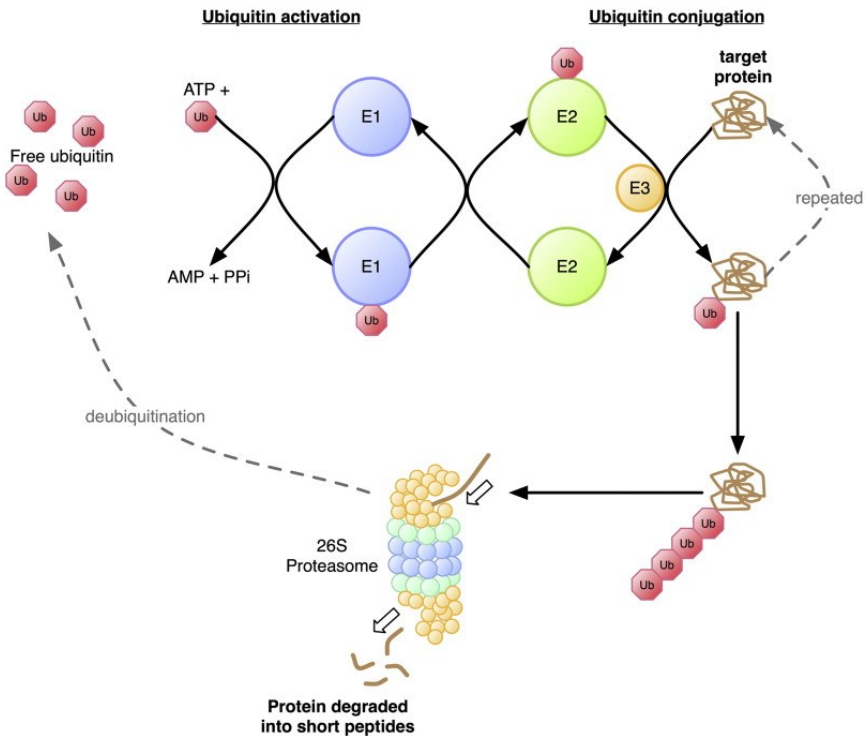


Figure 1.4 UPS mediated protein degradation. Diagrammatical representation of the UPS detailing the activation and conjugation of free Ub onto Ub-ligase targeted protein and

subsequent degradation by the 26S proteasome (Murton *et al*., 2008). *Abbreviation*: Ub, ubiquitin molecules; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub-ligase enzyme (ie. MAFbx and MuRF1).

1.5.1.1 MAFbx and MuRF1

The two muscle specific E3 ligases, F-box containing ubiquitin protein ligase (atrogin-1/MAFbx) and muscle RING finger 1 (MuRF1), were first identified and recognized as regulators responsible for muscle protein degradation under atrophy-stimulating conditions following transcript profiling in 2001. The discovery of atrogin-1/MAFbx was coincidentally achieved by two groups. One group has found through cDNA microarray analysis a unique gene fragment, which they named atrogin-1 being induced more than nine-fold in atrophying muscles of fasted mice. Whereas another study has applied the Gene-Tag differential display approach to select transcripts that were differentially regulated in immobilized rat muscle compared to the control muscle. These transcripts were then assayed for universality by Northern analysis using panels of mRNA prepared from muscles subjected to denervation, immobilization or unweighting from periods of 1 to 14 days. Two genes, MuRF1 (Muscle RING Finger 1) and MAFbx (Muscle Atrophy F-box), were found to be upregulated in all three models of atrophy and later analysis of rat and human tissues revealed their expression only in cardiac and skeletal muscles (Bodine *et al*., 2001a, Gomes *et al*., 2001). Subsequent knock-out studies have shown that inhibition of either ubiquitin ligases has markedly attenuated the amount of muscle loss. Through differential expression screening studies upregulated MAFbx and MuRF1 levels were detected in multiple models of muscle atrophy. Overall, their elevated expressions coexisted in 13 distinct animal

models of muscle atrophy, including hind limb suspension and denervation (Bodine *et al.,* 2001a, Gomes *et al*., 2001, Dehoux *et al*., 2003, Li *et al*., 2003, Deruisseau *et al*., 2005, Latres *et al*., 2005). A great amount of attention has been placed upon atrogin-1/MAFbx in the hope that they might serve as potential targets for pharmacologic intervention.

E3 ubiquitin ligases function to convey specificity to ubiquitination by transferring ubiquitin from E2 ubiquitin-conjugating enzymes to protein substrates. The targeting of E3 Ub ligases to their substrates is dependent on recognition of specific amino acid sequence and /or specific structural and phosphorylated domains. MuRF1 is characterized by its N-terminal RING-finger domain, which controls E3 ubiquitin-ligase activity. The current known substrates for MuRF1 in skeletal muscle include myosin heavy chain, creatine kinase as well as possible myofibrillar proteins titin, nebulin and myosin light chain II. MAFbx contains an F-box domain, a characteristic motif seen in a family of E3 ubiquitin ligases called the Skp1, Cullin1 and F-box-containing complex (SCF complex). This family of proteins establishes their E3 ubiquitin ligase activity by binding together. With MAFbx its F-box domain enables it to interact with Skp1. Protein substrates for MAFbx have been suggested to include calcineurin, eIF3f and MyoD, however neither protein has been demonstrated to be ubiquitinated by MAFbx in atrophy-induced muscle (Glass 2005).

As for the control of transcriptional regulation of MAFbx and MuRF1, limited information is available. Although data suggest that the expression of MAFbx and MuRF1 is regulated by the FOXO family of transcription factors via PI3K-Akt signaling, a recent human disuse study has shown their elevated mRNA levels being induced by pathways other than PI3K-Akt (Gustafsson *et al*., 2010)**.** The literature also points MuRF1 to be under the control of the nuclear factor-kB (NF-kB) pathway but it is unknown whether such a finding is relevant in the human situation.

Based from the vast majority of data on MAFbx and MuRF1, a classical upregulation of these two E3 ligases is consistently observed in animal models of disuse following denervation, hindlimb suspension, or space-flight in hibernating squirrels, mice and rats in comparison to a variable finding of MAFbx and MuRF1 expression changes in humans. Several human disuse studies have detected an upregulated transcription of the two E3 ligases, with the earliest occurring after 3 days of unloading (Gustafsson *et al*., 2010). Others have thus far reported no change or even a reduction in MAFbx and MuRF1 mRNA levels (Jones *et al*., 2004, Ogawa *et al*., 2006, de Boer *et al*., 2007b, Salanova *et al*., 2008). The discrepancies between animal and human data could be accounted as the result of the severity of disuse procedures employed in animals being usually greater than those applied to humans (Murton *et al*., 2008). On the other hand, the number of human muscle biopsies acquired is minimized in a way to limit invasive procedures being employed in humans. As a result information regarding the temporal expression of these two ubiquitin ligases is scarce with just a lone report examining the levels of MAFbx and MuRF1 at time points of 10 and 21 days, where MURF1 was the only one elevated at day 10 and at day 21 both of their mRNA levels were reduced (de Boer *et al*., 2007a). A possible explanation to this could be the timing of muscle sampling failed to coincide with the transiently induced response of MAFbx and MuRF1. Thus a comprehensive view of the temporal expression of these two ligases especially during the early days into unloading is needed. Despite the fact that to date overwhelming studies have examined MAFbx and MuRF1 at the transcriptional level there is a general absence of their protein data. Recently two human studies have demonstrated that the expression level of MURF1 does not correspond to either condition of muscle atrophy or hypertrophy (Leger *et al*., 2006b, Mascher *et al*., 2008). Moreover, Leger and colleagues have shown increases of the amounts of atrogin-1 mRNA and protein in response

to 8 week of resistance training and vice versa during detraining (Leger *et al*., 2006b). In summary, the functional significance of both ligases to the human condition of muscle disuse atrophy is yet to be justified.

1.5.2 Lysosomal proteolysis

Lysosomal degradation pathway is another major intracellular proteolytic system in mammalian cells. Unlike the well known UPS the role of lysosomal proteolysis in muscle atrophy is only beginning to be elucidated. As membrane-bounded vesicles consisting of an acidic lumen that encloses high concentrations of hydrolases, lysosomes therefore are organized intracellular compartments dedicated to the degradation of a variety of macromolecules.

Schematically, the activation of lysosomal degradation involves initially the sequestration of substrates into vacuoles called autophagosomes. This process of delivering intracellular protein substrates to lysosomes is termed autophagy. Substrates are delivered into the lysosomes by means of different forms of autophagy known as microautophagy, crinophagy, chaperone-mediated autophagy or macroautophagy (Blommaart *et al*., 1997). After autophagosomes fuse with lysosomes, lysosomal hydrolases begin to digest the content of vacuoles. Lysosomes are well adapted to proteolytic functions in the way that upon delivery of protein substrates for degradation the luminal acidic pH and high ratio of cysteine/cystine concentration help to weaken their intermolecular charge-charge interactions and promote further breakdown of some disulfide bonds. This enables the structurally intact proteins to unfold and so becoming more susceptible to proteolysis by lysosomal proteases. The degradation end-products will be

selectively delivered to the cytoplasm from lysosomes via membrane transporters where they are reused during cellular metabolism.

The proteolytic capacity of lysosomes is primarily determined by the major lysosomal proteases, cathepsins. Cathepsins are a group of enzymes that functions in protein hydrolysis and are comprised of endopeptidases (to cleave inner peptide bonds of proteins) and exopeptidases (to hydrolyse C-or N-terminal ends). These peptidases can again be categorised into cysteine (there are 11 human cysteine cathepsins, *i.e*., B,C,F,H,K,L,O,S,V,X and W), aspartic (cathepsins D and E) and serine (cathepsins A and G) peptidase families (Turk *et al*., 2012). Cathepsins B, L H and D are ubiquitous lysosomal peptidases that are expressed in many tissues. A relatively low concentration of cathepsins is present in skeletal muscle compared to other tissues with high rates of protein turnover. While within skeletal muscles, slow-twitch oxidative muscles typically exhibit higher levels of cathepsins than fast-twitch glycolytic muscles (Bechet *et al*., 2005). Initial morphological indications of the presence of cathepsins within muscle fibers were achieved via histochemical and immunohoistochemical investigations, where lysosomal vesicles containing immunoreactive cathepsins B and L were observed to present inside myofibres, in perinuclear regions beneath the sarcolemma and also found diffusely throughout the sarcoplasm (Stauber *et al*., 1985, Taylor *et al*., 1987) .Cathepsin B was found to be located in discrete vesicles in all muscle types and was more frequently observed at the periphery of the myofibre beneath the sarcolemma (Stauber and Ong 1981, Stauber and Ong 1982).

Although all cathepsins are able to degrade purified myofibrillar proteins the specificity of hydrolysis differs from one to another (Katunuma and Kominami 1983, Dufour *et al*., 1989). Whereas cathepsin B hydrolyses myosin heavy chain, troponin T, troponin I and tropomyosin (Schwartz and Bird 1977**,** Noda *et al*., 1981), cathepsin L degrades the widest range of

myofibrillar proteins, including troponin T, I and C, nebulin, titin and tropomyosin, with the exception of troponin V (Matsukura *et al*., 1981). Despite these findings, to date the importance of lysosomal proteolysis in muscle atrophy has received little attention since the inhibition of lysosomal function with agents that block lysosomal acidification or directly inhibit cathepsins has failed to reduce myofibrillar protein content or total protein degradation rate of atrophying muscles. However, a current notion suggests that cathepsins do not degrade cytosolic proteins like myofibrils but rather their major role is to degrade membrane proteins such as receptors and transporters. Evidences have shown that the lysosomal proteolytic pathway functions together with ubiquitin-proteasomal pathway to specifically degrade a number of mammalian receptors and ion channels. Lysosomal pathway appears likely to contribute to the atrophied muscle phenotype. Therefore one would not expect cathepsin inhibition to exert much effect on total or myofibrillar protein levels.

Lysosomal proteolysis has been reported to function in conjunction with other protein degradation mechanisms among different models of muscle wasting. With the aid of differential display technology and transcriptome analyses overexpression of markers of lysosomal pathway have been confirmed to present in various muscle atrophic conditions. Amongst all observations of increased capacity for lysosomal proteolysis during muscle atrophy the upregulation of cathepsin L expression has been recognized as a general marker (Fujino *et al*., 2009). A systematic induction of cathepsin L mRNA has been observed through transcriptome analyses of mRNA contents in atrophying muscles. Other lysosomal cysteine cathepsins are also involved in muscle protein degradation such as cathepsin B and D, however their gene expressions are not observed in all models of muscle wasting and generally appear less pronounced than those of

cathepsin L (Temparis *et al*., 1994, Bechet *et al*., 2005). Consequently, these findings support the idea that lysosomal degradation is involved in control of muscle mass.

Review of the literature illustrates that limited information exists regarding the temporal implication and myocellular specificity of lysosomal proteolysis in muscle atrophy, which is potentially important as slow- and fast-type muscles fibres seem to experience different magnitude and time course of atrophy with unloading. Within the available animal unloading data, increased cathepsin level and activity have been observed but the vast majority data show marked expression of various isoforms of cathepsin mRNAs. Elevated expressions of cathepsins L, B and D have all been reported to associate with reduced loading (Taillandier *et al*., 1996, Sacheck *et al*., 2007). In addition, a very recent study revealed upregulated expressions of many lysosomal proteases, lysosomal glucosidases and lysosomal lipases in a tenotomy–induced muscle atrophy model (Bialek *et al*., 2011). However studies suggest that the changes in cathepsin mRNA levels do not necessarily indicate similar modifications in lysosomal cathepsin activities. This reflects possible control of post-translational sorting of newly synthesized cathepsins to endosomes/lysosomes. Other evidences in support of the lysosomal involvement include morphological signs. Microscopic studies revealed the presence of secondary lysosomes in rat skeletal muscle at 24 and 28 hours of hindlimb suspension (Ferreira *et al*., 2008). A recent experiment using a pan-cathepsin peptide substrate cleavage assay to assess specific cathepsin activity in rat muscle has reported an increase in activity from both tenotomy-treated and cast-immobilized animals (Bialek *et al*., 2011).

The research focus on the regulatory networks that control the activation of lysosomal proteolytic mechanism in muscle atrophy is at its beginning. In vivo data have identified mTOR as regulators of autophagy-related genes via upstream IGF1/Akt signaling or directly by levels of

amino acids. Moreover, reduced IGF1/AKT signaling also happens to activate autophagy through a mTOR independent mechanism involving FOXO3. Two reports have now demonstrated an activation of lysosomal proteolysis by FOXO3 via the transcription of autophagy-related genes (Mammucari *et al*., 2007, Zhao *et al*., 2007).

In summary, the lysosomal proteolytic pathway might partially explain the increased protein breakdown in animal models of disuse atrophy but its implication in human disuse atrophy is not known since there is generally an absence of data.

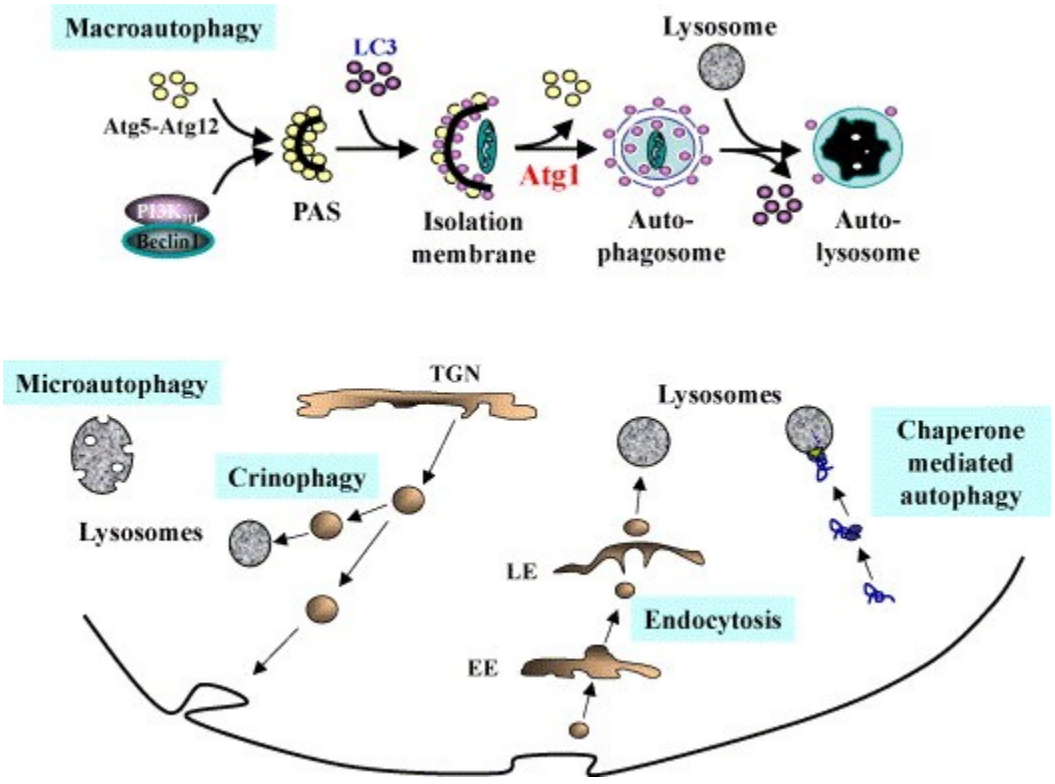


Figure 1.5 Schematic representation of the major pathways delivering cellular substrates to lysosomes. Cytosolic proteins may enter the lysosome by microautophagy (invagination of the lysosomal membrane), or by direct transfer through the lysosomal membrane when they contain

a KFERQ motif (chaperone-mediated autophagy). Crinophagy targets secreted proteins to lysosomes, while membrane lipids and proteins may reach lysosomes by endocytosis. Macroautophagy engulfs whole portions of cytoplasm together with various organelles (Bechet *et al*., 2005, Sandri *et al*., 2013). *Abbreviations*: Atg 1, autophagy related 1; Atg 5, autophagy related 5; Atg 12, autophagy related 12; PAS, phagophore assembly site; LC3, microtubule-associated protein 1 light chain 3; TGN, trans-Golgi network; LE, late endosome; EE, early endosome.

1.6 Summary and thesis directions

In summary of a review of the literature, the typical consequences underlying disuse atrophy consist of a shift in the contractile and metabolic phenotype from slow-oxidative toward that of a fast-twitch muscle and a reduction of muscle size and volume as a result of a decreased rate of protein synthesis and/or increased rate of protein degradation. Among the signaling pathways responsible for protein synthesis, costameric protein, focal adhesion kinase (FAK) has been recently identified as an upstream element of the mechano-sensory pathway to muscle protein synthesis of p70S6K activation, which is Akt-independent (Klossner *et al*., 2009). Current data has supported the idea that during load-dependent muscle remodeling costamere regulation is involved. This renders components of costameres potential targets of early modifications to reduced load-bearing of skeletal muscle. On the other hand, among the four main mechanisms responsible for protein degradation, the ubiquitin proteasome pathway has been recognised as the principal regulator of skeletal muscle atrophy. The activity level of chymotrypsin-like enzyme of 20S proteasome is a direct index of proteolytic activity. To present, the majority of findings on

the molecular mechanisms of human disuse atrophy have been reported based on longer periods (up to 60 days) of unloading regime (Jones *et al*., 2004, Ogawa *et al*., 2006, de Boer *et al*., 2007b, Salanova *et al*., 2008). The earliest documented molecular adjustments in human skeletal muscle with unloading involved pre-translational regulation of protein degradation, protein synthesis and mitochondrial metabolism at the transcript level (Chen *et al*., 2007). No study has yet investigated the onset of myocellular adjustments and how those aforementioned markers of disuse atrophy might be modified at the protein level early on in unloaded human skeletal muscle. In the past few years, components of the ubiquitin-proteasome pathway, especially the E3 ubiquitin ligases MAFbx and MuRF1, have been suggested to be major regulators of muscle mass and promoters of atrophy under muscle wasting conditions including animal models of disuse (Foletta *et al*., 2011). Ubiquitin-proteasome pathways especially MAFbx and MuRF1 have been extensively studied. Although a consistent elevation of both ubiquitin ligases has been observed in animals following various unloading conditions, their increased mRNA level has not always been reported in human skeletal muscle in the disuse studies performed to date (Jones *et al*., 2004, Ogawa *et al*., 2006, de Boer *et al*., 2007a, Salanova *et al*., 2008). A number of reports have detailed no change or a reduction the mRNA levels of MAFbx or MuRF1. One of the explanations for this inconsistency is the lack of a comprehensive examination of the temporal expression of these two E3 ligases in response to disuse in humans. A lone report examining temporal levels of MAFbx and MuRF1 mRNA has showed 10 days limb immobilization to result in elevated mRNA levels for MuRF1 but not MAFbx, however 11 further days of limb immobilization saw a subsequent fall in mRNA levels for both E3 ligases (de Boer *et al*., 2007a). As a result, it is currently not possible to determine the functional relevance of MAFbx and MuRF1 to the human condition of muscle disuse atrophy.

To date the majority of knowledge on the mechanisms underlying disuse induced skeletal muscle atrophy has beenobtained from animal studies (Thomason and Booth 1990, Murton *et al*., 2008). As a result, alterations of protein turnover facilitating the loss of muscle mass during disuse in humans require to be elucidated. Evidences indicative of a possible contribution of muscle protein breakdown in the etiology of human disuse atrophy has been demonstrated (Murton *et al*., 2008). Whereas, some reports have suggested the possible mechanism responsible for the resulting disuse induced atrophy in humans to involve predominantly depression of muscle protein synthesis (Ferrando *et al*., 1996, Paddon-Jones *et al*., 2006). Overall, an understanding of the regulatory mechanisms behind changes in muscle protein turnover associated with inactivity is important in developing effective countermeasures to prevent or attenuate disuse atrophy.

1.7 Aims and objectives

Aim:

The research aims to improve understanding of the cellular and molecular mechanisms that regulate the structural adaptations of skeletal muscle fibres in humans to disuse and to relay it to the concurrent functional alterations.

Objectives:

- To study the onset of disuse-induced protein level adjustments after 3 days unilateral limb suspension (ULLS)
- To examine the regulatory mechanisms governing changes in muscle protein turnover during the loss of muscle mass following 21 days of unloading

- To assess the role of costameres in the regulation of muscle size with altered muscle loading.

Towards this end we applied a number of techniques which are outlined in the following chapter.

2. Materials and Methods

2.1 Chemicals, Solvents and Reagents

Sigma (Poole, UK) was the provider of all general chemicals, solvents and reagents unless otherwise stated in specific methodology.

2.2 MHC II staining

Frozen slides carrying cryosections of 15 micrometer thickness were thaw for 30 minutes at room temperature. Cryosections were fixed for 15 minutes in cold acetone and rinsed three times in PBS (8g NaCl, 0.2g KCl, 1.44g Na_2PO_4, 0.24g KH_2PO_4 in 1L dH_2O at pH 7.4). Peroxidase activity was quenched for 10 minutes in 3% H_2O_2 containing 1ml of 30% H_2O_2 in 9ml of methanol. The sections were then blocked for 30 minutes in a humid chamber with 3% BSA consisting of 3g BSA in 100ml PBS. The blocking solution was removed and the sections were incubated with primary antibody monoclonal anti-skeletal myosin (fast, MHC II) MY-32 (M4276 Sigma) at a 1:400 dilution in 0.3% BSA (0.3g BSA in 100ml PBS) for 1 hour. 1ml of PBS was added to the slides for 5 minutes then aspirated. This procedure was repeated for four

times. Subsequently, secondary antibody, anti-mouse IgG (FAB specific) peroxidase conjugate (A9917 Sigma) was added at a 1:200 dilution in 0.3% BSA for 30 minutes. Again the slides were washed four times in 1ml of PBS. Substrate solution was prepared in the dark with a total solution of 2ml per slide containing 0.2ml AEC solution (1 AEC tablet (3-amino-9-ethylcarbazole) dissolved in the dark in 10ml dimethylformamide (DMF), 0.2ml imidazole buffer ((17g imidazole, 10.5g citric acid, 14.6g NaCl in 250ml dH_2O at pH 7.4), 1.6ml H_2O and 0.4µl H_2O_2 (30%)). The sections were incubated with the substrate solution in the dark and the immunoreactivity was stopped in between 10 to 30 minutes until the fast type fibres were stained uniformly and strongly red. The sections were then counterstained with hematoxylin (diluted Mayer's hematoxylin (Merck, Germany) at 1:5 with dH_2O) for 10 seconds. Colour was developed for 5 minutes in tab water. After that, slides were washed carefully in 80ml PBS-filled glass slide holder for 1 minute, then rinsed for another minute with 80ml dH_2O and imbedded in Aquatex (Merck, Germany). The sections were left to dry overnight, which were then used for fibre type percentage and fibre cross-sectional area analysis.

2.3 Fibre type percentage and fibre type cross-sectional area analysis

MHC II stained sections were recorded digitally at 10X magnification on an Axioskop 2 microscope (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom) using AxioVision software (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom). One field from the MHC II stained sections was assessed for cross-sectional area of stained (MHC II, fast type) and unstained (MHC I, slow type) fibres against a scale by manually recording the fibre periphery of each assessed fibre that was operated by the ARDOM software. The recorded cross-sectional area of

each fibre has contributed to the calculation of the overall mean cross-sectional area of slow and fast fibres by dividing the total area of either fibre type to the total number of that type of fibre measured. To obtain the fibre type percentage, either all stained fibres or unstained fibres on the section were counted and the following formula was applied.

$$\frac{\text{No of stained fibres}}{\text{Sum of stained and unstained fibres}} \times 100$$

2.4 Protein isolation and quantification

Muscle biopsies were cross-sectioned at 25-micrometer using a cryostat. Proteins were isolated with rotor stator mixer (ultraturrax, IKA Werke GmbH & Co. KG, Germany) into ice-cold modified radioimmunoprecipitation assay buffer (RIPA; 50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% sodium deoxycholate (90%), 1mM Na3VO4, 1µg/ml leupeptin, 2µg/ml pepstatin, 1µg/ml aprotinin, 0.1mM PMSF). Muscle protein was quantified with a bicinchoninic acid kit (BCA kit) against bovine serum albumin (BSA).

BCA reagents A and B were purchased from Pierce (Rockford, IL, USA). Reagent B (4% cupric sulphate) was mixed thoroughly in a 50ml eppendorf tube with reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M of sodium hydroxide) at a ratio of 1:50. Standards made with bovine serum albumin (BSA standards, Pierce) to determine the concentration of protein (mg/ml) were prepared with the following composition (see table);

RIPA (µl)	3	3	3	3	3	3
Standard BSA (µl)	0	5	10	15	20	25

H_2O (µl)	47	42	37	32	27	22

Each standard had a total volume of 50µl. Samples were prepared by mixing 3µl protein homogenate of each sample thoroughly with 47µl of dH_2O. Standard and sample tubes containing 50µl solution in total were mixed with 1ml of AB solution, which were then incubated at 37ºC for 30 minutes. The optical density of standard or sample solution was assessed in cuvettes by UV spectroscopy using a Biotech Photometer (WPA UV1101, Biochrom, Cambridge, UK) at 562nm. For the standard curve, the reading was set to zero with 0µl BSA (blank-corrected). The protein concentration was estimated against BSA standard curve using Excel. The standard curve was generated by plotting the 562nm measurement of each BSA standard against its known concentration in mg/ml. Sample concentration was calculated from the standard curve.

2.5 Immunoblotting

2.5.1 Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE)

Gels were prepared using a Mini-Protean III system (Biorad). The kit was assembled according to the manufacturer's instructions. Before pouring the gel the position of the upper level of the resolving gel was marked on the glass plate at 1cm below the bottom level of the comb. 7.5% resolving gel (7ml dH_2O, 3.5ml 1.5M Tris-HCl pH 8.9, 0.1% (w/v) SDS, 3.5ml 30% acrylamide 1% Bis, 42µl APS (0.046g+460µl dH_2O), 14µl TEMED) was poured between the clamped plates. 0.5ml of dH_2O was pipetted over the top of the gel to enable uniform polymerization by

preventing air from inhibiting the process at the surface of the gel. The gel was left to polymerize for 1 hour. Once polymerized, dH_2O was removed and 3% stacking gel (4.5ml dH_2O, 1.75ml 0.5M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.7ml 30% acrylamide 1% Bis, 42µl APS (0.046g+460µl dH_2O), 14µl TEMED) was over-layed. A 10 well-comb was inserted carefully into the unpolymerised stacking gel to ensure loading of the sample could take place. The combs were lifted evenly once the stacking gel had polymerized and the plates containing the gels were assembled in the gel running tank. The upper and lower chambers were filled with 1X running buffer (25mM Tris base, 192mM glycine, 2mM EDTA and 0.1% (w/v) SDS in dH_2O, pH 9.65). The total protein homogenate was denatured in Laemmli buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue) by heating for 5 minutes at 95°C. 15 micrograms of total muscle protein was loaded per sample lane by using a Hamilton syringe. The syringe was rinsed three times in running buffer before loading the next sample. Any empty wells were loaded with Laemmli buffer to avoid a discontinuous electric gradient across the gel. Molecular weights were determined by the incorporation of 5µl prestained molecular weight protein marker to one well per gel. Samples to be resolved were electrophoresed at first at 80 volts until samples left the stacking gel then ran the resolving gel at 120 volts using a Hoefer power supply (Hoefer,Inc., Holliston, MA, USA). The process was stopped when the sample buffer tracking dye had left the bottom of the resolving gel.

Protein quantification by immunoblotting can demonstrate a large inter-assay variability, which in my experience mainly comes from inequal development of the luminescent signal during the last stage of exposing the labeled membrane. In order to take this into account, samples were loaded in a paired manner (i.e. pre, post).

2.5.2 Transfer of proteins to hybond-c nitrocellulose

The glass plates were carefully disassembled without ripping the gels. The stacking gel was removed using a scraper. Meanwhile, a piece of Hybond-C nitrocellulose (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and six sheets of Whatman filter paper (Schleicher & Schuell, GmbH, Dassel, Germany) were cut to the size of the gel and immersed in transfer buffer (114mM glycine, 19mM Tris base, 20% methanol, pH8.3). One layer of foam pre-immersed in transfer buffer was placed on the black side of the blotting cassette. Three sheets of pre-immersed Whatman filter paper were placed on top of the foam, the gel was placed on top of the filter paper followed by the Hybond-C nitrocellulose membrane, a further three layers of pre-immersed Whatman filter paper were placed on top of that and finished with another layer of foam. At every stage the layers were rolled with a pipette to ensure no air bubbles were retained between layers. The gel sandwich stack held inside the blotting cassette was slided into the blotting unit in the chamber with black side (cathode) of the cassette being placed next to the cathode side of the unit. The chamber inside an ice filled polystyrene box was filled with more transfer buffer and a magnetic stirrer had been added. The proteins were left to transfer onto the nitrocellulose membrane overnight using a Hoefer power supply (Hoefer, Inc., Holliston, MA, USA).

2.5.3 Ponceau S staining

The transfer efficiency and equal loading of the proteins were validated by staining the membrane prior to immunodetection with a non-permanent dye called Ponceau S. Membranes

were incubated in 0.1% (w/v) Ponceau S solution with 5% (v/v) acetic acid (28.5ml dH_2O, 1.5ml acetic acid, 0.033g Ponceau S) for 5 minutes and washed with dH_2O to observe. Stained membrane was completely destained following washing with 1X TBST (9g NaCl (150mM), 20ml Tris base (20mM) pH7.5 in dH_2O containing 0.5ml Tween 20 (0.05% Tween 20)).

2.5.4 Immunodetection

The nitrocellulose membrane was blocked in a blocking solution containing 5% dried skimmed milk powder (Cadbury's Marvel Milk Powder, Sainsbury) and 2% BSA (Sigma, Fraction V grade) in 1X TBST at room temperature for 1 hour on a rocking platform. Subsequently, the membrane was incubated in a primary antibody solution diluted in blocking solution on a rocking platform at room temperature for 1 hour. The dilution factor was different according to the primary antibody used. Post-incubation, the membrane was washed four times in 1X TBST for 5 minutes per wash. The membrane was then agitated in HRP-conjugated secondary antibody solution diluted again in blocking solution (according to the primary antibody used) for 30 minutes. The blot was once again washed four times as described above. All incubations and washes were performed in a plastic box on a rocking platform.

2.5.5 ECL detection

Detection of biotin bound proteins was achieved using enhanced chemiluminescence (ECL) with an ECL detection kit (SuperSignal West Femto Chemiluminescent Substrate, Pierce, Rockford, IL, USA). The ECL substrate was made up from Enhancer solution and Stable Peroxide solution

in a ratio of 1:1 (500µl each per membrane), mixed and incubated for 5 minutes with gentle agitation according to manufacturer's instructions. These procedures were performed in the dark. Membranes were placed in between plastic sheets and paper towels were used to remove excess reaction solution off. The sheets were placed inside the scanning device. Briefly, reagent A decays to H_2O_2, the substrate for peroxidase. Reduction of the enzyme HRP is coupled to a light producing reaction by detection with reagent B. This contains lumino, which upon oxidation, produces blue light that can be detected on an imaging system. The signal recorded digitally was achieved by using the Biorad Imaging System (Hercules, CA,USA) supported by Quantity One 4.6.2 (Bio-Rad, Hercules, CA,USA).

2.6 Proteolytic activity assay

2.6.1 Sample preparation

Homogenates were prepared from 20-micrometer cryosections in 0.1M KH_2PO_4 buffer (pH7.2) containing 2mM EDTA on ice with the help of a Polytron mixer (Kinematica Switzerland) and were stored at -80°C. Protein concentration was quantified spectrophotometrically with the bicinchoninic acid protein assay kit (Perbio). For the quantification of enzyme activities, all samples were adjusted by adding KH_2PO_4 to achieve a final concentration of 2µg/µl.

2.6.2 Chymotrypsin-like protease activity assay

The measure of chymotrypsin-like enzyme activity of the 20S proteasome was based on the hydrolysis of a fluorogenic substrate essentially as described (Berthon *et al* 2007). 10µl of sample (20 micrograms of muscle extract) was preincubated in 980µl of imidazole buffer (60mM, pH 7.4) in pyrex tubes for 5 minutes at room temperature. The reaction was started by the addition of 10µl of the 10mM fluorogenic substrate, succinyl-leu-leu-val-tyr-7-amino-4-methylcoumarin (100µM) (Bachem, I-1395), in the dark to achieve a final reaction volume of 1ml. Substrate conversion was immediately assessed fluorometrically (excitation at 380nm and emission at 460nm) on a SFM25 fluorimeter (Kontron Instruments). All measures were done in duplicates, whereby each time four tubes were inserted into positions 1, 2, 3 and 4, and each tube's reading was taken every 15 seconds after the previous tube, in the order in which they were placed. Readings were consecutively taken in 1 minute intervals for each tube over the first 10 minutes of the reaction.

2.6.3 Cathepsin B/L activity assay

For cathepsin B/L activities, methods were used according to those described by (Duguez *et al*., 2003). 5µl of sample (10 micrograms of muscle extract) was preincubated in 985µl of sodium acetate buffer (100mM sodium acetate, 1mM EDTA, pH 6.0) in pyrex tubes for 5 minutes at room temperature. The assay was started by the addition of 10µl of the 10mM benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin (100µM) (Bachem, I-1160) in the dark. Then pyrex tubes with 1ml of total solution were immediately placed into a SFM25 fluorimeter (Kontron Instruments) and fluorescence was measured at the excitation and emission wavelengths of 380nm and 460nm, respectively. Measures on each sample were done in

duplicates, therefore, two samples were assessed at a time. Tubes were inserted into positions 1, 2, 3 and 4 inside the apparatus and each tube's reading was taken every 15 seconds after the previous tube, in the order in which they were positioned. Readings were consecutively taken at 1 minute intervals for each tube over the first 10 minutes of the reaction.

2.6.4 Enzyme activity analysis

Proteolytic activity was estimated based on a Michaelis-Menten type model of catalytic activity where the conversion rate (delta F) (i.e. the increase in fluorometric signal per time) is proportional to Vmax. Measures were repeated in 1 minute intervals over the first 10 minutes of the reaction and the mean conversion rate was calculated. Since the mean delta F value is in AU unit/min, proteolytic activity (Vmax) was calculated by dividing the mean delta F by the gradient of the standard curve of 7-amino-4-methylcoumarin drawn from dilutions in the range of 0-150 pmol/ml.

To prepare 7-amino-4-methylcoumarin (AMC) standard curve, 7mg AMC was resuspended in 10ml DMSO to achieve a concentration of 4mM and this stock solution was then diluted 1:400 to give 10µM AMC. The table below shows the differential volumes of reaction buffer and AMC that were used for the dilution range of 0-150 pmol/ml. The tubes were covered by aluminium foil at all times and experiments were performed at room temperature.

AMC (pmol)	0	30	60	90	130	150
AMC (10µM) (µl)	0	3	6	9	13	15
Reaction buffer (µl): (imidazole buffer (60mM, pH 7.4) or sodium acetate buffer (100mM sodium acetate, 1mM EDTA, pH 6.0))	1000	997	994	991	987	985

For a standard curve every two tubes containing the same amount of AMC were measured at one time by SFM25 fluorimeter (Kontron Instruments) at excitation and emission wavelengths of 380nm and 460nm, respectively. Each time a calibration curve was redone for a new reaction buffer that was made.

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Figure 2.2 Michaelis Menton equation, which defines algebraically the curve expressing the relationship between substrate concentration and reaction rate that has the same general shape for most enzymes (a rectangular hyperbola).

Abbreviation: V_0, initial velocity; $V_{max,}$ maximum velocity; $K_{m,}$ the Michaelis constant; [s], substrate concentration.

Leonor Michaelis and Maud Menten in 1913 proposed the general theory of enzyme action. They postulated that in an enzyme-catalyzed reaction, the enzyme first combines reversibly with its substrate to form an enzyme-substrate complex (ES) in a relatively fast reversible step. The ES complex then breaks down in a slower second step to yield the free enzyme and reaction product, where the rate-limiting step in the reaction is the breakdown of ES complex to the free enzyme and product. Therefore the overall rate of an enzymatic reaction is proportional to the concentration of the ES complex. The maximum initial rate (Vmax) is observed at high substrate concentration when virtually all the enzyme is present as ES and the concentration of E is

vanishingly small. Under these conditions, Vmax is directly proportional to enzyme concentration. At high [s] the equation simplifies to $V_0=V_{max}$ (Nelson and Cox 2000).

3. Early changes in protein expression in human muscle with unloading are muscle specific

3.1 Introduction

The maintenance of skeletal muscle mass calls for continued mechanical stress (Goldspink *et al*., 1995). This load dependence is illustrated by the decrease in size or volume of antigravity muscles after unloading induced by real or simulated microgravity (Mayet-Sornay and Desplanches., 1996, Ohira *et al*., 2002). The consequences of unloading are evident at the molecular level in human skeletal muscle within 3 days (Gustafsson *et al*., 2010, Tesch *et al*., 2008). Reductions in the MCSA of muscle fibres are detectable within 4-5 days of unloading with immobilization (Suetta *et al*., 2012, Wall *et al*., 2014). In rodents, atrophy occurs concurrently with a shift toward faster fibres type (Desplanches *et al*., 1987, Fitts *et al*., 2001). Evidence suggets that the reduced energy expenditure associated with chronic unloading is accompanied by compromised aerobic capacity and mitochondrial function (Desplanches *et al*., 1987, Adams *et al*., 2003, Flueck *et al*., 2005).

The earliest documented molecular adjustments in unloaded human skeletal muscle involve pre-translational regulation of protein degradation and protein synthesis. For instance, levels of nine transcripts involved in control of proteosomal protein degradation (E2 conjugating enzymes, E3

ligases, ubiquitin-specific proteases) and inhibition of protein synthesis (4E-BP1) in vastus lateralis muscle are increased within 2-3 days of unilateral limb suspension (ULLS) while those involved in mitochondrial metabolism are down-regulated (Chen *et al*., 2007, Gustafsson *et al*., 2010, Reich *et al*., 2010). Also amongst the general lack of changes in content as well as phosphorylation state of many measured anabolic signalling factors (AKT, TSC-2, p70S6K, 4EBP1, eIF4E and eEF2) in the early phase of unloading, there is marked alterations in FAK, such that a 30% decrease in FAK phosphorylation and a 20% decrease in content have been reported after 10 days of ULLS (Goldspink *et al*., 1995, Rennie *et al*., 2010). FAK phosphorylation at tyrosine residue Y397 have been demonstrated to regulate myofibrillar protein synthesis as well as the slow-oxidative expression program in a load-dependent fashion (Durieux *et al*., 2009). Phosphorylation of Y397 enables FAK to develop its full catalytic activity, phosphorylate auxiliary sites (e.g. Tyr576/577) and enter interactions with further binding partners (Schlaepfer *et al.,* 1999). pY397 FAK has also been shown to play important roles in the assembly and the turnover of focal adhesions that connect the cytoskeleton in the cell interior to the extracellular matrix and responsible for mechano-sensing in a number of cell types (Bershadsky *et al.,* 2003, Schlaepfer *et al*., 2004, Grounds *et al.,* 2005, Chiquet *et al*., 2009).

Focal adhesion complexes of the sarcolemma (costameres) (Huijing 1999, Ervasti *et al*., 2003, Grounds *et al*., 2005, Quach *et al*., 2006) are targets of early modifications with reduced load bearing of skeletal muscle (Rybakova *et al*., 2000, Anastasi *et al*., 2008). Studies have demonstrated that the concentrations of the costamere components paxillin and meta-vinculin are altered in rodent skeletal muscle following 7 days of unloading (Gordon *et al*., 2001, Chopard *et al*., 2002). These observations coincide with changes in the amount and activation status (Gordon *et al*., 2001, de Boer *et al*., 2007a) of FAK. Interestingly, the unloading induced concentration

changes of costamere components in rat happen to differ between muscles types according to their initial fibre type composition. Such that within one week of unloading paxillin and FAK are reduced in mixed and fast type muscles, *m. plantaris* and *m. gastrocnemius*, but the opposite is true in the slow soleus muscle (Gordon *et al*., 2001). Moreover, meta-vinculin concentration is increased after 3 weeks of unloading in slow type muscle but decreased in fast type muscle (Chopard *et al*., 2002). Similarly, the concentration of meta-vinculin is known to increase in soleus but not in vastus lateralis muscle after 56 days of bedrest (Chopard *et al*., 2005), which mirrors the differential modulation of the expression of proteolysis-related gene transcripts, in slow-type compared with mixed-type muscles after 3 days of unloading (Goldspink *et al*., 1995). In light of these results, the possible relation between muscle-specific costameric remodelling and factors that set the slow-oxidative phenotype during slow-to-fast fiber transformation induced by muscle unloading should be investigated.

In a skeletal muscle, the expressions of mitochondrial and contractile proteins can be correlated to the fractional synthesis rates of these proteins (Adey *et al*., 2000, Lopes *et al*., 2006). During unloading, there is a decrement in both mitochondrial and slow type myosin contents (Desplanches *et al.,* 1987, Ferretti *et al*., 1997), indicating that underlying proteins are targeted for proteolytic degradation. The diminished drive for the maintenance of a slow-oxidative phenotype of anti-gravity muscle with unloading is corroborated by a correlated down-regulation of respiratory chain constituents in rat soleus muscle (Flueck *et al*., 2005). Such findings suggest that the correlation pattern between mitochondrial, contractile and proteolytic elements is likely to be modified during muscle unloading.

3.1.1 Hypothesis

In the current study, we investigated whether 3 days of unloading initiated fibre transformation at the protein level. We hypothesized that human anti-gravity muscles would undergo fast muscle remodelling in response to unloading by unilateral limb suspension (ULLS), and that this could be detected within three days of the start of the unloading phase. Specifically, we hypothesised that this fast muscle remodelling would involve altered expression of costamere components, which are related to mechanosensory muscle remodelling via the organization of the attachment of muscle organelles and gene expression (Grounds *et al*., 2005, Quach and Rando 2006, Klossner *et al*., 2013), factors of the mitochondrial respiratory chain and the slow type myosin heavy chain. We further speculated that these responses would differ between the phenotypically distinct leg muscles, *m. vastus lateralis* and *m. soleus*. and would reflect differences in the activity of chymotrypsin, a main contributor to the 20S proteasome, being increased within 5 days of unloading in the rat (Berthon *et al*., 2007), and/or expression of costameric proteins. Subsequently, we assessed whether quantitative, linear relationships existed between unloading induced changes in chymotrypsin activity and fibre MCSA and between the levels of selected proteins that define the slow oxidative muscle phenotype and whose expression is subject to regulation by FAK protein and its pY397 content (Durieux *et al*., 2009, Klossner *et al*., 2013).

3.2 Methods

3.2.1 Subjects

Healthy men (mean ± SD; 25 ± 5 yr, 183 ± 3 cm, 76 ± 8 kg) were recruited from the metropolitan area of Stockholm. The subject sample ranged from physically active to sedentary individuals. Subjects were screened for any history of lower limb pathology, neuromuscular disorder, or cardiovascular disease. Written consent was obtained from each subject after being informed of the procedures, risks, and potential benefits associated with the experiments. The study protocol was approved by the Ethics Committee at the Karolinska Institutet and conducted in accordance with the Declaration of Helsinki (1964).

3.2.2 Unloading

Unilateral limb suspension (ULLS) was accomplished as previously described (Tesch *et al*., 2008, Gustafsson *et al.,* 2010). In brief, upright or ambulatory activities were aided by short-length crutches with handgrip and forearm support distal to the elbow (Swereco Rehab AB, Sollentuna, Sweden). The right foot was equipped with a shoe outfitted with a 10 cm thick sole in order to remove weight-bearing load from the left unloaded limb. There were no straps attached to the shoe restraining ankle- or knee-joint movement. The subjects lived at home and maintained their normal occupational tasks throughout the experimental period. Compliance was encouraged through daily interaction with one of the investigators. Two weeks prior to the ULLS intervention, all subjects underwent four sessions to practice walking on crutches and daily tasks associated with the ULLS intervention. The subjects refrained from any strenuous physical

activity three days prior to the onset of ULLS yet maintained their normal dietary habits. To assure compliance, all subjects were interviewed daily via telephone or in person.

3.2.3 Collection of muscle samples

Muscle biopsies were obtained from the right leg prior to ULLS and from the left leg after completing 72 hrs of ULLS and before resuming any weight-bearing activity. Following an overnight fast and after injection of local anaesthetic (Carbocain®) and skin incision, biopsies were obtained from vastus lateralis and soleus muscle using a 5 mm Bergström needle. Samples were cleansed of excess blood, connective tissue, and fat and then frozen in liquid nitrogen and stored at -80°C until further analysis.

3.2.4 Immunoblotting

Muscle biopsies were cross-sectioned at 25 micrometers using a cryostat; proteins were isolated with rotor stator mixer (ultraturrax, IKA Werke GmbH & Co. KG, Germany) into ice-cold modified radio immunoprecipitation buffer (RIPA; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate at 90%, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 2 µg/ml pepstatin, 1 µg/ml aprotinin, and 0.1 mM PMSF; Sigma, Buchs, Switzerland). Muscle

protein was quantified with a bicinchoninic acid kit (Pierce) against bovine serum albumine (BSA). Total protein was denatured in Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2%-mercaptoethanol, and 0.1% bromphenol blue) by heating for 5 min at 95°C. Equal amounts of total muscle protein per lane (i.e. 20 micrograms) were resolved via 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean III system (Biorad). The loading was in a paired design with four pre-/post-ULLS samples from SOL or VL muscle of subjects per gel loaded in adjacent lanes. Proteins were blotted onto nitrocellulose membrane (Amersham) and blotting efficiency and equal loading were verified by Ponceau S staining. Membranes were subjected to immunodetection with specific first antibodies and horseradish peroxidase-(HRP)-coupled secondary antibodies. For the detection of FAK-related proteins, this involved the polyclonal C-terminal antibody from animal "Lulu" as published (Flueck *et al*., 1999) and anti-rabbit HRP antibody (ICN Biomedicals GMBH, Germany). For vinculin this included a described monoclonal antibody (Goldspink *et al*., 1995) and anti-mouse HRP antibody (ICN). For detection of the mitochondrial proteins succinate dehydrogenase complex, subunit A (SDHA); ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 (ATP5A1); ubiquinol-cytochrome c reductase core protein I (UQCRC1) and NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9 (NDUFA9), an antibody mix (Molecular Probes/Invitrogen Ltd, Paisley, UK) was used. For the detection of slow-type myosin heavy chain, MHCI, a monoclonal antibody (Sigma Chemicals, Buchs, Switzerland), and anti-mouse HRP antibody (ICN Biomedicals GMBH, Germany) were used.

Signal detection was carried out with enhanced chemiluminescence (Femto kit, Pierce) and quantified with a Chemidoc system running under Quantity One software (Bio-Rad, Life Science Research, Hercules, CA, USA). The signal intensity of the respective band was estimated with

the "volume rectangular tool" and corrected versus the background of a band of equal height and size (area) in an empty sample lane. Background-corrected data were normalized to the mean values of the pre-ULLS samples for the respective gel; the values therefore reflect relative expression levels per total muscle protein. In addition, blots combining sample pairs from soleus (SOL) and vastua lateralis (VL) muscle were run on the same gel to compare and adjust relative protein content between the two muscle groups.

The content of FAK-pY397 per total protein was assessed as described (Klossner *et al*., 2009). In brief, the soluble fraction of 500 micrograms of total protein in 1 ml RIPA buffer was subjected to immunoprecipitation over night with 1 mg FAK-pY397-specific antibody (Invitrogen) and 50 microliters of a 10% slurry of protein A Sepharose (Sigma) at 4°C under continuous rotation using an Intellimixer (Progen Scientific). The precipitate of a 2-minute spin at 5000 g at 4°C was washed twice with 500- microliter RIPA, separated by 7.5% SDS-PAGE, western blotted onto nitrocellulose, and divided into two parts at the height of 85 kDa before being subjected to immunodetection with FAK-specific antibody as described above.

3.2.5 Proteosomal activity

Homogenates were prepared from 20-micrometer cryosections in 0.1 M KH_2PO_4 buffer (pH7.2) containing 2 mM EDTA on ice with the help of a polytron mixer (Kinematica, Switzerland). Protein concentration was quantified spectrophotometrically with the bicinchoninic acid protein assay kit (Perbio) and adjusted to 2 mg/ml. Samples were distributed in aliquots and stored at -

80°C. The quantification of chymotrypsin-like enzyme activity of the 20S proteasome, representing the catalytic core of the proteasome, was based on the hydrolysis of a fluorogenic substrate essentially as described (Berthon *et al*., 2007). In brief, 20 micrograms of muscle extract were incubated in a final volume of 1 ml imidazole buffer (60 mM; pH 7.4). The reaction was started by the addition of 100 μM of the fluorogenic substrate succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (Bachem, I-1395). Substrate conversion was assessed fluorometrically (excitation at 380nm and emission at 460nm) on a SFM25 fluorometer (Kontron Instruments). Proteolytic activity was estimated based on a Michaelis Menten type model of catalytic activity where the conversion rate (i.e., the increase in fluorometric signal per time) is proportional to Vmax. Measures were repeated in minute intervals over the first 10 minutes of the reaction and the mean conversion rate calculated. Proteolytic activity (i.e., Vmax) was then calculated versus standard curve of amido-4-methylcoumarin drawn from dilutions in the range of 0-150 pmol/ml.

3.2.6 Fibre-type percentage and fibre type cross-sectional area analysis

15-micrometer cryosections of VL and SOL muscle were incubated with a 1:400 dilution of mouse monoclonal antibody against fast myosin heavy chain (Clone MY-32, Sigma-aldrich) in 0.3% BSA in phosphate buffered saline (PBS), then reacted with a 1:2000 dilution of horseradish peroxidase -conjugated anti-mouse IgG (ICN). Immunoreactivity was detected with substrate solution, 3-amino-9-ethylcarbazole in dimethylformamide (Sigma Chemicals, Buchs, Switzerland). Nuclei were counterstained with hematoxylin and embedded in Aquatex (Merck,

Germany). Sections were then processed to assess fibre-type composition and cross-sectional area of slow- and fast-type fibres.

Myosin heavy chain-stained sections were recorded digitally at 10x magnification on Axioskop 2 microscope (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom) that was operated with AxioVision software (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom). Subsequently, one field from fast-type myosin heavy chain-stained sections was assessed for cross-sectional area of stained (fast-type) and unstained (slow-type) fibres against a scale by manually recording the periphery of each assessed fibre within the ARDOM software (Flueck *et al*., 2011). This differentiation was based on the observation that the large majority of muscle fibres in human vastus lateralis musle are pure type I or type II fibres (Flueck *et al*., 2011). These numbers were used to calculate the percentage and mean cross-sectional area of slow- and fast-type muscle fibres. The area content of slow-type fibres was calculated using the formula [area content of slow-type fibre percentage of slow-type fibres x mean cross-sectional area of slow-type fibers/100%]. On average, 159 slow- and 36 fast-type muscle fibres were counted per muscle cross section of SOL and an average of 88 slow- and 87 fast-type muscle fibres were counted per muscle cross sectionof VL.

3.2.7 Statistical analysis

Post vs. pre changes and interaction effects of muscle type (SOL, VL) were assessed with a repeated-measures ANOVA with as within factor time (pre/post). In the case of interactions,

differences were localized with the post hoc test of Fisher or Wilcoxon signed rank test, dependent on whether the data were normally distributed or not, respectively (Statistica 9.1). The differences were visualized as the delta between post vs. pre values. The post-hoc test was relaxed to a one-tailed test in cases where a single-sided hypothesis could be formulated, i.e. the increase in meta-vinculin (Adams *et al*., 2003) and mitochondrial protein (Quach and Rando, 2006). Effects with p-values $p< 0.05$ were considered significant and $0.05 \leq p < 0.10$ were viewed as a trend. All correlations of normalised values were calculated using Statistica 9.1 using Pearson correlations and displayed as correlation matrices (p-value weighted). Pearson correlations, visualized with Cluster and Tree and exported into Powerpoint (MS-Office) as described (Bey *et al*., 2003). For both SOL and VL n=8 pre and n=8 post, with the only exception for mitochondrial protein expressions in VL where n=7.

3.3 Results

3.3.1 Muscle specific fibre type composition

The soleus muscle had 32% more type I fibres than the *m. vastus lateralis* before ULLS ($p=0.0045$, ANOVA with post-hoc test of Fisher; Fig. 3.1). Type II fibres showed a trend for increased mean cross sectional area (MCSA) compared to type I fibres in both VL ($p=0.09$) and

SOL (p=0.09). MCSA of type I and II fibres in *m. vastus lateralis* and *m. soleus* was not altered post 3 days ULLS (Table 3.1).

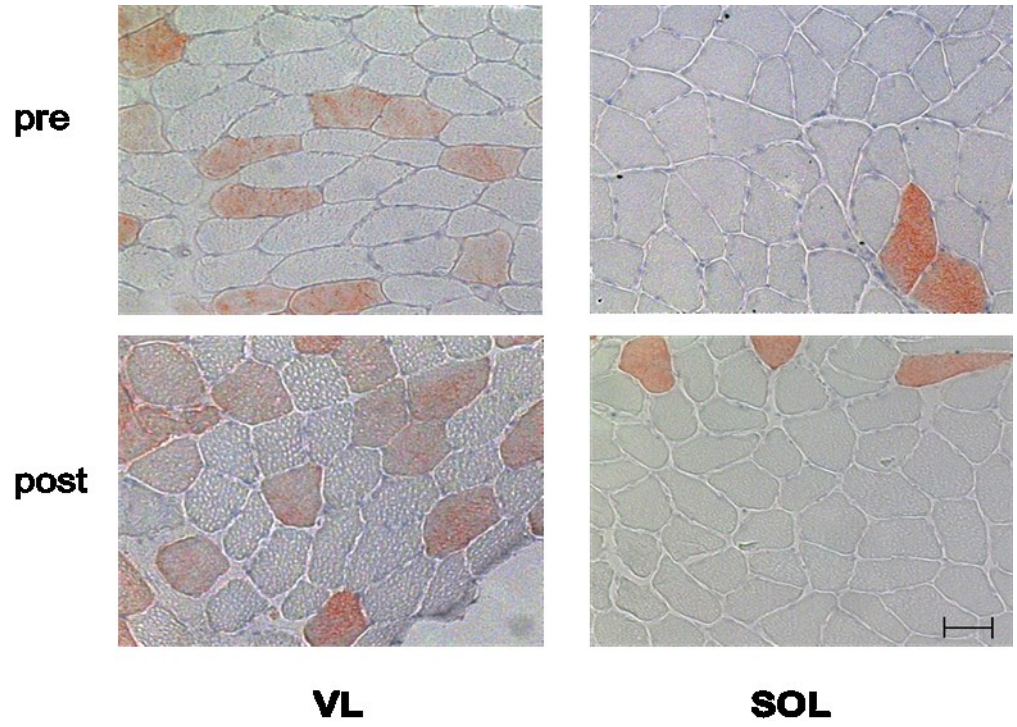


Figure 3.1 Microscopic pictures of muscle cross sections of VL and SOL pre and post ULLS after staining for type II myosin type heavy chain (orange). Nuclei appear in blue.

	pre	post
m. vastus lateralis		
slow type:	5275 ± 442	4446 ± 581
fast type:	6225 ± 292	5180 ± 540

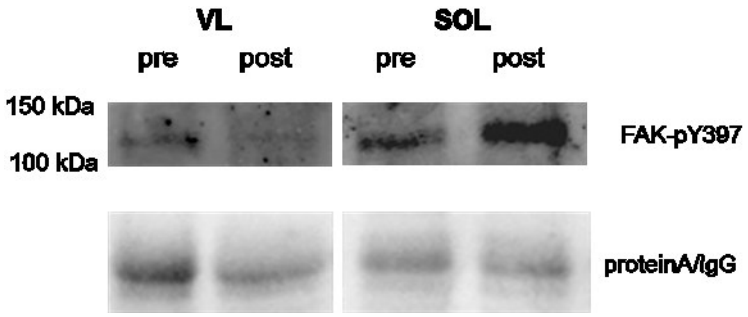
slow type%	51 ± 7%	43 ± 6%
m. soleus		
slow type:	6330 ± 479	5728 ± 452
fast type:	7352 ± 382	6889 ± 943
slow type%	86 ± 4%	97 ± 4%

Table 3.1 Mean and standard error of the mean of the fibre cross sectional area of slow and fast type muscle fibres, and the percentage of slow type muscle fibres in the two studied anti-gravity muscles pre and post 3 days of ULLS. No significant difference was identified (two tailed Fisher-test).

3.3.2 Changes in protein expression after 3 days unloading

FAK-pY397 content per total protein was 20% reduced in VL but not altered in SOL (Fig. 3.2). Figure 3.3 shows representative immunoblots of the detected mitochondrial, costameric and sarcomeric proteins (MHC1). For individual proteins, an increased content of meta-vinculin was apparent in *m. soleus* after 3 days of ULLS ($p=0.03$), but not in *m. vastus lateralis* ($p=0.23$; Fig. 3.4). There was also a trend ($p=0.059$) for an elevation of combined mitochondrial protein content in *m. vastus lateralis*.

A)



B)

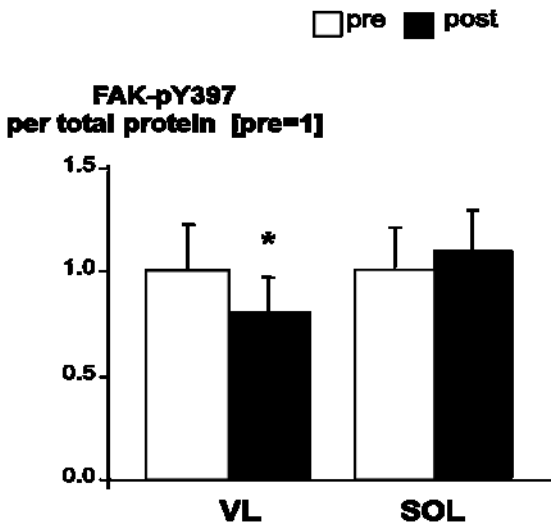


Figure 3.2 A) Top, example of immunoprecipitated FAK-pY397 from total homogenate of VL and SOL pre and post ULLS for two subjects. The position of FAK-pY3967 is indicated. Bottom, the amount of immunoglobulin/protein is shown as a loading control in the panel below. B) Bar graph showing mean and standard error of FAK-pY397 levels in SOL and VL pre and post 3 days of ULLS. * denotes $p < 0.05$ (one-tailed Wilcoxon-test).

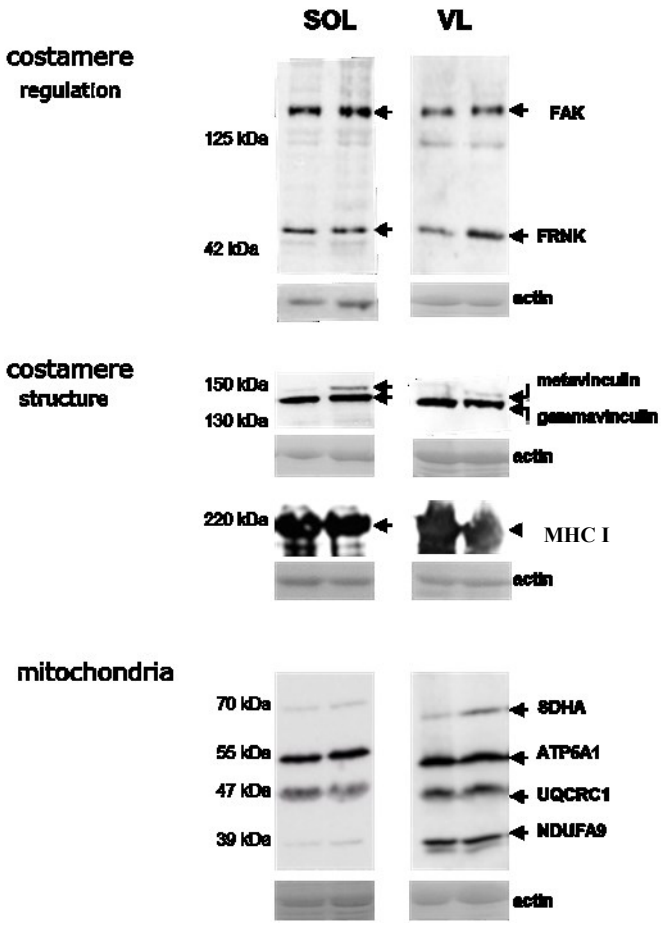


Figure 3.3 Representative immunoblots visualizing the detected proteins in *m. soleus* and *m. vastus lateralis*.

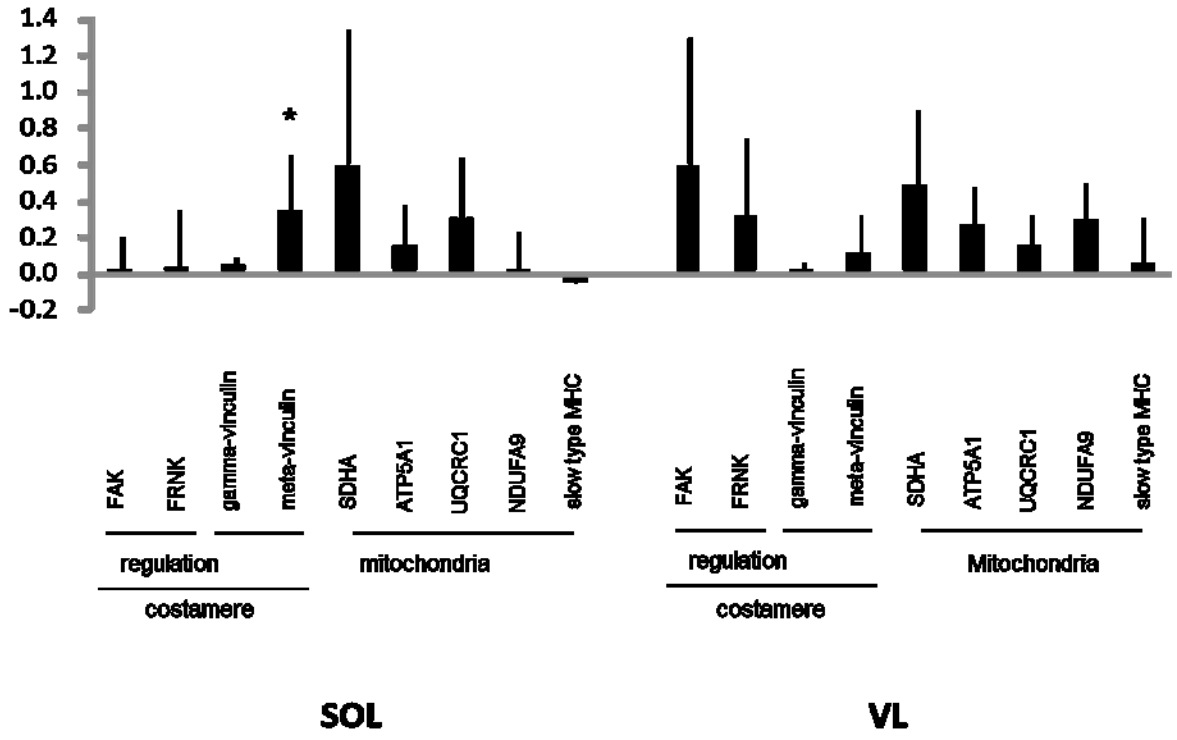


Figure 3.4 Mean and standard error of protein level changes with 3 days of ULLS in VL and SOL. Asterisk indicates those gene ontologies and proteins which were significantly affected by ULLS. *denotes $p < 0.05$ (Fisher test).

3.3.3 Proteosomal activity

We followed the activity of chymotrypsin, as this enzyme is a major component of the ubiquitin-proteasome pathway, which is mainly responsible for the degradation of sarcomeric proteins seen in unloading (Ikemoto *et al*., 2001, Capetanaki 2002, Schlaepfer *et al*., 2004, Polge *et al.,* 2011). Chymotrypsin activity in *m. vastus lateralis* was not altered after 3 days of ULLS ($p=0.72$) nor was its activity changed in *m. soleus* ($p=0.85$).

3.3.4 Changes in various protein components were associated with changes in chymotrypsin activity

Fold changes in chymotrypsin activity correlated negatively with fold changes in ATP5A1 (r=-0.80, p=0.02) in the VL but not in the SOL. No other significant correlations were found: UQCRC1 (r=-0.61, p=0.10), NDUFA9 (r=-0.78, p=0.22) and the area content of slow type fibres (r=-0.65, p=0.08) in VL.

3.3.5 Deregulated coordination of expression

Muscle specific changes with unloading were also reflected by modified linear relationships between proteins that define the slow oxidative muscle phenotype. At a threshold of $r \geq 0.70$ and $p<0.05$ this concerned a loss of positive correlations in SOL after 3 days of ULLS between respiratory chain constituents SDHA, ATP5A1 and UQCRC1 and slow myosin heavy chain (MHC1). Similarly, negative correlations between mitochondrial proteins and FAK-pY397 were lost while positive correlations developed respectively to FAK and FRNK after unloading.

In the VL, unloading affected correlations between mitochondria factors and structural components of costameres. After ULLS, negative correlations appeared between the vinculin isoforms and mitochondrial proteins. FAK-pY397 positively correlated with FAK/FRNK. Moreover, the positive linear relationship observed between FAK-pY397 and MHC1 prior to ULLS was inverted (Fig. 3.5).

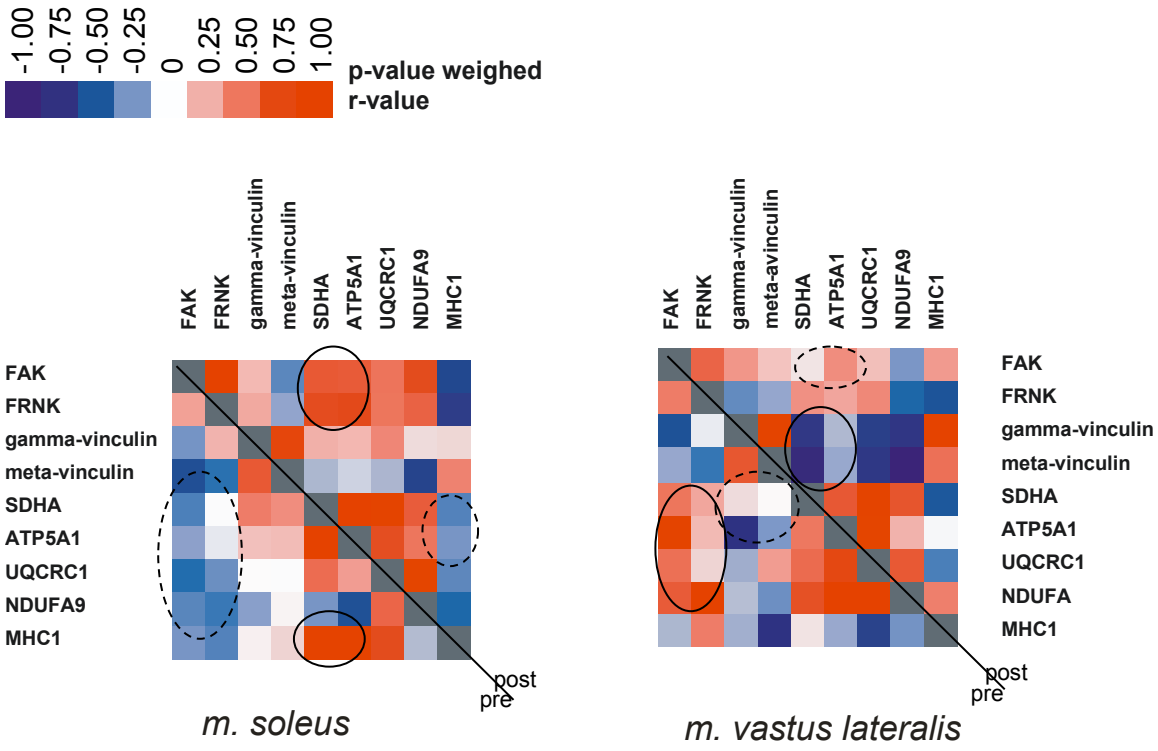


Figure 3.5 Correlation matrices visualizing the changes in linear relationships in the studied muscles with 3 days of ULLS. Transitions can be visualized by comparing relationships pre vs. post ULLS along the symmetry axes. Circles denote relationships of interest. R-values are given in colour coded boxes.

3.4 Discussion

3.4.1 Protein and fibre size alterations do not reach significance after 3 days of ULLS

Reduced weight bearing induces muscles loss mainly by decreasing anabolic drive because of decrased protein synthesis and increased proteolysis (Ohira *et al*., 2002). The consequences of unloading are evident at the molecular level in rodent and human anti-gravity muscles within the early days of unloading. (Bershadsky *et al*., 2003, Berthon *et al*., 2007). The quantitative importance of modified gene expression for the early structural changes in human muscle fibres that lead to atrophy is not fully understood (Adams *et al*., 2003, Schlaepfer *et al*., 2004). Our investigation is the first to corroborate the notion of early molecular changes at the protein level after discontinued weight bearing in human antigravity muscles. The study demonstrated a muscle-specific transition in the correlation pattern of factors that reflect the slow oxidative phenotype and a reduced activity status of FAK (Fig. 3.2). Consistent with the previously reported time pattern of muscle loss after reduced weight bearing (LeBlanc *et al.,* 1995), we did not find significant alterations in MCSA of muscle fibre types after 3 days of ULLS (Table 3.1).

3.4.2 Relationship between mitochondrial proteins and slow myosin heavy chain

The validity of assessing expressional changes by correlation analysis is supported by the linear relationships in the slow soleus muscle. For the soleus muscles of our subjects, which contained 32% more slow fibres than the *m.vastus lateralis*, significant correlations were identified between the protein content of slow myosin heavy chain and mitochondrial proteins (SDHA,

ATP5A1, and UQCRC1; $r<0.7$, $p<0.05$). It has previously been reported that mitochondrial respiratory chain components exist in equal proportion (Lopes *et al*., 2006) and that fractional synthesis rates of myosin heavy chain and mitochondrial protein are correlated (Adey *et al*., 2000). However, a correlation between mitochondrial proteins-and slow myosin heavy chain in human tissue has to the best of our knowledge not been reported before.

3.4.3 Ubiquitin-proteasomal-mediated proteolysis and fibre type transformation

Despite an absence of significant post vs. pre chymotrypsin-like enzyme activity changes, there was a reduction in the area content of slow type fibres. This change, and that in the expression of two mitochondrial proteins (ie. ATP5A1, UQCRC1), tended to be correlated negatively with fold changes in chymotrypsin activity only in the vastus lateralis ($r>0.6$, $p\leq 0.10$). This latter enzyme constitutes a main component of the proteasome machinery for muscle atrophy (LeBlanc *et al*., 1995, Gustafsson *et al*., 2010). Such observation mirror the more pronounced upregulation of mRNAs for atrogin-1 and MuRF-1 in *m.vastus lateralis* compared to *m.soleus* after 3 days unloading during a recent human study (Goldspink *et al*., 1995).

3.4.4 Correlations between the slow oxidative characteristics of fibre types in unloading

Changes in metabolic and contractile composition of muscle fibres have been seen after 2 weeks of simulated microgravity in rats and humans (Desplanches *et al*., 1987, Chopard *et al*., 2005). In this study, there was a loss of correlation between slow myosin heavy chain and mitochondrial protein (SDHA, ATP5A1, and UQCRC1) levels in the slow soleus muscle following ULLS.

Moreover, there was a trend for an increased expression of meta-vinculin post ULLS that has been suggested to indicate slow-to-fast fibre transformation (Bey *et al*., 2003). Such a selective increase in metavinculin in the predominantly slow type soleus but not the mixed vastus lateralis after 3 days of ULLS corresponds to a previous finding on the effect of 84 days of bedrest (Bey *et al*., 2003). Overall, the above mentioned observations support the notion of elevated metavinculin content being an early muscle-specific marker of the phenotypic response to unloading (ie. a slow-to-fast fibre type transformation), prior to significant changes in MCSA. Despite down-regulated phosphorylation of the auto-regulatory site Y397 of FAK, FAK protein content was not affected during early unloading. Interestingly FAK-pY397 content was only reduced in vastus lateralis muscle (Fig. 3.2), corresponding with the larger degree of molecular alterations seen in this muscle than in soleus muscle (Goldspink *et al*., 1995). Mechano-regulated Y397 phosphorylation of FAK has also been shown to lie upstream of the regulation of ribosome biogenesis by p70S6K which is essential for regulation of muscle mass (Huijing 1999).

3.5 Conclusions

First signs of fibre transformation might be observed in human anti-gravity muscle after 3 days of unloading as shown by an increase in meta-vinculin level and a loss of significant positive correlations between markers of the slow, oxidative phenotype in a slow-type muscle.

4. Effects of 21 days unloading on the regulation of anabolic and catabolic signaling in human vastus lateralis muscle

4.1 Introduction

A loss of muscle mass in response to actual and simulated microgravity exposure has been well documented (Tsika *et al*., 1987, Fitts *et al*., 2000, Ohira *et al*., 2002, Adams *et al*., 2003, Jones *et al*., 2004, de Boer *et al*., 2007a). In general, a reduction in muscle mass is the result of either a decreased rate of protein synthesis and/or increased rate of protein degradation (Judge *et al*., 2007, Fujino *et al*., 2009, Marimuthu *et al*., 2010). To date, the majority of knowledge of the regulatory mechanisms underlying muscle disuse atrophy has been generated from animal-based approaches (Thomason and Booth 1990, Murton *et al.,* 2008). As a result, the facilitative change causing the loss of muscle mass with disuse in humans still need to be fully elucidated. Observations of increased amounts of ubiquitin-protein conjugates and 3-methylhistidine suggest a possible contribution of muscle protein breakdown in the etiology of human disuse atrophy. On the other hand, others have speculated that the decline in muscle protein synthesis is the mechanism solely responsible for disuse induced atrophy in humans (Ferrando *et al.,* 1996, Paddon-Jones *et al*., 2006).

Among the anabolic mechanisms, the Akt/mTOR signaling cascade has been considered to date as the most well documented molecular pathway responsible for protein synthesis. Experimental evidence suggests that mechanical stimuli can lead to increased protein synthesis via two initial triggers, increased growth factor concentration or direct mechanical tension. Akt/mTOR

signaling happens to integrate the growth factor-dependent control of muscle mass, whereas the latter form of stimulation has not been well characterized. It is now widely recognized that there is a mechano-dependent signalling pathway to modulate protein synthesis in load-dependent skeletal muscle remodelling. Evidence currently sheds light through muscle-targeted transgenesis in a rat hindlimb unloading and reloading model, identifying focal adhesion kinase (FAK) as an upstream element of a mechano-sensory pathway to muscle protein synthesis of p70S6K activation, which is Akt-mTOR independent (Klossner *et al*., 2009). Further attention is needed to assess whether this pathway is responsible for eliciting the observed decline in muscle protein synthesis following human skeletal muscle disuse.

For potential mechanisms regulating protein degradation, an animal unloading study has demonstrated that a reduction in muscle mass is accompanied by increased expression of components within the lysosomal and ubiquitin-proteasomal systems (Ferreira *et al*., 2009). The fact that inhibitors of lysosomal function fail to block the degradation of myofibrillar components in atrophying muscles has resulted in limited data available on lysosomal contributions to muscle atrophy. However, an increased capacity for lysosomal proteolysis has been demonstrated in these data from animal models of various types of atrophy, including disuse atrophy. The role of lysosomal proteolytic pathway in disuse atrophy is underlined by recent investigations on cathepsin expression, where cathepsin L and cathpesin B genes appear upregulated (Bechet *et al.,* 2005). Since variations in cathepsin mRNA levels are not systematically correlated with similar modifications in cathepsin activities (Bechet *et al*., 2005), the need to measure the actual activity of cathepsin isoforms warrants attention. Currently, data have shown that the major function of lysosomes is to degrade membrane proteins rather than cytosolic proteins like myofibrils. Therefore, one would not expect their inhibition to have much

effect on myofibrillar protein and total protein degradation rates. In fact, in vivo data has shown that lysosomal and ubquitin-proteasomal pathways operate in conjunction to degrade specific membrane protein substrates such as receptors and ion channels (Bechet *et al*., 2005, Zhao *et al*., 2007). Thus, it is necessary to examine the involvement of the lysosomal pathway in human disuse atrophy.

In contrast to a limited data available on lysosomal involvement in disuse atrophy, ubquitin-proteasome pathway has been extensively studied. As a result, a substantial body of evidence implicates UPS to be the principle regulator of skeletal muscle atrophy. Numerous studies carried out on differing models of human muscle disuse atrophy have focused their attention on two muscle-specific Ub-ligases, MuRF1 and MAFbx. Contrary to a classical elevation of MuRF1 and MAFbx mRNA in animal models of disuse, the human data is inconsistent. Due to a general absence of protein measurements for these two E3-ligases, and the lack of assessments on other representative elements of the UPS, it is currently difficult to discern the actual importance of UPS in the human conditions of muscle disuse atrophy.

4.1.1 Aim and hypothesis

Our aim was to investigate the selected mechanisms implicated in the loss of muscle mass after a chronic unloading period of 21 days in humans. In order to obtain more information on the effects of disuse in the context of likely decreases in muscle protein synthesis as well as possible increases in muscle protein degradation, we examined muscle fibre morphology and potential factors associated with anabolic signalling (ie. protein content of FAK and p70S6K), and markers representative of the catabolic pathways (ie. cathepsin B/L enzyme activities,

chymotrypsin-like enzyme activity) in response to disuse. We hypothesized that total protein levels of these anabolic signalling molecules might change to a state to reflect reduced translational capacity, while the catabolic enzyme activity levels would increase in association with elevated proteolytic capacity.

4.2 Methods

4.2.1 Subjects

Nine healthy adult men were recruited from the Manchester area. The subject sample ranged from physically active to sedentary individuals. Prior to the experiments, participants were screened for any history of lower limb pathology, neuromuscular disorder or cardiovascular disease. Written consent was obtained from each participant after being informed of the procedures, risks and potential benefits associated with the experiments. The study protocol was approved by the Ethics Committee at Manchester Metropolitan University.

4.2.2 Induction of atrophy

Unilateral limb suspension (ULLS) was accomplished as previously described (de Boer *et al*., 2007a). Prior to the ULLS intervention, all participants took time to practise walking on crutches. In brief, the non-dominant leg of each participant was fitted with a platform shoe and the domain leg was kept in a slightly flexed position using straps suspending the foot above the ground while

walking with crutches. The participants had to walk on crutches for the whole duration of the 21-day suspension period. They were asked to refrain from loading the leg in any way, including by driving vehicles, yet maintained their normal dietary habits and their normal occupational tasks throughout the experimental period.

4.2.3 Collection of muscle samples

Biopsies (50mg) of vastus lateralis muscle were taken prior to ULLS and after 21 days of ULLS before resuming any weight-bearing activity by use of the Bergström technique (Mayet-Sornay and Desplanches 1996). After making skin and fascia incisions under local anaesthesia (1% lignocaine), muscle biopsies were obtained from the *m.vastus lateralis* using a 5 mm Bergström needle. Muscle samples were cleansed of excess blood, connective tissue and fat, and then frozen in liquid nitrogen and stored at -80°C until further analysis.

4.2.4 Immunoblotting

Muscle biopsies were cross sectioned at 25 micrometers using a cryostat, proteins were isolated with rotor stator mixer (Ultra-Turrax, IKA Werke GmbH & Co. KG, Germany) into ice-cold modified radio immuno precipitation buffer (RIPA; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate at 90%, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 2 µg/ml pepstatin, 1 µg/ml aprotinin, and 0.1 mM PMSF; Sigma, Buchs, Switzerland), where an amount of 10 mm^3 tissue corresponding to 2 mg of protein was pooled and mixed in 200 µl RIPA buffer. Muscle protein was quantified with a bicinchoninic acid kit (Pierce) against bovine serum

albumine (BSA). Total protein homogenate of a 2 mg/ml concentration was denatured in Laemmli buffer (50mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 2%-mercaptoethanol, 0.1% bromphenol blue) by heating for 5 minutes at 95°C. 20 micrograms of total muscle protein per sample lane were separated using 7.5% sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean III system (Biorad). Proteins were blotted onto nitrocellulose membrane (Amersham) and blotting efficiency and equal loading were verified by Ponceau S staining. Membranes were then incubated in 5% low fat milk in TBS with 0.1% tween. Subsequently, membranes were subjected to immunodetection with specific first antibodies and horseradish peroxidase (HRP)-coupled secondary antibodies**.** For the detection of FAK this involved polyclonal C-terminal antibody 'Lulu' and anti-rabbit HRP antibody (ICN Biomedicals GMBH, Germany). As for the detection of p70S6K this included polyclonal antibody p70S6K (C-18) (Santa Cruz, USA) and anti-rabbit HRP antibody (Santa Cruz, USA). Signal detection was carried out with enhanced chemoluminescence (Femto kit, Pierce) and quantified with a Chemidoc system running under Quantity One software (Bio-Rad, Life Science Research, Hercules, CA, USA). The signal intensity of relevant bands was estimated with the 'volume rectangular tool' and corrected versus the background of a band of equal height and size (area) in an empty sample lane. Separation of equal amounts of total protein (20 micrograms per lane) in a paired design (loading pattern) with SDS-PAGE that is samples from the time course of one subject (pre and post ULLS) were loaded on the same 7.5% SDS-PAGE gel. Each background-corrected band value was normalized to its corresponding Ponceau S stained actin band signal intensity on the respective gel; the values therefore reflect relative expression levels per total muscle protein in arbitrary units. Due to an insufficient amount of biopsy sample

available, we were unable to perform other tests, such as assessment of the phosphorylated S6K and FAK.

4.2.5 Enzyme activities

Homogenates were prepared from 20-micrometer cryosections in 0.1 M KH_2PO_4 buffer (pH 7.2) containing 2 mM EDTA on ice with the help of a Polytron mixer (Kinematica Switzerland). Protein concentration was quantified spectrophotometrically with the bicinchoninic acid protein assay kit (Perbio) and adjusted to 2 mg/ml. Samples were distributed in aliquots and stored at -80°C. The quantification of chymotrypsin-like enzyme activity of the 20S proteasome was based on the hydrolysis of a fluorogenic substrate essentially as described (Berthon *et al*., 2007). In brief, 20 micrograms of muscle extract were incubated in a final volume of 1 ml imidazole buffer (60 mM, pH 7.4). The reaction was started by the addition of 100 µM of the fluorogenic substrate succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (Bachem, I-1395). For cathepsin B/L activities, 10 micrograms of muscle extract were incubated in a final volume of l ml sodium acetate buffer (100 mM, pH 9.0) (Duguez *et al*., 2003). The assay was started by the addition of 10 mM benzyloxycarbonyl-Phe-Arg-amido-4-methylcoumarin (Bachem, I-1160). Substrate conversion was assessed fluorometrically (excitation at 380nm and emission at 460nm) on a SFM25 fluorimeter (Kontron Instruments). Proteolytic activity was estimated based on a Michaelis-Menten type model of catalytic activity where the conversion rate (i.e. the increase in fluorometric signal per time) is proportional to Vmax. Measures were repeated in minute intervals over the first 10 minutes of the reaction and the mean conversion rate calculated.

Proteolytic activity (Vmax) was then calculated vs. standard curve of amido-4-methylcoumarin drawn from dilutions in the range of 0-150 pmol/ml.

4.2.6 Immunocytochemistry

15-micrometer cryosections of VL and SOL muscle were incubated with a 1:400 dilution of mouse monoclonal anti-fast myosin heavy chain antibody (MHCII) Clone MY-32 (Sigma-aldrich) in 0.3% BSA in phosphate buffered saline (PBS), then reacted with a 1:2000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (ICN). Immunoreactivity was detected with substrate solution, 3-amino-9-ethylcarbazole in dimethylformamide (Sigma Chemicals, Buchs, Switzerland). Sections were nuclei counterstained with hematoxylin and embedded in Aquatex (Merck, Germany). Sections were then used for fibre % and fibre cross-sectional area analysis.

4.2.7 Fibre type percentage and cross-sectional area analysis

Type I and type II myosin heavy chain stained sections were recorded digitally at 10X magnification on an Axioskop 2 microscope (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom) that was operated with AxioVision software (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom). Subsequently, one field from type II myosin heavy chain stained sections were assessed for cross-sectional area of stained (fast-type) and unstained (slow-type) fibers against a scale by manually recording the fibre periphery of each assessed fiber within the ARDOM software (Flueck *et al*., 2011). These numbers were used to calculate the percentage and mean cross-sectional area of slow and fast type muscle fibres. The area content of slow type

fibres was calculated using the formula: [area content of slow-type fibre = percentage of slow-type fibres x mean cross-sectional area of slow-type fibers x 100].

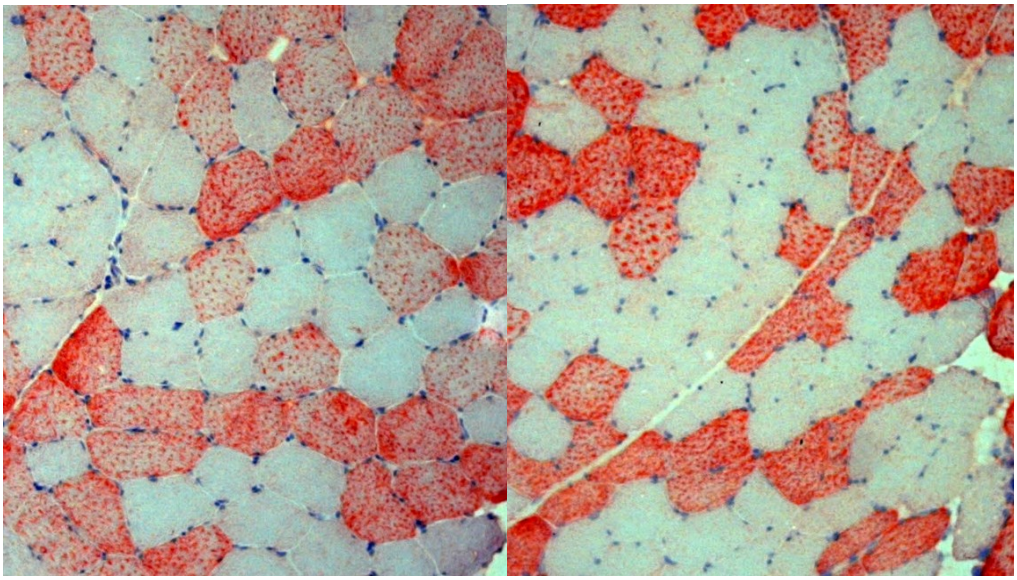
4.2.8 Statistical analysis

Post vs. pre changes were assessed with repeated ANOVA and effects were localized with a post hoc test in function of the result of the Shapiro-Wilks test for normality (Statistica 9.1). In cases where the assumption of a normal distribution was rejected, a nonparametric one-tailed Wilcoxon test was employed to assess the post vs. pre effects. In any other cases, a student's paired t-test was used to analyse data. A one-tailed t-test was employed in situations where single sided hypothesis could be formulated i.e. the increase in chymotrypsin-like and cathepsin B/L activities. For all data used n=9 pre and n=9 post. Significance was assigned at $p<0.05$.

4.3 Results

4.3.1 Muscle fibre type composition

m.vastus lateralis had a typical fibre type distribution, with 26% more type II fibres than type I fibres (p=0.0003; two-tailed paired Student's T-test). Type II fibres had a significantly elevated mean cross sectional area (MCSA) compared to type I fibres (p=0.008) (Table 4.1).



Pre Post

Figure 4.1 Microscopic images of muscle cross sections of *m.vastus lateralis* pre and post 21 days of ULLS after staining for type II myosin type heavy chain (orange). Nuclei were counterstained with hematoxylin (blue).

	pre	post
m. vastus lateralis		
slow type:	4824±386	4158 ± 253
fast type:	5612 ± 404	4625 ± 254

Table 4.1 Mean and standard error of the mean of the fibre cross sectional area of slow and fast type muscle fibres in m. vastus lateralis pre and post 3 weeks ULLS. Significant difference was identified (two tailed Fisher-test). Type I fibres were reduced 14% in MCSA post ULLS compared to that of the pre samples from the same leg (p=0.030), whereas type II fibres showed a significant 21% lower MCSA. (p=0.006).

4.3.2 Response to unloading

There was a main effect of ULLS observed between parameters assessed combined as indices of negative (i.e. chymotrypsin-like enzyme of 20S proteasome and cathepsin B/L enzyme activities) and positive (i.e. type I fibre MCSA, type II fibre MCSA, protein content of FAK and p70S6K) protein balance (p=0.0001; repeated ANOVA Statistica 10.0).

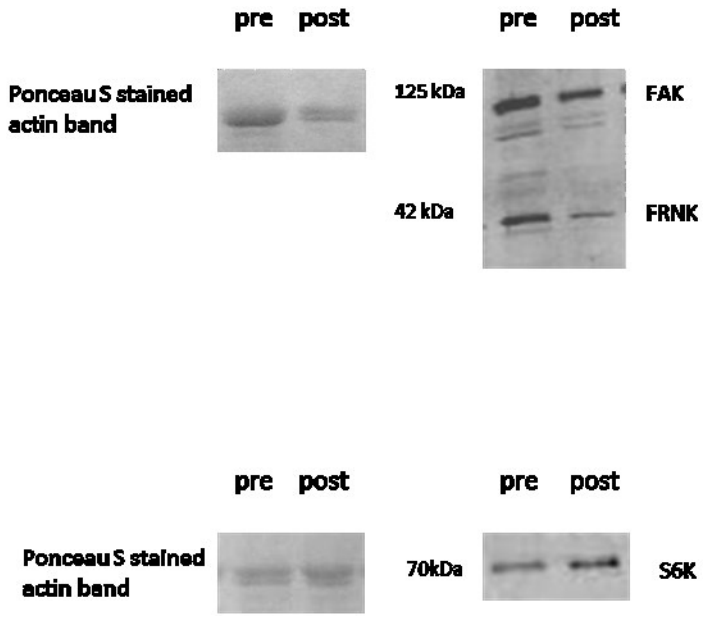


Figure 4.2 Representative immunoblots visualizing the detected proteins, FAK and p70S6K from total homogenate of *m.vastus lateralis* pre- and post-ULLS for the same subject. Ponceau S stained actin bands are shown as a loading control.

4.3.3 Fibre atrophy with unloading

Both type I and type II fibres showed a significantly lower MCSA after 21 days of ULLS. Type I fibres were reduced 14% in MCSA post ULLS compared to that of the pre samples from the same leg (p=0.030), whereas type II fibres showed a significant 21% lower MCSA (p=0.006) (Fig. 4.3). The reported muscle fibre atrophy is fibre type independent (p=0.297, two-tailed paired Student's T-test). Type II fibre continued to show significance for a larger MCSA than that of type I fibres post ULLS (p=0.043).

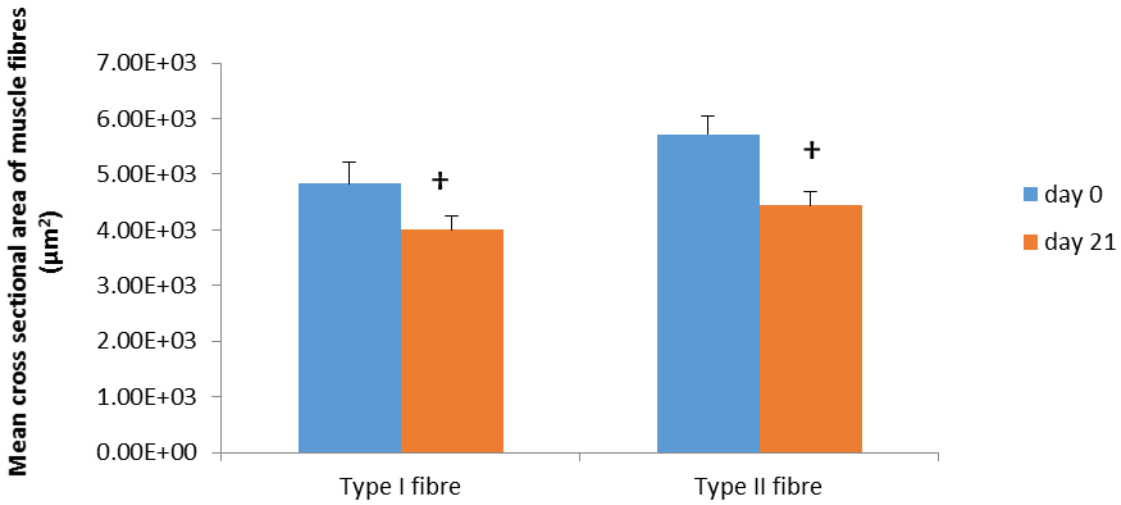
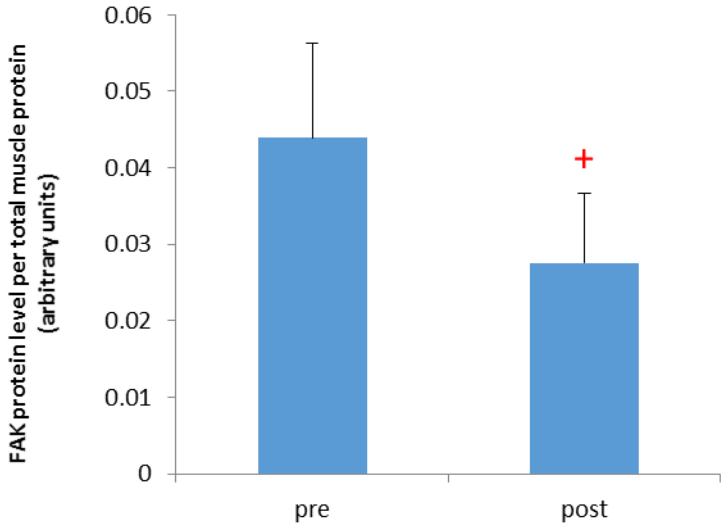


Figure 4.3 Bar graph showing mean and standard error of the mean of the fibre cross-sectional area of slow and fast type muscle fibres in *m. vastus lateralis* pre and post 3 weeks of ULLS. * $p<0.05$ identified (two-tailed Fisher-test).

4.3.4 Effects of unloading on protein expression

Figure 4.2 shows representative immunoblots of the measured anabolic signaling proteins. After 21 days of ULLS, there was a trend for a reduction of FAK protein content ($p=0.07$, two-tailed paired Student's T-test). Moreover, FAK protein content developed a positive correspondence with type I fibre percentage as well as the percentage area content of type I fibres post ULLS ($r=0.64$ and $r=0.62$ respectively). The total protein level of p70S6K was unchanged with unloading ($p=0.20$) (Fig. 4.4).



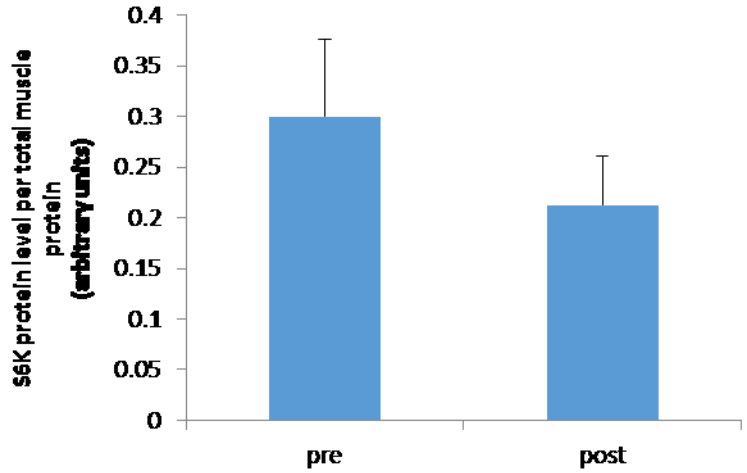


Figure 4.4 Bar graph showing mean and standard error of FAK and p70S6K protein level changes with 21 days of ULLS in *m.vastus lateralis*. + $p<0.10$ (two-tailed paired Student's T-test).

4.3.5 Proteasomal activity

In order to determine whether there were changes in indices of proteolytic activity in *m.vastus lateralis* after 27 days of ULLS, we assessed the activity of chymotrypsin-like enzyme of 20S proteasome of the ubiquitin-proteasome pathway known as the main contributor to target sarcomeric proteins, and also that of cathepsin B/L, the primary enzymes of the lysosomal pathway to function in skeletal muscle degradation. Cathepsin B/L and chymotrypsin-like enzymes showed a trend for elevated activities post ULLS ($p=0.057$ and $p=0.057$ respectively; one-tailed Wilcoxon) (Fig 4.5). The percentage change in activity of Cathepsin B/L following

unloading was positively correlated with that of chymotrypsin-like enzyme and there was a strong positive correlation between the activity levels of both enzymes post ULLS (r=0.92 and r=0.95 respectively).

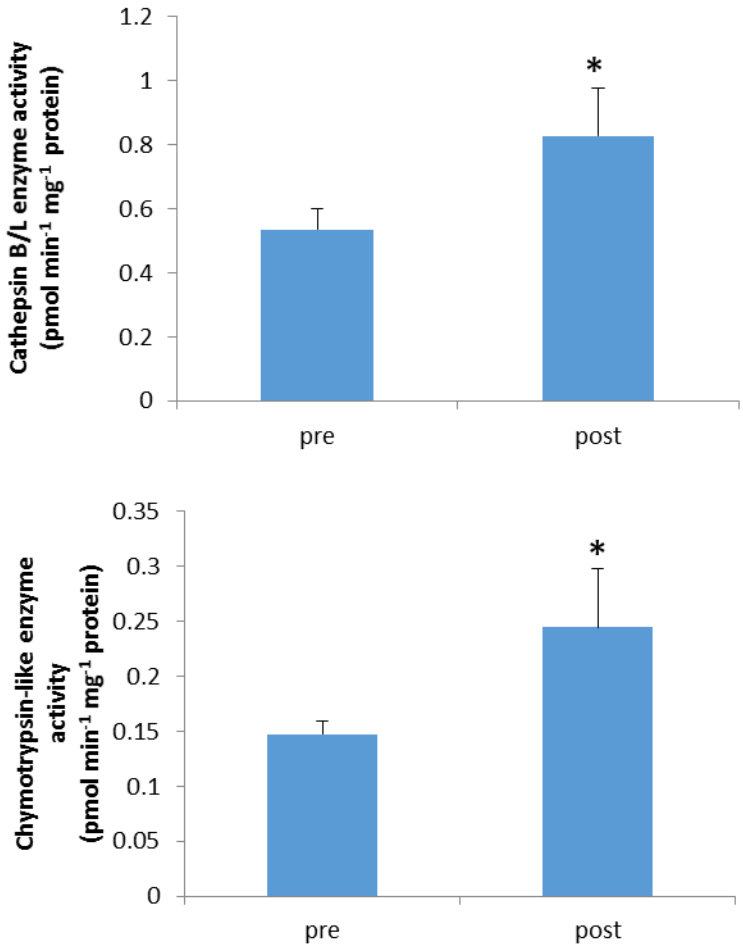


Figure 4.5 Effect of 21 days ULLS on chymotrypsin-like enzyme activity of 20S proteasome and cathepsin B/L enzyme activities. Enzyme activities are expressed as pmol min^{-1} mg^{-1} protein (n=9). Values are means ± SE. *Pre* control group; *Post* unilateral limb suspension for 21 days, * $p<0.10$ (one-tailed Wilcoxon).

4.4 Discussion

4.4.1 A decrease in the mean cross-sectional area of both fibre types are detected in *m.vastus lateralis* following 21 days of ULLS.

Animal studies of disuse have shown that the mechanisms responsible for the muscle atrophy involve a decrease of protein synthesis accompanied by an increase of protein degradation (Fujino *et al.,* 2009). Few investigations to date have been carried out in humans (Murton *et al*., 2008). As a result, the actual regulatory mechanisms governing human conditions of disuse atrophy are unresolved. Our investigation suggests that after 21 days of muscle deconditioning, the observed muscle fibre atrophy is associated with elevated proteolytic activity and reduced anabolic signaling.

In general, this study is limited by a small sample size and an inter-subject variability. There was a significant main effect indicative of unloading induced differential response between factors representing negative and positive protein balance. The structural alterations in muscle submitted to unloading are illustrated first by muscle atrophy. After 21 days ULLS, we observed a 14% decrease in MCSA of type I fibres and a 21% reduction in that of type II fibres in *m.vastus lateralis*, which is consistent with the general concept that anti-gravity extensor muscles of the lower limb experience the greatest atrophy in response to inactivity and unloading (Belavy *et al*., 2009). But the magnitude of reduction of our data seems to be greater than those findings from previous work, with observed CSA measures in the order of an 8-9% decline after 16-28 days of ULLS (Narici and de Boer 2011). However, non-significant reductions in MCSA of type I and type II muscle fibres of *m.vastus lateralis* have been reported to amount to 11 and 24 % after 5

days of spaceflight. The reduction in MCSA of both fibre types was fibre-type independent ($p=0.297$). This fibre independence is supported by an earlier experiment showing the rates of atrophy being unrelated to the proportion for type I muscle fibres (Blottner *et al*., 2006). Similarly, another study has reported a preferential atrophy of the slow-oxidative type I fibres in rats, but not in humans. The author has suggested that atrophy of type II fibres is comparable, if not greater than, that of slow type I fibres (Fitts *et al*., 2000).

4.4.2 A reduced protein level of FAK happens to positively correlate with the percentage number and area content of type I fibres.

Although it is widely recognized that mechanically induced tension plays a critical role in the regulation of the skeletal muscle mass (Hornberger and Chien 2006, Hornberger *et al*., 2006), the events linking mechanical stimuli to the anabolic signaling pathways remain still not thoroughly understood. To date, focal adhesion kinase (FAK) has been considered as a signaling molecule associated with mechano-transduction (Durieux *et al*., 2007**,** Klossner *et al*., 2009). Its tyrosine phosphorylation at residue 397 reflects the mechano-chemical coupling between mechanical stimulation of integrins and activation of intracellular signal transduction. The total amount and phosphorylation state of FAK are reportedly increased during load-induced muscle hypertrophy in conjunction with elevated myofibrillar content in vivo and in vitro. Based on our experiment, there was a trend for a decrease of FAK total protein, which corresponds to the observations of reduced levels of phosphorylated FAK and its protein content after 10 days ULLS intervention in humans in association with a 50% decrease in the fractional rate of myofibrillar protein synthesis (de Boer *et al*., 2007b). Our finding reinforces the notion of FAK as an upstream modulator of a

mechano-regulated pathway to protein synthesis (Klossner *et al*., 2009), and suggests that this protein is responsible for eliciting the decline in muscle protein synthesis through decreased anabolic signaling at 21 days of human skeletal muscle disuse atrophy.

In addition, the content of FAK was positively correlated with the percentage of type I fibres and the percentage area content of type I fibres at 21 days ULLS (r=0.64 and r=0.62 respectively). These correlations are the first human data to support the findings from animal studies. Whereby, sarcolemmal FAK concentration was found to correspond to the degree of muscle fibre recruitment for contraction with an elevated FAK content being found in slow-oxidative muscle fibres that experience a high degree of load-bearing activity. More recently, a muscle-targeted transgenic investigation in rat has identified FAK as a load-dependent governor of the slow-oxidative muscle phenotype (Klossner *et al*., 2009).

4.4.3 Protein content level of p70S6 kinase remains unchanged

The level of p70S6 kinase did not alter with unloading. Although we haven't measured the phosphorylated state of this protein, it was shown by another 21 days ULLS study that there was no alteration in either activity or content of p70S6K (de Boer *et al*., 2007b). (I have spent about two months last year to try to detect the phosphorylated state of s6k without the immunoprecipitation step but failed, and the rest remaining samples is not sufficient to carry out immunoprecipitation). However, this does not exclude its involvement as an upstream regulator of translation initiation with the decreased protein synthesis rate in disuse atrophy, but could rather suggest that the time point when the biopsies were taken did not coincide with the transient change of the protein. A decreased p70S6K phosphorylation has coincided with a

reduced translation rate in animal models of muscle disuse (Bodine *et al*., 2001b, Hornberger *et al*., 2001). In fact, Flueck and colleagues have demonstrated an existing temporal relationship between FAK and its downstream effector p70S6K, by which enhanced FAK signaling resulted in a delayed response of p70S6K (Klossner *et al*., 2009). Furthermore, an increase in the phosphorylation status of p70S6K was not observed after a resistance training programme. Its author has regarded the result as a response of negative feedback of p70S6K being auto-regulated by eIF-4E to reach cellular translational homoeostasis and to prevent uncontrolled growth, rather than an indication its inactivation during human skeletal muscle hypertrophy (Gautsch *et al*., 1998).

4.4.4 Chymotrypsin-like activity of the 20S proteasome and cathepsin B/L enzyme activities are elevated after 21 days of ULLS in humans.

This study is the first to show a trend of increase in chymotrypsin-like activity of the 20S proteasome in a human disuse model, which is in line with the data from animal disuse programs, where a classical activation of the ubiqutin–proteasome pathway has been indicated by reported increases in mRNA expression, protein level and activity of various UPS constituents (Berthon *et al*., 2007, Murton *et al*., 2008, Fujino *et al*., 2009). While in humans, elements of the UPS, notably levels of ubquitinated proteins and HC6 mRNA appear elevated in a bed rest and an immobilization studies respectively, the vast majority of human disuse experiments performed to date have been focused on mRNA expressions of two E3 ligases, MuRF1 and MAFbx. The resulting variable findings in regard to MAFbx and MuRF-1 levels and a general absence of their protein content measures render the role of UPS in muscle atrophy to be questioned (Murton *et*

al., 2008). Our observation based on assessing chymotrypsin-like activity of the 20S proteasome is indicative of a role for ubquitin-proteasome proteolysis in human chronic unloading. We also happen to be the first to have found a trend for an elevation of the cathepsin B/L enzyme activities in humans. A survey of the literature shows no studies to date have investigated the lysosomal degradation pathway under human disuse conditions. Our findings are supported by the available data from animal unloading programs showing marked increase in various isoforms of cathepsin mRNAs and increase in cathepsin protein or activity levels (Sakamoto and Goodyear 2002).

4.4.5 Evidences for close regulation of the ubiquitin-proteasome system and the lysosomal degradation pathway during disuse atrophy are observed.

Strong positive correlations were established between the activity levels of cathepsin B/L and chymotrypsin-like enzymes post ULLS as well as their percentage increase in activity. This observation reinforces the notion from in vitro and in vivo studies that the two main mechanisms responsible for the majority of cellular protein degradation, lysosomal proteolysis and UPS are being closely regulated and an interaction of both mechanisms has been proposed to govern muscle protein breakdown during muscle atrophy. Evidence has shown that lysosomal proteolytic pathway seems to operate in conjunction with the ubiquitin-proteawsome pathway for degradation of specific protein substrates (Bechet *et al*., 2005, Zhao *et al*., 2007). A coordinated stimulation of both degradation pathways accompanying a decrease of the net proteolytic rate have been observed in animal models of disuse. More importantly, two in vivo studies now implicate that FOXO3 transcription factor can cause marked atrophy through coordinately

regulating the transcription of components of lysosomal and ubquitin-poteasomal systems (Mammucari *et al*., 2007, Zhao *et al*., 2007).

4.5 Conclusion

After 21 days of unilateral unloading (ULLS), muscle fibre atrophy has been in association with decreased FAK protein levels and strongly correlated increases of lysosomal and ubiquitin-proteasomal proteolytic activity. The latter observation is indicative of the two proteolytic systems being coordinately regulated and possibly reflects their close interaction during protein degradation. In summary, the human disuse atrophy data favors the proposition, based on animal studies, that the regulatory mechanisms governing disuse atrophy involve decrease of protein synthesis and increase of protein degradation.

5. Costamere remodeling with muscle loading and unloading in healthy young men

5.1 Introduction

Skeletal muscle size shows a pronounced mechano-dependence, notably increasing in size with chronic overloading and decreasing in size with chronic unloading, as for example during experimental bedrest (Loughna *et al*., 1986). Changes in mean muscle cross-sectional area (CSA) and fascicle pennation angle can be observed within a few days of altered loading in humans (Narici and Maganaris 2007, Hackney and Ploutz-Snyder 2012). For instance, atrophy is detectable as early as after 7 days of bedrest (Ferrando *et al.*, 1995) in anti-gravity muscle *m. vastus lateralis*, and aggravates progressively with prolonged unloading until 6 months (Bloomfield 1997, Hackney and Ploutz-Snyder 2012). Similarly, hypertrophy of *m. vastus lateralis* can be detected after as few as 10 sessions of eccentric-type resistance exercise in 3 weeks, further increasing with the progression of the training period (Seynnes *et al*., 2007). Changes in muscle size are largely driven by modified content of myofibrils, which involves adaptations of both slow- and fast-type fibers (Fry 2004, Borina *et al*., 2010). With unloading, atrophy is observed for both slow type I, and fast type IIA and IIX muscle fibers (Bloomfield 1997, Fitts *et al*., 2001, Hackney and Ploutz-Snyder 2012). Based on changes in myosin heavy chain (MHC) expression with prolonged unloading, there may also be a conversion of slow- into fast-type muscle fibers, leading to a relative increase in fast- over slow-type MHC content (Bloomfield 1997, Hackney and Ploutz-Snyder 2012). An increase in nitrogen excretion suggests that altered myofibrillar makeup contributes to the observed muscle atrophy after 7 days of unloading in humans (Bloomfield 1997). More recently, the use of stable isotopes has enabled to show a 50% reduction in myofibrillar protein synthesis within 10 days of lower limb unloading in humans (de Boer *et al*., 2007a,b). Conversely, an increase in myofibrillar synthesis rate implicates the contribution of increased MHC synthesis to the elevated CSA of both slow- and fast-type muscle fibers after resistance-type exercise (Williamson *et al*., 2001, Fry, 2004,

Wilkinson *et al*., 2008). Despite its apparent importance (Adams *et al*., 2003, Chopard *et al*., 2009), the question arises of how such myofibrillar alterations are integrated between the molecular and the architectural level in muscle fibers that undergo adaptive changes.

Sites of focal adhesion in the sarcolemma (costameres) are critical for the attachment of myofibrils to the fiber periphery (Ervasti 2003, Grounds *et al*., 2005). They assemble through the binding of cytoskeletal and signaling molecules to the intracellular inside of integrin-type and/or dystrophin/sarcoglycan-type extracellular matrix receptors (Miyamoto *et al*., 1995, Ervasti 2003, Grounds *et al*., 2005, Trimarchi *et al*., 2006). Thereby, costameres provide an anchor for the intermediate filaments that hold sarcomeres in register and establish a physical link between neighboring muscle fibers via the interstitium (Pardo *et al*., 1983, Ervasti 2003, Grounds *et al*., 2005, Ramaswamy *et al*., 2011). Assembly of new costameres is essential for myofibrillogenesis in culture by providing a platform for the attachment of myofibril/myosins (Quach and Rando 2006). The association between costameres and myofibrils is of great interest given that mechanical factors exert important control over the expression of costamere components and fiber size in anti-gravity muscle (Flueck *et al*., 1999, Gordon *et al*., 2001, Chopard *et al*., 2002, Chopard *et al*., 2005, Anastasi *et al*., 2008). For example, expression of the costamere components gamma- and meta-vinculin, talin, and b1-integrin is compromised with prolonged muscle unloading and muscle inactivity when muscle sarcomeres are lost (Chopard *et al*., 2002, Chopard *et al*., 2005, Anastasi *et al*., 2008). By contrast, concomitant resistance-type exercise prevents alterations in costamere components in unloaded muscle (Chopard *et al*., 2005). Interestingly, costamere component expression also varies between fiber types (Shear and Bloch 1985, Bozyczko *et al*., 1989, Schröder *et al*., 1997, Williams *et al*., 2000, Flueck *et al*., 2002, Thoss *et al.,* 2013). Owing to their role in myofibril attachment, the mechano-regulation of

costamere components is suggested to reflect active phases of muscle remodeling and fiber-type transformation with altered loading.

The integrin-associated focal adhesion kinase (FAK) is instrumental for costamerogenesis in culture (Quach and Rando 2006). Overexpression of FRNK, which competes with FAK for binding to focal adhesions, implies that this involves the control over focal adhesion turnover via the interaction between focal adhesion components (Ilic *et al*., 1995, Schlaepfer *et al*., 2004). Phosphorylation of FAK at tyrosine residue 397 is an important control point of this regulation. It alters the capacity of FAK to interact with binding partners such as the tyrosine kinase c-src, enabling FAK to develop its full catalytic activity, phosphorylate auxiliary sites and enter interactions with further binding partners (Schlaepfer *et al*., 1999) that mediate the clustering of cytoskeletal and signaling proteins to integrins (Miyamoto *et al*., 1995). A number of observations highlight that both phosphorylation of Y397 and auxiliary sites, as well as the content of FAK, is load-regulated (Li *et al*., 1997, Aikawa *et al*., 2002, Torsoni *et al*., 2003, Lal *et al*., 2007). For instance, FAK-pY397 content is increased in relation to enhanced FAK protein content after the first day of chronic overload in rat soleus muscle (Flueck *et al.,* 1999, Gordon *et al*., 2001, Durieux *et al*., 2009). Conversely, muscle unloading downregulates FAK tyrosine phosphorylation within 7 days in rat and human skeletal muscle, and this is confounded by an upregulation of FAK protein content in rat muscle (Gordon *et al*., 2001, de Boer *et al*., 2007b). Likewise, components of the dystrophyin/sarcoglycan-type receptor demonstrate mechano-regulated expression and are involved in mechanical signal transduction in skeletal muscle (Barton 2006, Chopard *et al*., 2005). Increased FAK amount in rodent skeletal muscle appears also to promote the load-regulated activation of the serine/threonine kinase p70S6K (the positive regulator of protein synthesis), which is robustly associated with increased protein synthesis and

hypertrophy with muscle overload (Baar and Esser 1999, Terzis *et al*., 2008, Wilkinson *et al*., 2008, Klossner *et al.,* 2009, Carter and Flueck 2012).

We reasoned that loading-dependent plasticity of muscle size is integrated through costamere turnover via a load-regulated process that involves level alterations of FAK and FAK-pY397 concentration. The functional implication of FAK in the hypothesized mechano-regulation of costamere remodeling was studied by quantifying the concentration of the focal adhesion components, meta- and gamma-vinculin, beta 1 integrin, and p70S6K in relation to concentration changes in FAK, FAK-pY397 and its inhibitor FRNK during increased loading and unloading of human vastus lateralis muscle. We thus hypothesized that the changes in costamere components through the course of increased muscle loading with eccentric-type resistance training and unloading by bedrest would be inter-related with FAK-pY397. As costameres are connected to the cytoskeletal elements that hold sarcomeres in register, we expected that costamere protein levels exist in a constant proportion to muscle size (muscle thickness or CSA), except during phases of muscle remodeling characterized by the formation or removal of myofibrils (i.e. fiber transformation and atrophy). This was tested by comparing the changes in muscle size with those of costamere protein levels during the course of altered loading of m. vastus lateralis in humans. Time points for the sampling of bioptic material were selected on the basis of documented anatomical changes with the two loading interventions (Ferrando *et al*., 1995, de Boer *et al*., 2007a, Seynnes *et al*., 2007).

5.1.1 Aim and hypothesis

The aim of the study was to investigate the role of costameres in the regulation of muscle size with altered muscle loading. For this purpose we assessed the association of level changes in costameres components and its upstream regulator, FAK, with changes in muscle thickness and CSA following bedrest and resistance training. We hypothesized that changes in content of costamere components (beta 1 integrin, FAK, meta-vinculin, gamma-vinculin) with increased and reduced loading of human anti-gravity muscle would i) relate to changes in muscle size and molecular parameters of muscle size regulation (p70S6K, MHC1 and MHCIIA), ii) correspond to adjustments in activity and expression of FAK, and its negative regulator, FRNK, and iii) reflect the temporal response to reduced and increased loading.

5.2 Methods

5.2.1 Experimental design

Anti-gravitational muscle (m. vastus lateralis) of untrained human subjects was subjected either to unloading by bedrest (experimental Group 1) or to overloading by resistance training (experimental Group 2). In both groups, anatomical measures were taken to estimate muscle size and muscle fiber CSA, and a muscle sample was collected to determine the content of costameric proteins (FAK, FRNK, beta 1 integrin, meta-vinculin, gamma-vinculin) and the phosphorylation status of FAK at pY397, and where appropriate the content of p70S6K. All subjects gave their written informed consent to participation in the study, which had been approved by the Ethical Committee of the University of Primorska for subjects of Group 1 and by the local Ethics

Committee of Manchester Metropolitan University for subjects of Group 2. Muscle tissue was collected and stored under the United Kingdom's Human Tissue Act.

5.2.2 Bedrest (Group 1)

These experiments were carried out in the Orthopedska Bolnica Hospital in Valdoltra, Ankaran (Slovenia) under medical supervision. Nine healthy Caucasian young men (age: 24.3± 2.6 years; stature: 179.7±8.0 cm; body mass: 76.4±10.4 kg) from the Slovenia area were recruited for this study via advertisement in a student job portal and via word of mouth. Muscle thickness was determined after the 8th and 34th day of bedrest (see 'Muscle anatomy' below). On this occasion, a muscle biopsy sample was collected with a conchotome under local anaesthesia from the belly portion of the vastus lateralis muscle (~80-150 mg). Samples were snap-frozen in liquid nitrogen and stored until use at -80°C.

5.2.3 Resistance training (Group 2)

Six healthy Caucasian men (age: 22.3 ± 1.5 years; stature: 175.2 ± 3.4 cm; body mass 71.0 ± 4.6 kg) from the student population of Manchester Metropolitan University participated in this study. Unilateral leg extension was performed three times per week for 9 weeks on a flywheel ergometer (YoYo Technology, Sweden; Seynnes *et al*., 2007). In brief, resistance is produced from the inertia of the flywheel during both concentric and eccentric contraction phases, by virtue of the unwinding/rewinding of a strap connecting the flywheel to the lever arm. Each session consisted of one set of seven submaximal leg extensions as a warm-up, and was

proceeded by four sets of 10 maximal coupled concentric and eccentric contractions, with 2-min rest periods between sets. Subjects were tested for vastus lateralis muscle thickness and quadriceps femoris CSA on three occasions (see 'muscle anatomy' below): at baseline, 4 weeks before the first training session (pre-training); after the 10th training session (mid-training) and after the 27th training session (post-training). Bioptic samples were collected pre-training, mid- and post-training from lignocaine-anaesthetized vastus lateralis muscle. For the training samples the interval between the preceding exercise bout and muscle sampling was 4-6 h.

5.2.4 Muscle anatomy

Group 1

Muscle thickness was measured by using B-mode ultrasound scanning (Mylab 25, 13-4 MHz, linear array transducer probe LA523; Esaote Biomedica, Geneva, Italy). Scans were performed at mid-muscle length along the mid-sagittal axis of the vastus lateralis, perpendicular to the lower aponeurosis plane. Thickness was defined as the average of three equidistant measurements along the scan width, between the superficial and deep aponeuroses of the muscle. The consistency of ultrasound scanning between the testing sessions was ensured by recording the location and orientation of the transducer on transparent acetate paper.

Group 2

The methods for assessing maximum anatomical CSA of the vastus lateralis muscle have been described in detail elsewhere (Erskine *et al*., 2009). Briefly, the upper leg was scanned using a 0.2-T magnetic resonance imaging scanner (G-Scan, EsaoteBiomedica, Genoa, Italy), adhering

to a Turbo 3D T1-weighted sequence with the following scanning parameters: time of repetition 40 ms; time to echo 16 ms; matrix 256 x 256; field of view 180 mm x 180 mm; slice thickness 2.8 mm; interslice gap 0 mm. Contiguous axial slices were taken from the tibiofemoral joint to the iliac crest perpendicular to the femur, with the participant in the supine position. From the contiguous slices, the maximal anatomical CSA of the vastus lateralis muscle was identified and manually outlined (Osirix 2.7.5, Osirix Foundation, Geneva, Switzerland). Muscle thickness was quantified by ultrasound with the same approach used for Group 1.

5.2.5 Cross-sectional area of muscle fibers

Twelve-micrometer cryosections were prepared from muscle biopsies and subjected to fiber typing using mouse anti-type II MHC and Alexa Fluor 555-coupled secondary anti-mouse antibody (DAKO) essentially as described (Flueck *et al*., 1999, Gordon *et al*., 2001, Durieux *et al*., 2009, Klossner *et al*., 2009). The fluorescent signal was digitally recorded from different microscopic fields with standardized settings using a TCS SP5 confocal microscope (10 x objective and 4 x zoom; Leica Microsystem CMS, Milton Keynes, UK). Image files were exported and the CSA of stained (type II) and non-stained (type I) fibers in a given microscopic field was quantified using image J 1.6.0_33 J (http://imagej.nih.gov/ij). Numerical values for the same fiber type from the different microscopic fields of a muscle cross-section were pooled to calculate for each muscle biopsy the mean CSA of type II and type I fibers, and the percentage in muscle area covered by type I fibers (Flueck *et al*., 2011). On average, 45 and 84 type I and type II muscle fibers, respectively, were counted per muscle biopsy (subject). The mean coefficient of variance for the estimation of CSA per subject was 4%.

5.2.6 Immunodetection of FAK in sections

Staining of muscle sections for FAK was carried out with fluorescence essentially as described in Klossner et al. (2009). Twelve-micrometer cryosections were freshly prepared from pre- and post-unloading sample of subjects and assembled onto the same slides. Sections were left to dry and fixed with 4% paraformaldehyde/0.1% Triton X-100 for 30 min, quenched with 3% H_2O_2 and blocked in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Subsequently, sections were incubated with a 1:100 dilution of FAK C-terminal antibody (Flueck *et al*., 1999, Gordon *et al*., 2001, Chopard *et al*., 2002, Chopard *et al*., 2005, Anastasi *et al*., 2008) and mouse anti-type I MHC (Durieux *et al*., 2009) in 3% BSA-PBS overnight at 4°C, followed by washes in PBS and incubation with a 1:400 dilution of Alexa Fluor 488-coupled secondary anti-rabbit antibody (DAKO) and Alexa Fluor 555-coupled secondary anti-mouse antibody (DAKO). Nuclei were stained using 5-min incubations with TO-PRO3 (Invitrogen). Fluorescence and digital phase contrasts were recorded at standardized settings (10 x objective and 4 x zoom) using a TCS SP5 confocal microscope (Leica Microsystem CMS, Milton Keynes, UK).

Subsequently, image files were taken from different microscopic fields of a stained muscle section and exported as tiff files. The signal intensity of FAK at the sarcolemma was inspected and quantified using image J 1.6.0_33 J (http://imagej.nih.gov/ij). Fibers were categorized for fiber type and sarcolemmal FAK content (positive or negative). Fibers were classified as positive when staining was identified along at least two edges of the generally squared human muscle fibers, as described elsewhere (Flueck *et al*., 2002, Evans *et al*., 2008). The number of fibers in

each of the four categories (i.e. type I-FAK+, type I-FAK-; type II-FAK+, type II-FAK-) was determined for each muscle from counting of different, non-overlapping microscopic fields. Statistical significance of changes in the frequency of FAK staining with unloading was assessed essentially as previously described (Flueck *et al*., 2003). First, the frequency of FAK staining at the sarcolemma of all combined muscle fibers of a given biopsy was assessed for a difference pre- vs. post-unloading with a Chi^2-test ($p<0.05$). If statistical significance was reached, the percentage of FAK staining at the sarcolemma was calculated for type I and type II muscle fibers, respectively, and each muscle section, and compared for significance of a differences post- vs. pre-unloading with a two-tailed paired t-test. On average 23 and 59 type I and type II muscle fibers, respectively, were counted per muscle biopsy and subject.

5.2.7 Protein detection

Muscle was cryosectioned at 12 µm, and total homogenate was prepared in modified RIPA buffer (1% NP-40, 0.25% deoxycholate, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 2 µg/ml pepstatin, and 1 µg/ml aprotinin; all reagents were received from Sigma). Protein concentration was quantified with bicinchoninic acid assay reagents against BSA standard (Pierce). Twenty micrograms of protein was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Western blotted onto nitrocellulose (Schleicher & Schuell). Gel loading was in a paired design with pre-, mid- and post-intervention samples of three subjects being applied in adjacent lanes per gel. The membrane was stained with Ponceau S to verify equal loading from the intensity of detected protein bands, including the major signal at 40 kDa that corresponds to

skeletal alpha actin. Subsequently membranes were subjected to immunodetection with specific antibodies against slow-type MHC, fast-type MHC, FAK, FRNK, beta-1 integrin, gamma-vinculin, meta-vinculin, p70S6K, essentially as described (Flueck *et al*., 1999, Gordon *et al*., 2001, Durieux *et al*., 2009, Klossner *et al*., 2009). For the detection of fast-type myosin, the immunblot was stripped after signal development for slow-type myosin by incubating for 30 min at 65°C in (62.5 mM Tris, pH 6.8, 1% SDS, 0.7% beta-mercaptoethanol) with occasional shaking followed by extensive rinses in 0.5% Tween-20 in Tris-buffered saline. Content of FAK-pY397 was assessed in FAK-immunoprecipitates from the supernatant of 1 mg total protein as described (Klossner *et al*., 2009). Signal detection was carried out with enhanced chemoluminescence using a Geldoc system that was operated using Quantity One 1-D analysis software 4.6.1 (Bio-Rad Laboratories, Hemel Hempstead, UK). Signal intensity of the protein bands was determined using the rectangle density mode and background from an empty sample lane of equal size was subtracted. Background-corrected data were normalized to the mean values of the pre-samples for the respective gel; the values therefore reflect relative expression levels per total muscle protein.

5.2.8 Statistics

Alterations in 'muscle parameters' (muscle thickness, CSA, FAK, FRNK, FAK-pY397, meta-vinculin, gamma-vinculin, p70S6K, MHCI, MHCIIA) through the 'time course' (pre-, mid-, post-) of the particular 'loading condition' (i.e. unloading and overloading) were assessed with a repeated ANOVA for the repeated factor 'time course' using Statistica 9 (StatsoftInc, Tulsa, USA). A Wilcoxon test was used to localize the effect between time points of muscle

unloading/overloading. Effects were called significant at $p < 0.05$. Pearson correlations were calculated to identify linear relationships that were deemed biologically significant if $r > 0.65$ and $p < 0.10$.

5.3 Results

5.3.1 Changes in muscle thickness and cross-sectional area

Table 5.1A and B, respectively, show the changes in muscle thickness with unloading by bedrest (Group 1) and overloading with eccentric-type of resistance training (Group 2). Muscle unloading produced a progressive reduction in thickness of vastus lateralis muscle (Table 5.1A). This was reflected by atrophy of type I muscle fibers (Table 5.2A). A tendency ($p=0.08$ and 0.06 for changes mid- and post-bedrest, respectively) for a reduction in mean CSA of type II fibers was also observed (Table 5.2A).

Muscle overload increased the thickness and maximal anatomical CSA of vastus lateralis muscle (Table 5.1B). Neither the daily nor sessional percentage change in muscle thickness differed between the first and second intervention phase, either for bedrest ($p=0.58$) or resistance training ($p=0.72$). Mean CSA of type I (+34%), but not type II, muscle fibers was increased with resistance training, and this differed between the first and second phase of training (Table 5.2B). The percentage of type I fibers was 40 and 41%, respectively, before bedrest and resistance training, and this was not affected by the intervention ($P>0.19$). Changes in CSA of type I and type II muscle fibers over all interventions were correlated with those of muscle thickness (Fig.

5.5A). Mid-way into the combined response to unloading or overloading, only the changes in CSA of type I fibers were correlated with those of muscle thickness (Fig. 5.5B).

A)

	Pre	Mid	Post
Muscle thickness (mm) (bed rest)	22.1 ±1.8	21.6 ±1.9*	19.0±1.6*
Muscle thickness (mm) (resistance training)	23.1±1.6	24.7±1.5	24.9 ±1.6*

B)

	Mid vs. pre	Post vs. mid
Delta (%) of muscle thickness (bed rest)	-2.7 ±0.9	-16.8 ±2.4†
Delta (% per session)	-0.34±0.11	-0.49±0.07
Delta(%) of muscle thickness (resistance training)	3.7±2.2	13.6±2.7
Delta (% per session)	0.37±0.22	0.51±0.10

Table 5.1 Opposite changes in muscle size with reduced and increased muscle loading. A) Median and standard error (SE) of muscle thickness (in mm). B) Percentage changes in muscle thickness of vastus lateralis after 8 days (mid) and 34 days (post) of unloading by bed rest and percentage changes in muscle thickness of vastus lateralis after 10 sessions (mid) and 27 sessions

(post) of overloading by resistance type training (n=6). * denote $p<0.05$ vs. pre intervention levels (Wilcoxon-test). †, significant effect of' 'time'(repeated ANOVA).

A)

	Pre	Mid	Post
Type I mean CSA (µm²) (bed rest)	6113±734	4515±633*	4244±884*
Type II mean CSA(µm²) (bed rest)	7245±1256	5898±1049+	5663±1397+
Type I mean CSA (µm²) (resistance training)	4530±1151	4690±1200+	6058±1095*
Type II mean CSA(µm²) (resistance training)	6135±1337	6353±798	6863±744

B)

	Mid vs. pre	Post vs. mid
Delta (%) of Type I mean CSA (bed rest)	-27.1± 2.0	-29.5± 8.7
Delta (%) of Type II mean CSA (bed rest)	-10.0±7.9	-14.8±7.5
Delta (%) of Type I mean CSA (resistance training)	8.5±2.5	16.1±13.1†
Delta (%) of Type II mean CSA (resistance training)	3.6±8.7	11.9±11.1†

Table 5.2 Fibre cross sectional area with altered muscle loading. A) Median and standard error (SE) of mean CSA of type I and type II muscle fibres in vastus lateralis. B) Percentage changes in mean CSA of type I and type II fibres in vastus lateralis after 8 days (mid) and 34 days (post) of bed rest (n=4) and 10 sessions (mid) and 27 sessions (post) into resistance type training (n=5). *and + denote $p<0.05$ and $0.05\leq p<0.10$ vs. pre intervention levels (Wilcoxon- test). †, significant effect of 'time'(repeated ANOVA).

5.3.2 Molecular factors of muscle size regulation during bed rest and resistance training

Molecular parameters of muscle size regulation (p70S6K, MHCI, MHCIIA) demonstrated an interaction effect between the 'time point' x 'loading condition' ($p=0.04$). Bed rest did not modify the content of MHCI or MHCII, or p70S6K (Fig. 5.1A,B,E). The content of the slow MHC, MHC I, increased at the mid-point of resistance training, and p70S6K protein was reduced in the end- vs. the mid-point of training (Fig. 5.1C,D,F).

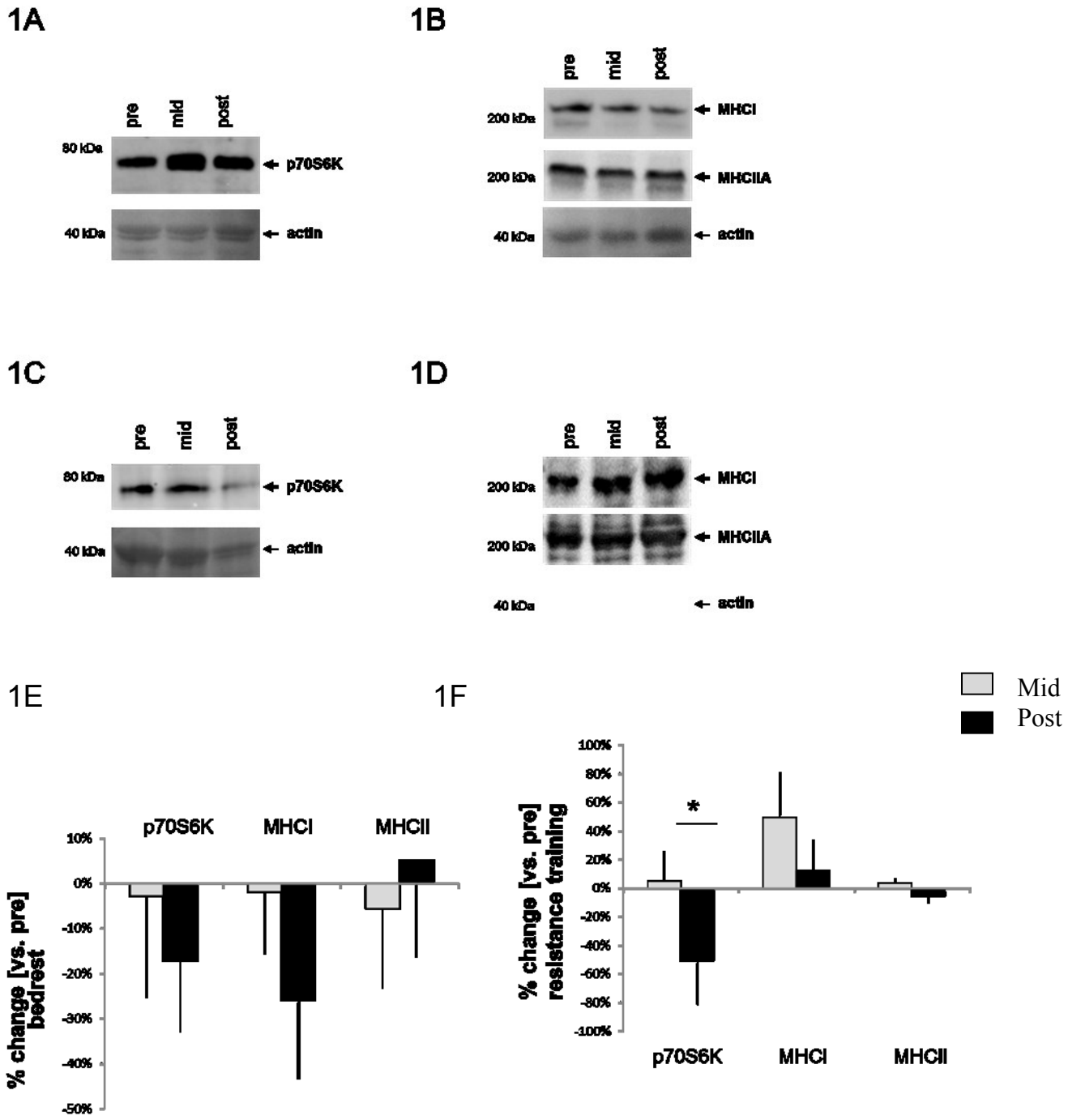


Figure 5.1 Level alteration of factors related to muscle size with modified muscle loading.

A-D) Representative detection of p70S6K (A,C) and MHCI and MHCII (B,D) in western blots

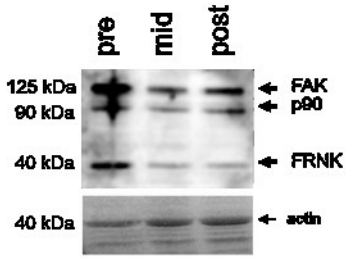
of 20 microgram total protein in muscle homogenate during the course of unloading (A,B) or overload (C,D). E, F) Median and SE of changes in p70S6K, MHCI and MHCII protein levels per total protein during the time course of bedrest (B; n=9) and resistance training (D; n=6). * and + denote $p < 0.05$ and $0.05 \leq p < 0.10$ for the indicated comparison (Wilcoxon-test).

5.3.3 Costameric protein expression with altered muscle loading

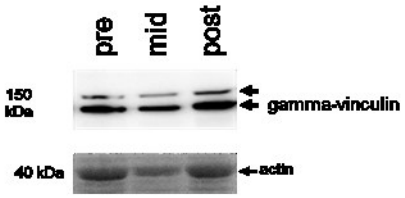
There was an interaction effect between the 'time point' x 'loading condition' for the two parameters associated with costameres, FAK-pY397 and meta-vinculin. Levels of both parameters were not altered in response to bedrest (Fig. 5.2). With muscle overload a biphasic response of both, FAK-pY397 and meta-vinculin, and gamma-vinculin content was evident; all increasing between the mid- and end-point of resistance training (Fig. 5.3B,C,E,F). Changes in meta-vinculin and FAK-pY397 content were correlated over all sampled data points (r=0.80), and this was pronounced when only changes at the end of the intervention were compared (r=0.85). Muscle overload also increased beta 1 integrin mid- but not at the end of resistance training (Fig. 5.3D,F).

Levels of FAK and its inhibitor, FRNK, per total protein were not altered by muscle overload, but the content of both was reduced at the mid- and end-point of the unloading protocol (Fig. 5.2A,E). Microscopic examination visualized staining of FAK at the sarcolemma and locations in the sarcoplasma (Fig. 5.4). Sarcolemmal FAK immunoreactivity was significantly reduced after bedrest in type II muscle fibers (Fig. 5.4D).

2A


pre
mid
post
125 kDa
90 kDa
40 kDa
40 kDa
FAK
p90
FRNK
actin

2C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mid
post
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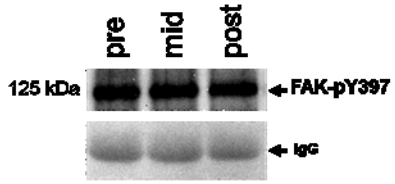
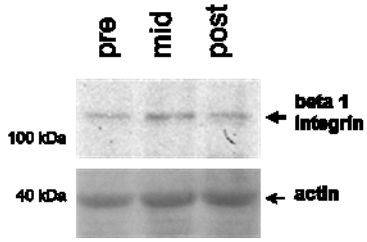
40 kDa
gamma-vinculin
actin

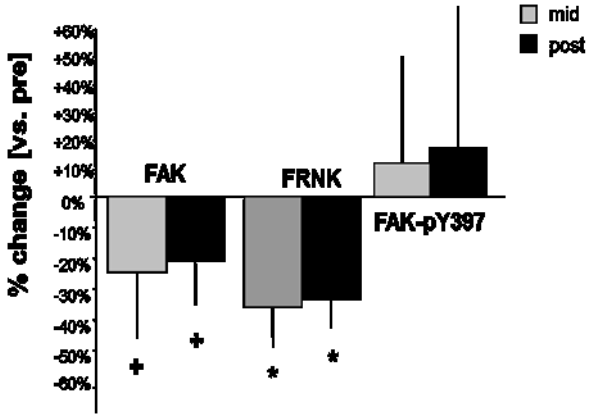
2B


pre
mid
post
125 kDa
FAK-pY397
IgG

2D


pre
mid
post
100 kDa
40 kDa
beta 1 integrin
actin

2E


mid
post
% change [vs. pre]
+60%
+50%
+40%
+30%
+20%
+10%
0%
-10%
-20%
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-40%
-50%
-60%
FAK
FRNK
FAK-pY397
+
+
*
*

2F

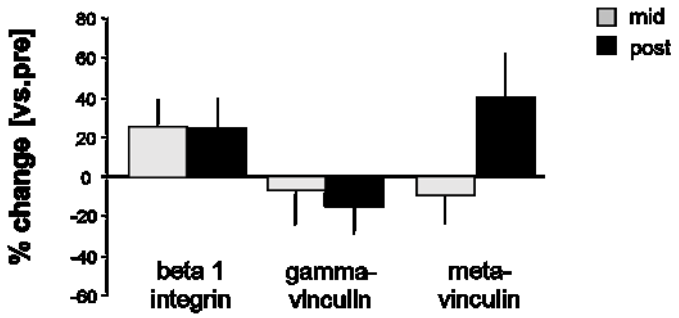
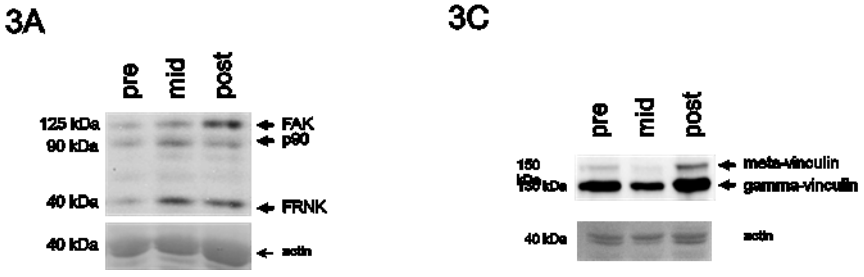
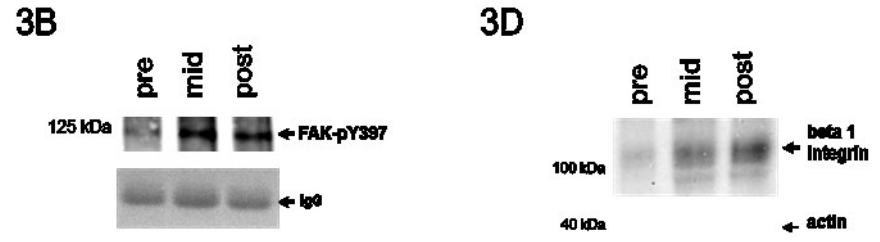
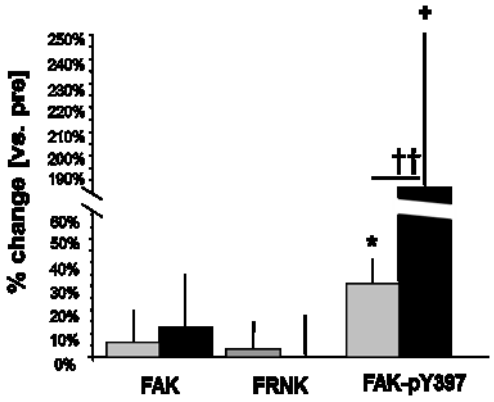


Figure 5.2 Alterations in FAK in human vastuslateralis muscle with unloading. A,D) Representative detection of FAK and FRNK (A), vinculin isoforms (C) and beta 1 integrin (D) in western blots of 20 microgram total protein, and pY397 phosphorylated FAK in immunoprecipitates from 1 milligram soluble protein (B) of *vastus lateralis* muscle prior, mid and post bedrest. At the bottom of each panel the respective loading control (i.e. sarcomeric actin or IgG on the Ponceau S stained membrane) is shown. E, F) Median and SE of percentage changes in the content of FAK-related regulatory (E) and structural costamere proteins (F) per muscle protein during the time course of bedrest (n=9). + and * denote $0.05 \leq p < 0.10$ and $p < 0.05$ vs. pre intervention levels (Wilcoxon-test).





3E



3F

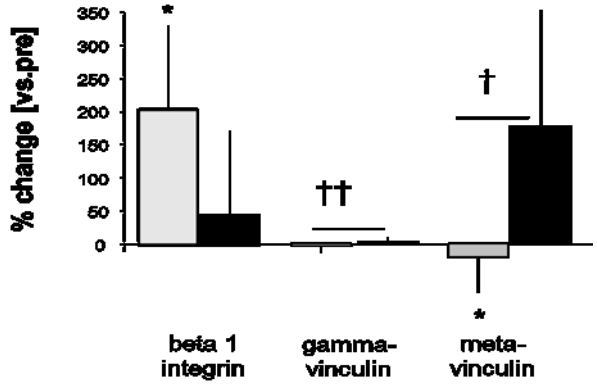


Figure 5.3 Alterations in FAK in vastuslateralis muscle with increased loading. A-D) Representative detection of FAK and FRNK (A), vinculin isoforms (C) and beta 1 integrin (D), and FAK-pY397 (B) in *vastus lateralis* muscle prior, mid and resistance training. Respective

loading controls are shown at the bottom of each panel. E, F) Median and SE of changes in the content of FAK-related regulatory (E) and structural costamere proteins (F) per muscle protein during the time course of resistance training (n=6). + and * denote $0.05 \leq p < 0.10$ and $p < 0.05$ vs. pre intervention levels (Wilcoxon-test). † and †† denote $0.05 \leq p < 0.10$ and $p < 0.05$, respectively, between fold changes mid vs. post the intervention (Wilcoxon-test).

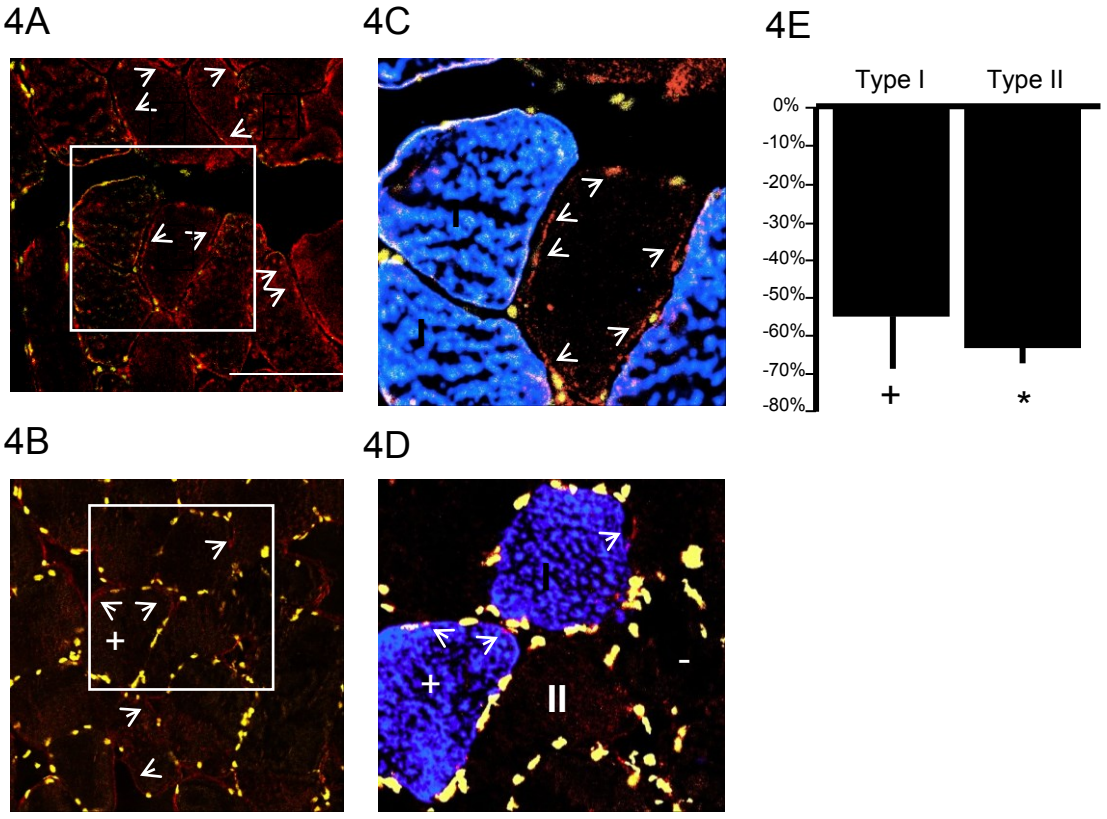


Figure 5.4 Localization of FAK to the sarcolemma.

A, B) Example of a FAK-stained (red) section of *vastus lateralis* muscle in a subject before (A) and after (B) bedrest.Bar, indicates 100 micrometer. C, D) Enlarged images visualizing FAK signal(red) and type I myosin heavy chain-stained muscle fibers (blue) pre (C) and post unloading in *vastus lateralis* muscle of the same subject (D). The frames in panel A and B indicate the region enlarged in panel C and D, respectively. I, and II indicate examples of type I

and type II muscle fibers. + and - denote examples of FAK-positive and negativemuscle fibers.Examples of FAK-positive staining are indicated with white arrows.Nuclei appear in yellow. E) Bar graph showing median + SE of unloading-induced changes in the percentage of type I, and type II, muscle fibers showing FAK-immuno-reactivity. 1030 fibers from 4 subjects were counted. * denotes $p < 0.05$ post vs. pre bedrest levels (paired T-test).

5.3.4 Associations between muscular changes with altered loading

A number of linear relationships were identified between alterations in costamere protein content and muscle structure in the mid-point of atrophy and hypertrophy (Fig. 5.5B). This concerned correlations between percentage changes in beta 1 integrin content and muscle thickness ($r=0.76$). Similarly, changes in FAK-pY397, meta-vinculin and b1 integrin content were correlated to changes in CSA of type I fibers after resistance training ($r>0.65$).The changes in percentage of muscle area covered by type I fibers were positively correlated to changes in the costamere components gamma-vinculin, b1 integrin and type IIA MHC mid-way into altered loading, i.e. $r=0.66$, 0.65 and 0.96, respectively. Conversely, changes in FAK-pY397 content mid-way into altered loading were negatively correlated to those of MHCI content ($r=-0.67$).

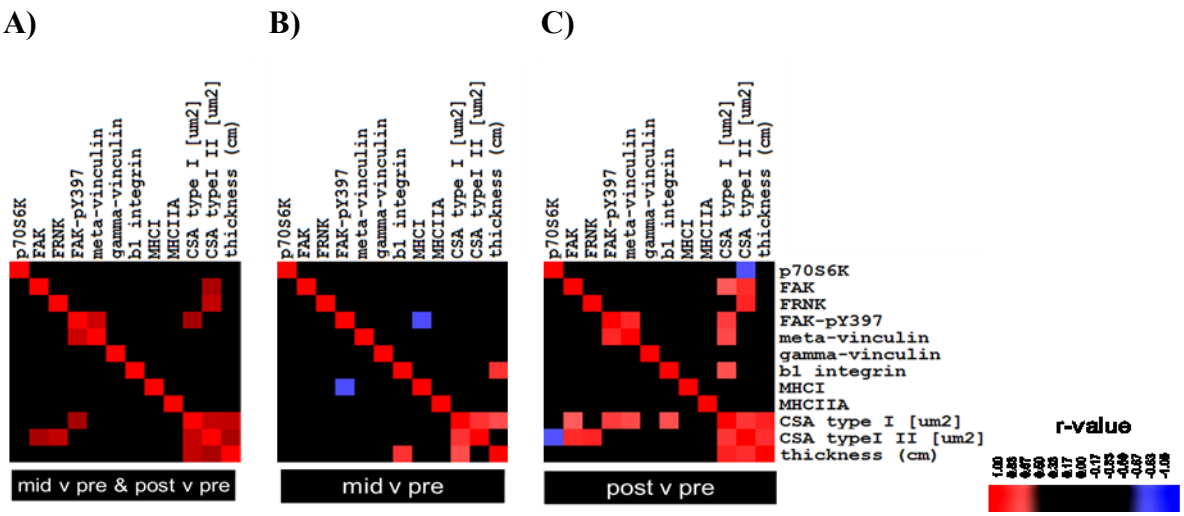


Figure 5.5 Inter-relationship between muscular changes with altered loading.

A-C) Correlation matrix of changes in assessed parametersin a given phase, i.e. 'mid vs. pre' (A), 'post vs. pre' and 'mid vs. post' combined (B) and 'post vs. pre' (C) for the combined interventions. R-values are given in color-coding with those below 0.65 being blinded.

5.4 Discussion

5.4.1 An early and preferential hypertrophy of type I fibres was detected following resistance-training

Skeletal muscle mass is highly sensitive to alterations in mechanical loading (Flueck and Goldspink 2010). A number of signaling factors/pathways, i.e. IGF-AKT-mTOR-p70S6K, FOXO, NF-KB, are implicated in the control of muscle anabolism and catabolism (Wilkinson *et al*., 2008, McCarthy and Esser 2010, Schiaffino and Mammucari 2011), whereby p70S6K is a

robust predictor for skeletal muscle growth (Baar and Esser 1999, Terzis *et al*., 2008). The anatomical basis for load-dependent signal regulation of muscle hypertrophy and atrophy is currently poorly understood despite the widespread view that this issue is considered of great importance (Adams *et al*., 2003, Chopard *et al*., 2009). Costameres, which reinforce the muscle fiber – extracellular matrix composite (Gullberg *et al*., 1998, Huijing 1999), are prime candidates for the organization of muscle signaling as they represent sites where the mechanical stress of force transmission is integrated into signaling (Ervasti 2003, Grounds *et al*., 2005). In order to address the role of costameres in the regulation of muscle size by mechanical loading, we assessed the association of level changes in the costamere's components and its upstream regulator, FAK, with changes in muscle thickness and CSA following bedrest and resistance training.

The present findings imply that the response to our resistance training paradigm involves an early and preferential hypertrophy of type I fibers. This is indicated by the tendency for an increase in the CSA of type I muscle fibers after 10 training sessions (3 weeks) concomitantly with the selective increase in content of MHCI at this mid-point of resistance training (Fig. 5.1F; Table 5.2B). With completion of the second phase of training, when training-induced increases in CSA of type II fibers were larger than in the first phase of training, changes in MHCI protein content fell to pre-training levels (compare Fig. 5.1F with Table 5.2B). Myofibrils form the larger part of muscle material (Luthi *et al*., 1986). It follows that the absence of significant changes in MHCI content per total protein in the second phase of hypertrophy with resistance-type training reflects an increased incorporation of slow-type myofibrils being offset by elevations in CSA of type II myofibrils. This view is in line with slightly higher values of correlation between changes in muscle thickness and CSA of type I fibers compared with those

of type II fibers. It remains to be assessed to what extent this difference in type I and type II fiber hypertrophy reflects the earlier time point of our study compared with other investigations (Williamson *et al*., 2001, West *et al*., 2010) and/or the contribution of slow concentric- and eccentric-type contractions in the employed training paradigm (Seynnes *et al*., 2007).

5.4.2 FAK protein level changes were found to differ between bed rest and resistance-type training

Similar to our expectations, changes in the costamere regulator, FAK (Quach and Rando 2006), differed between bed rest and resistance-type training (Figs. 5.2E and 5.3E). The down-regulation of FAK protein levels with unloading, which was reflected by reduced FAK C-terminal immunoreactivity at the sarcolemma (Fig. 5.4D), reproduces our previous observations of load-regulated FAK expression and recruitment-related FAK staining at the sarcolemma (Gordon *et al*., 2001, Flueck *et al*., 2002, de Boer *et al*., 2007b, Evans *et al*., 2008). It is inconsistent with previous findings regarding the non-significant reduction in total FAK content with 10 days of unilateral limb suspension (de Boer *et al*., 2007b). Our findings in Chapter 4 have also shown a reduction in FAK protein expression following 21 days of ULLS. Interestingly changes in the proxy of focal adhesion turnover in culture, i.e. FAK-pY397 content (Dumbauld *et al*., 2010), over all data points and at the end of resistance training correlated with changes in metavinculin (r=0.80; 0.85), and both parameters demonstrated an increase with resistance training (Fig. 5.3E,F). These findings are an indication of the implication of modified fiber adhesion in muscle remodeling with altered loading in men.

5.4.3 Concentration changes of costameric protein per total protein are inversely related to the changes in fiber radius

Because myofibrillar mass largely contributes to total protein that serves as a reference for the estimation of total protein content, the increase in myofibrils with hypertrophy may camouflage (or dilute) any effect of resistance training on the amount of FAK. In this regard it is helpful to consider the geometric effects of atrophy and hypertrophy on the concentration of costameric proteins. As costameres reside at the fiber surface they scale to the fiber radius (i.e. $2\pi r$). By contrast, fiber volume that is the reference for total protein content is related to the square of the radius (i.e. πr^2). As a consequence, changes in fiber radius are expected to result in concentration changes of costameric protein per total protein that are inversely related to the change in fiber radius. Assuming that the expression of costamere components is not affected, the latter relationship is expected to result in negative correlations between individual changes in costamere proteins and in muscle (fiber) volume. This is in agreement with our finding of a close correlation ($r=-0.86$) between changes in vastus lateralis thickness and meta-vinculin content mid-way into bedrest. As a consequence, the absence of a significant increase in the concentration of structural components of costameres with bedrest may indicate that their expression is affected, yet this does not manifest at the level of content vs. total protein. Conversely, hypertrophy induced by resistance training could have been expected to result in a reduction of the concentration of costameric proteins. However, we detected an increase, which further emphasizes that expressional adjustments of costameres are load-dependent.

5.4.4 Costamere components and muscle anatomy

The observed regulation of costamere components is of interest when put into the context of time course of changes in muscle (fiber) CSA with reduced and increased muscle loading. This comparison demonstrates that adjustments in the level of costamere components differ between the mid- and end-point of resistance training, and are matched to changes in muscle anatomy. The observed changes in costamere component content and micro- and macroscopic estimates of hypertrophy were specifically inter-related during the first phase of resistance training. For instance, the changes in metavinculin, b1 integrin and FAK-pY397 content were correlated to changes in mean CSA of type I fibres with resistance training ($r>0.65$). We also noticed correlations between the changes in the costamere component metavinculin and changes in muscle thickness mid-way into resistance training ($r=0.79$) and FAK with CSA post resistance training (data not shown). In addition, beta 1 integrin and muscle thickness were correlated up to the mid-phase, but not the end, of the combined response to overload and unloading (r-value of 0.76 vs. 0.09). Taken together, the observations suggest that a possible structural reinforcement of costameres in the late phase of muscle hypertrophy contributes to the attachment of newly synthesized myofibrils after the integrin anchor is laid down, which is in line with the reported dependence of fiber growth in culture on costamere-mediated attachment of nascent myofibrils (Quach and Rando 2006, Konieczny *et al*., 2008).

5.4.5 A decrease in the level of p70s6k was detected in the second phase of training

The altered modality of muscle size regulation at the two time points of resistance training is supported by the reduced level of the serine/threonine kinase p70s6k in the second phase of training (Fig. 5.1C,D). It has been shown that post-translational activation of p70s6k is enhanced

after different types of training in both the trained and untrained state (Wilkinson *et al*., 2008) and is positively correlated with the degree of hypertrophy with resistance training (Terzis *et al*., 2008). Surprisingly, changes in p70s6k content correlated negatively with changes in CSA of type II fibres after altered loading (r=-0.68; Fig. 5.5), and changes in muscle thickness after resistance training (r=-0.78) and after bed rest (r=-0.68). To our understanding it is currently unknown whether signaling is maintained through the prolonged response to altered muscle loading. Our data now suggest that expressional regulation of p70s6k may contribute to muscle protein accretion during resistance training in men (Coffey *et al*., 2006).

5.4.6 Changes in content of costamere components in antigravity muscle can be regarded as markers for load-dependent muscle remodeling

It has been previously suggested that maintenance of nitric oxide signaling mechanisms and changes in protein turnover can be regarded as biomarkers for human skeletal muscle atrophy with long-term bedrest (Salanova *et al*., 2008). We show here that changes in costamere-associated muscle parameters, meta-vinculin, FAK-pY397 and MHCI, serve as indicators of muscle remodeling with altered loading in vastus lateralis muscle. This finding relates to the fiber type-specific localization of FAK and meta-vinculin to the sarcolemma (Flueck *et al*., 2002, Thoss *et al*., 2013). The observation is of interest, as one isoform of NO synthase as well is associated with costameres (Baum *et al*., 2000). Thus, our findings reinforce the notion that costamere components can serve as markers of load-dependent muscle remodeling.

5.4.7 Limitations

A limitation of our study was that we did not quantify the localization of FAK at the sarcolemma as a function of muscle overload. This was due to an insufficient amount of high- quality bioptic sample to perform this characterization *post-hoc*. Also, we acknowledge that we did not assess an extensive list of costamere proteins for level alterations with increased and reduced muscle loading. Despite these constraints, we believe our findings provide novel information on the load-regulated content of structural (i.e. beta 1 integrin, vinculin and meta-vinculin) and regulatory factors of costameres in human subjects in relation to changes of muscle micro- and macro-structure.

5.5 Conclusion

Our observations point to a novel role for the organization of fiber adhesion in the mechano-regulation of muscle size in men. Notably, two phases of costamere remodeling with resistance training can be distinguished by the expression of the integrin anchor and vinculin isoforms, and CSA of muscle fibers, but not at the macroscopic level by muscle thickness.

6. Summaries, discussions and future directions

6.1 The effect of short-term unloading on protein expression patterns in two phenotypically distinct human leg muscles, *m.vastus lateralis* and *m.soleus*

6.1.1 Summary of findings

The study detailed in Chapter 3 was set out to explore potential early molecular changes at the protein level after only 3 days of reduced muscle loading. In line with our expectation, no significant change in MCSA of fibres was observed at such an early stage of unloading. Findings have demonstrated for the first time that elements of sarcolemmal focal adhesion complexes, i.e. costameres, are targets of early muscle fibre adaptation to reduced load-bearing, with indication for structural modification happening at just 3 days of ULLS. We found a tendency toward increased metavinculin content in soleus following unloading, which corresponds to the de novo expression of this protein in rat soleus after hindlimb suspension, as originally reported by Chopard and colleagues (Chopard *et al*., 2002). The group later detected the same adjustment in a long-term bed rest study (Chopard *et al*., 2005), where selective high protein content of metavinculin in a slow type muscle, soleus, rather than in a mixed type muscle, vastus lateralis, has been equally confirmed by my study in Chapter 3. The distinctive overexpression of metavinculin in slow-type muscle at unloading has been suggested as a sign of fibre type transformation in a slow-to-fast phenotype shift (Chopard *et al*., 2002).

Upon detailed inspection of correlations between assessed factors, an overall muscle type dependent deregulation of protein expression in response to short-term unloading has been identified, before this is detected at the single-protein level. In particular, the present study demonstrated a muscle-specific transition in the correlation pattern of factors that reflect the slow

oxidative phenotype. For instance, there was a positive correlation between the levels of MHCI and mitochondrial proteins (SDHA, UQCRC1, and ATP5A1), and such typical relationship of a slow-oxidative muscle was later lost following unloading.

This experiment is the first to have assessed chymotrypsin-like enzyme activity in human skeletal muscle during disuse situations.Whilst there were no significant alterations of its activity detected following 3 days of unloading in either muscles, our correlation analysis revealed the presence of a linear relationship between fold changes in the activity of chymotrypsin that constitutes a main component of the proteasome and the MCSA of type II fibres in the VL muscle after 3 days of ULLS (r=-0.71). Our finding is compatible with the previously identified upregulation of the mRNAs for the ubiquitin ligases, atrogin-1 and MuRF-1 (which direct the activity of the proteasomal pathway) in the VL compared with the SOL (Gustafsson *et al*., 2010), suggesting an enhanced capacity for proteasomal degradation of myofibrillar protein in VL muscle with unloading.

6.1.2 Future direction

An increased level of metavinculin has been observed in soleus following a short-period of ULLS. Given costamere's inherent structure for force transmission, as targets of early muscle fibre adaptation to reduced load-bearing, costameres might be structurally important for the regulation and initiation of mechano-dependent signalling, It is still very early to ascertain the true sgnificance of such finding since little information has been available to clarify the specific and distinct role of metavinculin as well as other components of costameres in skeletal muscle under atrophic conditions. Amongst the few existing studies, altered expressions of metavinculin

as well as other components of costameres during reduced functional demand have been reported, which suggest that these sarcolemmal focal adhesion proteins are targets of load-dependent muscle remodeling (Ervasti 2003, Chopard *et al*., 2005, Quach and Rando 2006). Further analysis on costameric proteins should serve to provide a better understanding of their functions in skeletal muscle fibre physiology. In particular, research focused on the role of costameres in functional and structural adaptations of skeletal muscle to altered mechanical strain is required. Although, we failed to observe significant change in chymotrypsin activity, interestingly, fold changes of its activity happened to negatively correlate with area content of slow type fibres and levels of two mitochondrial proteins (i.e. ATP5A1, UQCRC1) in VL but not in SOL, such observations could imply that the ubiquitin proteasome system functions to regulate slow-oxidative fibre type in a muscle dependent manner at 3 days of human unilateral limb unloading. The muscle-specific response of early UPS activation is in line with Gustaffsson's work at 3 days of ULLS, where increased mRNA levels of MAFbx and MuRF1 were reported in vastus lateralis but not in soleus (Gustaffsson *et al*., 2010). In fact, UPS-mediated human skeletal muscle proteolysis is also evident from a previous study subjected to another short-term ULLS whereby 2 days of immobilization triggered elevated expressions of MAFbx and MuRF1 (Reich *et al*., 2010). However, according to the literature, the actual role of UPS in human disuse atrophy has not been fully elucidated due to a general lack of its enzymes' protein and activity measures. Future studies should incorporate these assessments.

It is well known that human skeltal mucle exhibits a load-dependent structural and functional plasticity, whereby, typical adjustment of metabolic and contratile compositions of muscle fibres manifests beyond two weeks of weightlless. However, according to the literature, majority of the data were generated over extended periods of muscle unloading as a result the onset of such

myocellular change wth unloading has not been clearly defined. The earliest documented molecular modification to date in humans involves mRNA epression alterations as a result, the quantitative significance of these pre-translational regulation, at early stages of disuse prior to the apparent muscle loss is not known (Chen *et al*., 2007, Gustafsson *et al*., 2010). The study detailed in chapter 3 was set out to explore potential early molecular changes at the protein level after only 3 days of reduced muscle loading. Given the results for Chapter 3, adjustments of assessed factors appear to have taken place earlier than previously reported. However, the outcome has been identified based on correlation analysis, hence future research providing information at the single protein levels on these factors is mmportannt in order to provide direct indications for their involvement. Such research should concentrate on understanding the events of the first few days of human muscle disuse. It was hypothesized that gene expression and signalling events known to control the atrophic process in cell culture and animal models could effectively take place in human skeletal muscle following just a few days of non-weight bearing, an understanding of early alterations in the underlying cellular and molecular basis of muscle adaptations to short-term unloading is pertinent in a way to promote the development of effective countermeasures and rehabilitation methods in situations where musculoskeletal unloading is a component.

The results of our study show typical muscle-specific adaptive response to short-term unloading, Consistent with our observation, another 3 days ULLS study has identified differential protein turnover and pretranslational responses in the two phenotypically distinct muscles, *m.vastus lateralis* and *m.soleus* (Gustafsson *et al*., 2010). The author concluded that regulatory mechanisms, which initiate disuse induced muscle loss, are influenced by phenotype and/or function and he failed in an attempt to identify the underlyig mechanisms responsible for the

muslce specific atrophy in humans. Collectively, our work together with that from Gustaffsson's would hence demonstrate that regulatory machanisms, which initiate the disuse-induced changes, are influenced by phenotype and /or muscle function and could possibly involve multiple signalling pathways during initial stages of unloading. Future studies should aim to identify these signaling pathways. For this purpose, in part, potential transient changes and differential time courses for protein phosphorylation and gene expression should be acknowledged, such that early down-regulation of PI3K-Akt signalling has been reported to return to baseline level within around 72 hours whereas MAFbx and MuRF1 have a more sustained upregulation of over 10 days (de Boer *et al*., 2007b). Therefore, experimental attention will need to focus on assessing the temporal regulation of potential signalling pathways, especially during the initial period of disuse. Aspects like the number of subjects or biopsies should be taken into careful consideration in future experiemntal designs.

6.2 The regulation of anabolic and catabolic processes of human skeletal muscle in response to long-term unweighting

6.2.1 Summary of findings

In chapter 4 we have assessed several markers of muscle protein synthesis and degradation at 21 days of ULLS. Distinct muscle atrophy has been confirmed by mean values of muscle fibre cross sectional area (MCSA), with a 14% decrease in type I fibre MCSA and a 21 % decrease in type II fibres MCSA of *m.vastus lateralis* after ULLS, which is consistent with the general concept

that anti-gravity extensor muscles of the lower-limb experience the greatest atrophy in response to inactivity and unloading (Belavy *et al*., 2009). The reductions in MCSA of both fibre types were fibre-type independent (p=0.297), such observation is supported by an earlier experiment showing the rates of atrophy being unrelated to the proportion of type I muscle fibres (Blottner *et al*., 2006). Similarly another study has reported a preferential atrophy of the slow-oxidative type I fibres in rats, but not in humans. The author suggested that atrophy of fast type II fibres was comparable, if not greater than, that of slow type I fibres (Fitts *et al.,* 2000).

At the molecular level, there was a trend for a decrease in total focal adhesion kinase (FAK) protein, which happens to correspond with the observation of reduced levels of phosphorylated FAK and its protein content after 10 days ULLS intervention in humans accompanying a 50% decrease in the fractional rate of myofibrillar protein synthesis (de Boer *et al*., 2007b). We have also found that the content of FAK was positively correlated with the percentage of type I fibres and the percentage area content of slow fibres post ULLS. These correlations are the first human evidence to support the findings from animal studies of FAK as a load-dependent regulator of the slow-oxidative muscle phenotype. Our data reinforces the notion that FAK is an upstream modulator of a mechano-dependent anabolic pathway (Klossner *et al*., 2009). In fact, Flueck and colleagues have demonstrated from a rat muscle targeted transgenesis model that the downstream effector of the load-dependent FAK signaling in control of muscle mass is S6K (Klossner *et al*., 2009). In chapter 4, the level of p70S6 Kinase did not alter at 21 days of ULLS. Although we haven't measured the phosphorylated state of this protein due to the small size of the biopsies, which prohibited further biochemical molecular analysis, another recent study has reported no significant change with either phosphorylation of p70S6K at thr389 or its total content after 21 days ULLS (De Boer *et al.,* 2007a).

Apart from the anabolic signaling elements assessed, this study is the first to report a trend of increased chymotrypsin-like activity of the 20S proteasome as well as a trend of elevated cathepsin B/L enzyme activities in a disuse model in humans. These obervations are in line with the findings from animal unloading programs (Taillandier *et al*., 1996, Lecker *et al*., 2004, Ferreira *et al*., 2009, Fujino *et al*., 2009). In addition, strong positive correlations were found between the activity levels of cathepsin B/L and chymotrypsin-like enzymes post ULLS. Evidence has accumulated to show that both proteolytic pathways cooperate to degrade specific protein substrates and their activation corresponds with the net reduced proteolytic rate in animal models of disuse. More importantly, two in vivo studies now implicate thta FOXO transcription factors function to increase transcription of components of lysosomal and ubiquitin-proteasomal protein degradation systems (Attaix and Bechet 2007).

6.2.2 Future direction

In Chapter 4, we have observed a tendency for a reduction in total FAK protein content following 21 days of unloading. Currently, the majority of studies conducted were performed on rodents, with just two studies available to have assessed FAK in the reduced loading situations in human skeletal muscle (de Boer *et al*., 2007b, Glover *et al*., 2008). Interestingly, in both studies the only change detected among the rest of measured signaling proteins was that of FAK, where de Boer's group observed a 30% decrease in FAK phosphorylation at Tyr 576/577 and a 20% decrease in its content after 10 days of ULLS and likewise to Glover's group a reduced level of phosphorylated FAK (Tyr 576/577) was detected after 14 days of unilateral knee immobilization. In addition, much of immobilization-induced muscle loss observed in the two studies was the

results of a decline in post-absorptive muscle protein synthesis (de Boer *et al*., 2007b, Glover *et al*., 2008). Collectively, these data closely reflect FAK's role based on cell and animal experiments as a mechano-transducer that induces downstream signaling molecules regulating protein synthesis. Future work will need to examine the temporal regulation of FAK activity under human skeletal muscle atrophic conditions. A better understanding to the mechanisms of FAK induced load-dependent anabolic signaling with particular focus on its regulation and downstream effectors in skeletal muscle are needed. These will help to verify the functional significance of FAK in human disuse muscle atrophy (Sakuma *et al*., 2009, Spangenburg 2009). S6k has been reported to be a downstream effector of the load-dependent FAK signaling in control of muscle mass. Suprisingly, we have not observed a change in S6K levels after 21 days of ULLS. Our observation has coincided with findings from another recent study, where no significant change with either phosphorylation of p70S6K at Thr 389 or its total content also after 21 days of ULLS was observed (de Boer *et al*., 2007b). The critical nature of S6K in muscle was initally demonstrated in the S6K knockout mice whereby the cross-sectional area of the individual muscle fibres was significantly smaller than that of fibres from the wild-type animals (Ohanna *et al*., 2005). In vitro studies have confirmed that S6K functions to promote skeletal muscle growth via controls over protein synthesis for increased translation capacity through phosphorylating downstream targets including S6 (Pende 2006). Furthermore, findings from several altered loading studies have shown that S6K activation is related to load-induced muscle mass increase and its decreased phosphorylation happens to correlate with reduced translation rate (Bodine *et al*., 2001b, Hornberger *et al*., 2001). However, only a few experiments have studied the relevance of animal findings of the functional role of S6K as an upstream

regulator of translation initiation in association with load-dependent skeletal muscle mass remodelling in humans. An accurate answer calls for further research in the human situation. Moreover, discrepancies have been oberved between animal and human data to date, as a result the role of S6K in load-dependent skeletal muscle remodelling remains unclear.

In the future, researchers should consider the need for careful interpretation of the activation of S6K. The importance for such work to be undertaken is reinforced by a study, which has reported an increase in phosphorylated (thr421/ser424) S6K, when phosphorylation of S6 was rather unchanged. Such observations suggest the phosphorylation at thr42 1and ser424 as only the partial phosphorylation of s6k instead of a full phosphorylation to account for the activation of the enzyme (Koopman *et al*., 2006). Therefore, reports so far on increased phosphorylation of S6K are controversial in the way that selected phosphorylation sites might not be necessary or sufficient to indicate true s6k activation. Other poorly understood phospohrylation events contribute to s6k regulation. However, P70s6k phosphorylation in the 389 threonine end has been considered as a marker for increased protein synthesis induced by mechanical stimulation in animals (Baar and Esser 1999). Further interpretation must await new data on variables such as final activation of s6k (specially related to phosphorylation on residues responsible for the full activation of the enzymes) and protein synthesis or indirect markers of protein synthesis (eg. phosphorylation of ribosomal protein S6) to provide detailed information on the importance of s6k's role during human skeletal muscle unloading.

As part to understand the role of s6k in human disuse atrophy, the signaling mechanisms of its regulation need to be defined. Dufner and colleagues have proposed that current known signaling factors including PI3K, PDK1 and mTOR only partially activate p70s6k in vivo. Additional signaling pathway is responsible for full p70s6k activation status (Dufner *et al*., 1999). Further

research should aim to identify those regulatory molecules as well as to verify their relevance in human situations like the aforementioned case in FAK.On the other hand, other signaling systems might appear to compensate to fully rescue proliferation under chronic inactivation of s6k. Therefore it is highly advisable that future investigations should consider all the key components related to potential signaling pathways with consequences on protein synthesis.An effort to further explore alternative candidate pathways controlling human skeletal muscle wasting processes is required (Gustafsson *et al*., 2010).

Furthermore, findings highlight the need to define its signaling activation patterns. Since unobserved change in S6K could simply reflect the time point when the biopsies were taken did not coincide with the transient change of the protein as suggested by the existing temporal relationship between s6k and its upstream regulator FAK through which enhanced FAK signaling triggers a delayed response of p70S6k py397. Differential time courses of activation or differential extents of signaling responses could explain the inconsistent comparison between findings from human subjects versus those observed in studies with rodents. In addition, it is interesting to note that according to one particular human study, the unchanged phosphorylation status of p70S6K observed following resistance training has been regarded as a response of negative feedback of p70S6k being auto-regulated by eIF-4E to reach cellular translational homoeostasis in a way to prevent uncontrolled growth rather than an indication of its inactivation during human skeletal muscle hypertrophy, giving rise to the idea that there might be a negative feedback of a similar sort to prevent uncontrolled muscle loss (Gautsch *et al*., 1998).

It has been well established that a change in muscle mass is the result of an imbalance between protein synthesis and protein degradation. To date, the underlying regulatory mechanisms employed in human skeletal muscle disuse atrophy has not been resolved. The notion that disuse

atrophy of human skeletal muscle is primarily facilitated by compromised protein synthesis has been established for 20 years. However, increased protein breakdown have been indicated by measures of indirect indices of proteolytic activity in animal disuse atrophy studies where signs to suggest lysosomal degradation as well as ubiquitin proteasome proteolysis were observed (Taillander *et al*., 1996, Judge *et al*., 2007). Accumulating evidences have shown that both proteolytic pathways cooperate to degrade specific protein substrates and two in vivo studies now implicate that FOXO transcription factors function to increase transcription of components of the lysosomal and the ubiquitin-proteasome systems (Attaix and Bechet 2007). As a result, one of the aims of Chapter 4 was to measure the activity of these proteolytic systems in human disuse atrophy. We found that there was a tendency for an increased activity of cathepsins B/L, which is the first to be reported in a human disuse model. To date, very few studies have investigated the involvement of the lysosomal system in muscle atrophy and in light of the current findings future research should work to establish the role of lysosomal system in disuse atrophy of human skeletal muscle.

Our results also showed a tendency to increased proteasome activity. In comparison to the wealth of knowledge gained from animal models concerning the function of UPS in load-dependent skeletal muscle remodeling, studies on humans are at a relatively early stage, with majority of the findings based predominantly upon transcriptional events. More specifically, it is the expression of the two E3 ubiquitin ligases, MAFbx and MuRF1, as the major regulators of muscle mass in various atrophic conditions including immobilization that were intensively examined (Murton *et al*., 2008, Foletta *et al*., 2011). In contrast to that of animal data, the distinctive increase in MAFbx and MuRF1 mRNA levels has not been consistently observed in humans (Murton *et al*., 2008). Apart from the question of whether a chosen animal model truly

represents the human situation, the inconsistency in findings could be explained by the fact that the timing of muscle sampling has failed to coincide with transient elevations of the E3 ligases and/or that only transcriptional changes have mainly thus far been examined. Therefore, differential, time -dependent changes at the transient level should be taken into consideration when coming to interpret these data. Above all, there is a major absence of protein measurements. Given the important findings from a couple of muscle wasting studies, where a dissociation between altered muscle proteolysis and genes expression changes of MAFbx and MuRF1 was observed, the presumption that their increased transcript levels represent concomitant rise in muscle proteolysis might prove unwarranted. As a result, the availability of protein content data is especially pertinent. Future study should assess both E3 ligases at the translational levels. Moreover, attention should focus on various other components of the ubiquitin-proteasome system. These approaches could help discern the functional relevance of UPS-mediated degradation to the human condition of muscle disuse atrophy.

To date, the role of muscle protein breakdown in human muscle disuse atrophy remains equivocal. Our data from Chapter 4 revealed a likely involvement of both arms of muscle protein turnover, i.e., suppressed protein synthesis and activated protein breakdown at 21 days after ULLS. Such observation shares the same general view as those proposed in animal unloading models, where the underlying mechanism involves a combination of reduced protein synthesis and increased protein degradation, but it is not in concordance with some documented findings from human studies, where results demonstrate the notion that muscle atrophy is primarily facilitated by compromised protein synthesis. This suggestion has beeen established for 20 years. It was first reported in 1987 from a 7-week human immobilization experiment showing a profound fall in protein synthesis with no rise in protein breakdown, which was later confirmed

through disuse studies during the 14 to28-day time period (Gibson *et al*., 1987, Ferrando *et al*., 1996, Paddon-Jones *et al*., 2006). Recently, de Boer and colleagues have detected a significantly reduced myofibrillar protein synthetic rate of 50% over 10 days with its lesser attenuation by 21 days and a degree of 0.5% muscle atrophy per day over 23 days based on ULLS in young healthy men. The author concluded that considering the large decline in rate of myofibrillar protein synthesis it was unlikely for a rise in muscle protein degradation to take place over the entire period (de boer *et al*., 2007b). On the contrary, there is less substantial evidence to support increased proteolytic activity being involved in disuse induced human muscle loss. However, several human studies have also observed upregulation of E3 ligases expressions, a component of UPS, in different periods of disuse, which is in line with the data from animal unloading programs (Taillandier *et al*., 1996, Lecker *et al*., 2004, Ferreira *et al*., 2009, Fujino *et al*., 2009). In fact, UPS-mediated human skeletal muscle proteolysis is evident from a previous study subjected to short-term ULLS whereby 2 days of immobilization triggered elevated expressions of MAFbx and MuRF1 (Reich *et al*., 2010). More recently, another human experiment has shown that ULLS triggers increased protein degradation as early as 3 days based on measures of indirect indices of proteolytic activity (Gustafsson *et al*., 2010). Overall, data highlight the pivital importance for detailed human studies to assess protein degradation in vivo. In order to discern the true relevance of protein breakdown in disuse atrophy, it is important for future research to provide more conclusive human data to address protein breakdown directly under muscle disuse atrophic conditions. There are essentially no direct approach to describe proteolysis in skeletal muscle loss since the required accurate and valid methodology to assess protein degradation in vivo in humans have unfortunately not been at hand. Previous report in 1987 discovered unchanged protein breakdown in association with the distinctive muscle atrophy

over an extended period of 7 weeks of unilateral lower limb suspension in humans that was based on data inferred from differences in leg muscle mass and rates of protein synthesis (Gibson *et al*., 1987). Until recently, a lone study has determined the muscle contractile rate by means of a semi-invasive microdialysis technique, which involved measures of intramuscular 3MH content in vivo, allowing for the quantitative determination of proteolysis of the contractile elements actin and myosin in individual muscles. The resulting finding was an elevated interstitial levels of 3MH as a result of increased myofibrillar proteolysis after 3-day human ULLS (Tesch *et al*., 2008).

6.3 Costamere remodeling with muscle loading and unloading in healthy young men

6.3.1 Summary of findings

The regulation of the content of structural and regulatory factors of costameres in human subjects in relation to changes of muscle micro- and macro-structure following bed rest and resistance training were assessed in Chapter 6. An early and preferential hypertrophy of type I fibres have been observed after just 10 sessions of resistance training, whereby a tendency for an elevation in the CSA of type I muscle fibres was indicated together with a selective increase in content of MHC I. The costamere regulator, FAK protein levels in the first phase happened to be down-regulated with unloading, which is in agreement with reduced FAK C-terminal immunoreactivity at the sarcolemma, such findings suggest a reduced capacity for FAK-regulated costamere remodelling during bed rest. Interestingly, adjustments in costamere components differed

between the mid- and end-point of resistance training. The observed regulation of costamere components is of interest when put in the context of time course of changes in muscle(fibre) CSA with reduced ad increased muscle loading. In particular, this comparison demonstrates that two phases of muscle remodelling were involved with resistance training. Thus, changes in costamere protein content and micro- and marcoscopic estimates of hypertrophy were specifically inter-related during the first phase of resistance training. For instance, changes in metavinculin levels and changes in muscle thickness were positively correlated at the mid-point of resistance training (r=0.79). Moreover, beta 1 integrin was correlated with muscle thickness only up to the mid-phase of the combined response to overload and unloading, such differences suggest that the upregulation of costameric proteins is related to a marked hypertrophic response after the 10th vs. 27th session of resistance exercise. An decrease in the level of p70S6k , a marker for muscle protein synthesis, in the second phase of training again support the observation of an altered modality of muscle size regulation at the two time points of resistance training.

6.3.2 Future directions

One of the findings reported in chapter 5 was that following just 10 resistance training sessions, there was an early and preferential hypertrophy of type I fibres. Future studies are needed to assess to what extent this difference in type I and type II fiber hypertrophy reflects the earlier time point of our study compared with other investigations (Williamson *et al*., 2001, West *et al*., 2010) and/or the contribution of slow concentric- and eccentric-type contractions in the employed training paradigm(Seynnes *et al*., 2007).

A number of signalling factors have been implicated in load-dependent skeletal muscle remodelling, amongst which p70S6K is a major marker for anabolic signaling in skeletal muscle. Our findings suggest that expressional regulation of p70S6K may contribute to muscle protein accretion during resistance training in men at an earlier time point. However, surprisingly, in contrast to the idea that p70S6K should be positively related to the degree of hypertrophy our data showed that changes in p70S6K content correlated negatively with changes in CSA of type II fibers after altered loading (r=-0.68) (post vs pre), and changes in muscle thickness after resistance training (r=-0.78) and after bed rest (r=-0.68). To our understanding, it is currently unknown whether S6K signalling is maintained through the prolonged response to altered muscle loading. Data from animal studies have suggested that the activation of S6K is related to gains in muscle mass induced by mechanical stimuli (Baar and Esser 1999, Nader and Esser 2001, Burry *et al*., 2007), and its low levels of phosphorylation are correlated with reduced translation rates (Bodine *et al*., 2001b, Hornberger *et al*., 2001). But, discrepancies exist between the observations made from animal studies and the few available human studies to date, such that one study has reported no significant change with either phosphorylation of p70S6K at thr389 or its total content in human skeletal muscle after 21 days ULLS (de Boer *et al.,* 2007b). Overall, findings highlight the need to define S6K's signalling activation patterns. Therefore, future research should aim to elucidate the time course of S6k activation during altered loading conditions in humans with reference to changes in muscle size. Since such inconsistent comparison between findings from human subjects versus those observed in studies with rodents could simply reflect the time point when the biopsies were taken did not coincide with the transient change of the protein.

In the present study, we observed a reduced FAK c-terminal immunoreactivity at the sarcolemma with unloading. This observation is in line with the recent data from a human study where protein content and phosphorylation status of FAK were reported to decrease with reduced loading (de Boer *et al*., 2007b). Load-regulated FAK expression has been demonstrated in various animal-based studies but generally not in humans due to very little human research being carried out to date (Gordon *et al*., 2001, Flueck *et al*., 2002., de Boer *et al*., 2007b, Evans *et al*., 2008). The level and phosphorylation of FAK have been reported to increase during load-induced skeletal muscle hypertrophy, which is associated with increased myofibrillar content in vivo and in vitro animal studies (Flueck *et al*., 1999, Carson and Wei 2000). However, we failed to detect an increase in FAK content after resistance training. We believe such finding could demonstrate the fact that the increase in myofibrils with hypertrophy in response to resistance training can have a dilution effect on the actual elevation for FAK since myofibrilar mass count for a major/main proportion of total protein within a muscle tissue. Unfortunately, we were unable to quantify the localization of FAK at the sarcolemma as a function of muscle overload because of an insufficient amount of high-quality bioptic sample to perform this characterization. In this regard, future research should aim to assess the localisation of FAK at the sarcolemma in humans during unladoing as well as reloading conditions.

The project has assessed level changes of a couple of costamere components in response to altered loading in humans. The results of which suggest that costamere components can serve as markers of load-dependent muscle remodelling and such notion can be supported by several lines of evidence generally from animal studies indicating that mechanical tension is critical in regulating costameric protein expression, stability and organisation. Costamere constituents talin and vinculin were shown to be upregulated in reponse to muscle contraction (Tidball *et al*.,

1999). In addition, it has been reported that the normal transverse banding pattern of several costameric proteins (dystrophin, syntrophin and dystroglycan) was disrupted in skeletal muscle following 3 days of denervation, but was restored when the muscles were electrically stimulated (Bezakova and Lomo 2001). Moreover, the expressions of metavinculin and vinculin, two major components of costameres changed greatly under conditions of reduced functional demand in rat skeletal muscle (Chopard *et al*., 2002). Furthermore, dystrophin, vinculin and aciculin contents in skeletal muscles were found to increase after 7 and 21 days denervation and after 6 days spaceflight (Rezvani *et al*., 1996). One of the limitations of the current study is that we have only measured few of costamere proteins amongst the other proteins found in costamere include talin and alpha-actinin. In fact, over the past 20 years, an extensive list of costamric proteins has been documented (Ervasti 2003). Future research should explore the effects of unloading and reloading on a range of costamere proreins for level alterations in humans.

Interestingly, our study has shown that the adjustments in the level of assessed costamere components (metavinculin, gamma-vinculin and beta1 integrin) happened to differ between the mid – and end-point of resistance training. By contrast, costamere protein level alterations did not change between the two time points of bed rest. To date the temporal response of costamere proteins to altered loading conditions has not been considered in humans. Whereas, data from animal models of disuse suggest that the changes in the relative contents of costamere components differ according to the duration of unloading. The relative vinculin content was observed to increase after 3 weeks of hindlimb suspension in rat skeletal muscles and more after 6 weeks, which was in agreement with results for the dystrophin glycoprotein complex from another study (Chopard *et al*., 2001). On the other hand, the relative metavinculin content has been shown to decrease by 20% after 3 weeks unloading but returned to control levels 3 weeks

later (Chopard *et al*., 2002). Given these observations, future research should aim to assess the temporal response of costameric components under altered loading conditions in humans.

Our study is the first to identify a function for FAK-mediated costamere remodeling in the load-dependent regulation of muscle size in men through the association of level changes in costameres components with changes in muscle structure and molecular parameters of muscle size regulation following bed rest and resistance training. The observed regulation of costamere components is of interest when put in the context of time course of changes in muscle (fiber) CSA with reduced and increased muscle loading. Findings are in support for an altered modality of muscle size regulation at the two time points of resistance training. These findings could suggest a possible structural reinforcement of costameres in the late phase of muscle hypertrophy. Future experimentation should aim to better understand the regulation and function of FAK-mediated costamere turnover in load-dependent skeletal muscle plasticity.

7. References

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8. Appendix 1 Copy of peer reviewed journal article including data from chapter 5 (Li *et al*., 2013).

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