

**INTERACTION OF LOADING AND FEEDING ON SKELETAL MUSCLE
ANABOLIC SIGNALING AND PROTEIN TURNOVER IN HUMANS**

INTERACTION OF LOADING AND FEEDING ON SKELETAL MUSCLE
ANABOLIC SIGNALING AND PROTEIN TURNOVER IN HUMANS

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TITLE: Interaction of Loading and Feeding on Skeletal Muscle Anabolic Signaling and Protein Turnover in Humans

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Abstract

Resistance exercise and amino acids independently and synergistically stimulate muscle protein synthesis. Unloading of skeletal muscle depresses fasted state muscle protein synthesis, but the effect on the fed state response is unknown. Elucidation of the signaling pathways underlying the regulation of these processes in humans is in its infancy.

Therefore, the purpose of this thesis was to determine how resistance exercise, feeding, and unloading interact to affect muscle protein turnover and its markers. In study 1 young men (N=9) underwent an acute bout of unilateral leg resistance exercise with or without feeding, with biopsies 6 h post exercise. Exercise dephosphorylated eIF2B ϵ and together with feeding potentiated the increase in phosphorylation of p70s6k and rps6. In study 2, 12 young people received primed constant infusions of $^{13}\text{C}_6$ -Phe in the fasted state and at one of two i.v. AA infusion rates (low, 42.5 mg/kg/h AA; high: 261 mg/kg/h AA) after 14 d of knee-brace mediated immobilization. Immobilization decreased fasted and fed state myofibrillar protein synthesis at both doses without obviously affecting translational signaling proteins. In study 3, two markers of muscle protein breakdown and oxidative damage were measured in 21 subjects (men, N=13, women, N=8) after 2 d and 14 d of knee-brace mediated immobilization. Protein ubiquitination was elevated after 2 d of immobilization but there was no sustained elevation in ubiquitination at 14 d or increases in the 14kDa actin fragment or protein carbonyls and 4-hydroxy-2-nonenal. These studies support the concept that the responses of human muscle to changes in loading are primarily at the level of protein synthesis, and the p70 pathway appears to play a role in mediating the hypertrophic response. The currently known static markers of translational

signaling and protein breakdown, however, are not very informative when attempting to account for an underlying molecular mechanism for disuse atrophy.

To the memory of my mother, Pirkko-Liisa, whose never ceasing encouragement got me to where I am today, and my niece, Ella Lise, whose adventure is just beginning

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Format and Organization of the Thesis

This thesis was prepared in the “sandwich format” according to the School of Graduate Studies’ guide for the Preparation of the Thesis. This thesis is comprised of a general introduction, three original research papers (Chapters 2-4), a general discussion and an appendix containing additional microarray findings from Study 3 that are not included in the manuscript. Studies 1 and 2 are published while Study 3 is in review with the candidate as first author.

Contribution to papers with multiple authorship

Publication

Glover, E.I., Oates, B.R., Tang, J.E., Moore, D.R., Tarnopolsky, M.A. & Phillips, S.M.

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E.I. Glover recruited subjects, coordinated the study and conducted the trials with assistance from the co-authors. The supervisor for this study was S.M. Phillips. Muscle biopsies were obtained by S.M. Phillips. B.R. Oates assisted with study trials (exercise, muscle and blood collection), western blot analysis and manuscript revision. J.E. Tang assisted with study trials (exercise, muscle and blood collection), western blot analysis and manuscript revision. D.R. Moore assisted with study trials and manuscript revision. T. Rerecich performed blood glucose, amino acid and insulin analyses. M.A. Tarnopolsky assisted in preparation and editing of the manuscript. Statistical analyses and manuscript preparation were completed by E.I. Glover.

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E.I. Glover recruited subjects, coordinated the study and conducted the trials with assistance from the co-authors. E.I. Glover processed samples, performed Western

blotting analysis, and GC-MS analysis of intracellular amino acid enrichments. The supervisor for this study was S.M. Phillips. Muscle biopsies were obtained by S.M. Phillips. B.R. Oates assisted with subject management, study trials (muscle and blood collection), and manuscript revision. J.E. Tang assisted with study design, trials (muscle and blood collection), GC-MS analysis of intracellular amino acid enrichments and manuscript revision. M.A. Tarnopolsky assisted with study design and manuscript preparation and revision and provided support for Western blot analysis. T. Rerecich performed blood glucose, amino acid and insulin analyses and GC-MS analysis of plasma amino acid enrichments. A. Selby assisted with preparation and IR-MS analysis of intramuscular amino acid enrichments. K. Smith performed IR-MS analysis of muscle amino acid enrichments. M.J. Rennie provided support for study sample preparation and analysis and assisted in the preparation and editing of the manuscript. Statistical analyses and manuscript preparation were completed by E.I. Glover.

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E.I. Glover participated in the study design and coordination, performed the immunoblotting, and assisted with the caspase assay. N. Yasuda participated in the study design and coordination, and carried out the MRI and fibre type analysis. A. Abadi participated in the caspase-3 assay, organized the microarray data (in the appendix) and review of the manuscript. M.A. Tarnopolsky conceived of the study, oversaw its design,

obtained muscle biopsies, provided support for analysis and reviewed the manuscript. S.M. Phillips participated in study design, obtained biopsies and review of the manuscript. Statistical analyses and manuscript preparation were completed by E.I. Glover.

List of abbreviations

3-MH	3-methyl histidine
4E-BP1	eukaryotic initiation factor 4E binding protein 1
4-HNE	4-hydroxy-2-nonenal
AA	amino acids
ACC β	acetyl-CoA-carboxylase β
AIDS	acquired immunodeficiency syndrome
Akt/PKB	Akt/protein kinase B
ALS	amyotrophic lateral sclerosis
AMPK	5'-AMP dependent protein kinase
ATG	autophagy-related protein 5
BCAA	branched chain amino acids
cbl	casitas B-cell lymphoma
CHO	carbohydrate
CSA	cross sectional area
DNPH	2,4-Dinitrophenylhydrazine
EAA	essential amino acids
eEF2	eukaryotic elongation factor 2
eIF	eukaryotic initiation factor
ERK	extracellular regulated kinase
FAK	focal adhesion kinase
FBXO40	F-box protein 40
FOXO	forkhead box, subgroup O
FSR	fractional synthetic rate
G β L	G-protein beta-subunit-like
GSK3 α/β	glycogen synthase kinase α/β
GTP	guanosine triphosphate
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IC	intracellular
IRS 1/2	insulin receptor substrates 1/2
JNK	c-Jun NH ₂ -terminal kinase
L-NAME	N (G)-nitro-L- arginine methyl ester
MAFbx	muscle atrophy F-box
MAPK	mitogen activated protein kinase
MHC	myosin heavy chain
MKB	MAPK specific phosphatases
MPB	muscle protein synthesis
MPS	muscle protein breakdown
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MuRF1	muscle ring finger 1
PDK	phosphatidylinositol-dependent protein kinase 1

PI3K	phosphatidylinositol-3-kinase
PP-2A	protein phosphatase 2A
PSMD11	proteasome 26S subunit non-ATPase
Rheb	ras homologue enriched in brain
ROS	reactive oxygen species
rps6	ribosomal protein s6
RUNX1	runt-related transcription factor 1
SCI	spinal cord injury
SLP1	synaptotagmin-like protein 1
TSC1/2	tuberous sclerosis complex 1/2
ULLS	unilateral lower limb suspension
USP	ubiquitin specific peptidase
Vps	vacuolar protein sorting

Chapter 1: Introduction to the Thesis

Introduction

In humans the mass of skeletal muscle is determined by the balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Figure 1). Diurnally, muscle proteins cycle between states of (loss) negative ($MPB > MPS$) and (gain) positive ($MPS > MPB$) net protein balance in response to the degree and frequency of the anabolic stimulus of feeding (Figure 1). In addition, muscular work can also affect the amplitude and duration of fed-state protein gain.

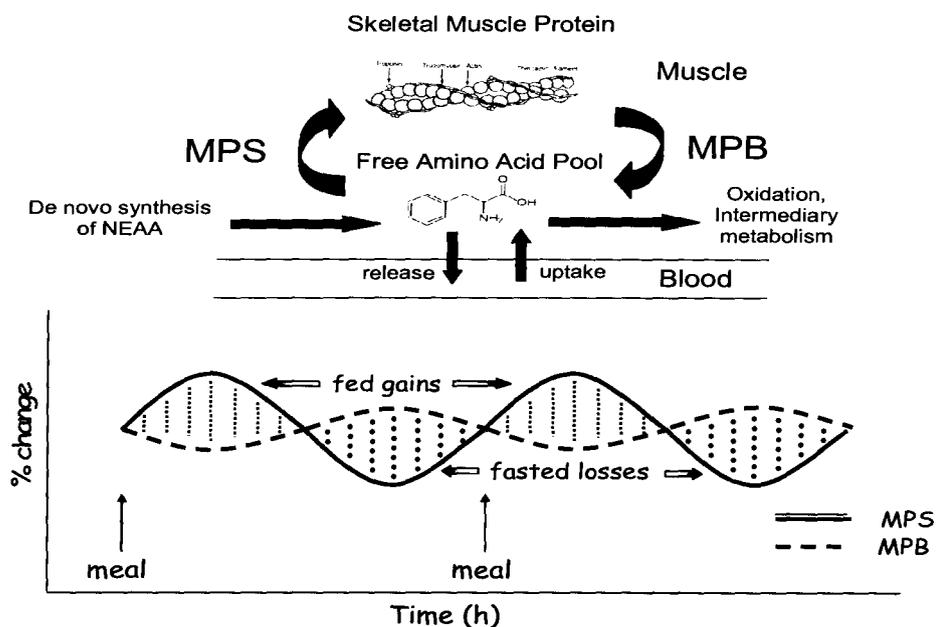


Figure 1. Protein turnover in muscle and diurnal changes in protein balance. Modified from (161; 167).

The impact of these effectors on MPS and MPB has been well documented in humans (167; 172; 175), although the underlying cellular signals that link the stimuli of feeding

and exercise to the responses in MPS and MPB have only recently begun to be mapped out. Until very recently most of these data have come from animal models (10; 28; 150); however, recent advances in human metabolic research have greatly advanced our understanding.

While the acute (167; 172; 175) and long term (training) (167; 172; 175) effects of increased loading (i.e., resistance exercise) have been extensively investigated, less is known about the other end of the muscular activity spectrum. In states of substantially decreased loading such as immobilization and bed rest or other conditions of disuse, loss of muscle mass (atrophy) occurs. Disuse atrophy in humans is evident as early as 7 d after unweighting or immobilization (21; 68; 177), implying that unloading rapidly shifts muscle protein balance in favour of net breakdown. Much data in the disuse atrophy field has been derived from animal and in vitro studies in which proteolysis or its markers are substantially upregulated (66; 104; 165). This has led to the prevailing paradigm in which increased MPB is proposed to be the primary change responsible for driving disuse-induced wasting.

Since the studies reported in this thesis were all carried out in humans the review of literature focused on data obtained from studies conducted in humans, characterising the acute protein turnover and signaling/proteolytic responses to resistance exercise and feeding, and uncomplicated disuse atrophy. Where human data is lacking, reference is made to findings from animal- and, when appropriate, cell-based models.

Regulation of Translation

Work conducted primarily in animals and cell culture over the last 10 years has revealed that insulin, muscle contraction and amino acids converge at various points on a common signaling pathway, the PI3K-Akt-mTOR pathway (Figure 2) (23; 27; 227), and that protein synthesis is primarily regulated at the stage of translation initiation, although the eukaryotic elongation factor2 (eEF2), a factor obviously involved in peptide chain elongation, has also been identified as a target of the mTOR and MAPK signaling pathways (227). It should be cautioned that the relevance of these pathways and interactions to the human situation is currently, at best, unclear.

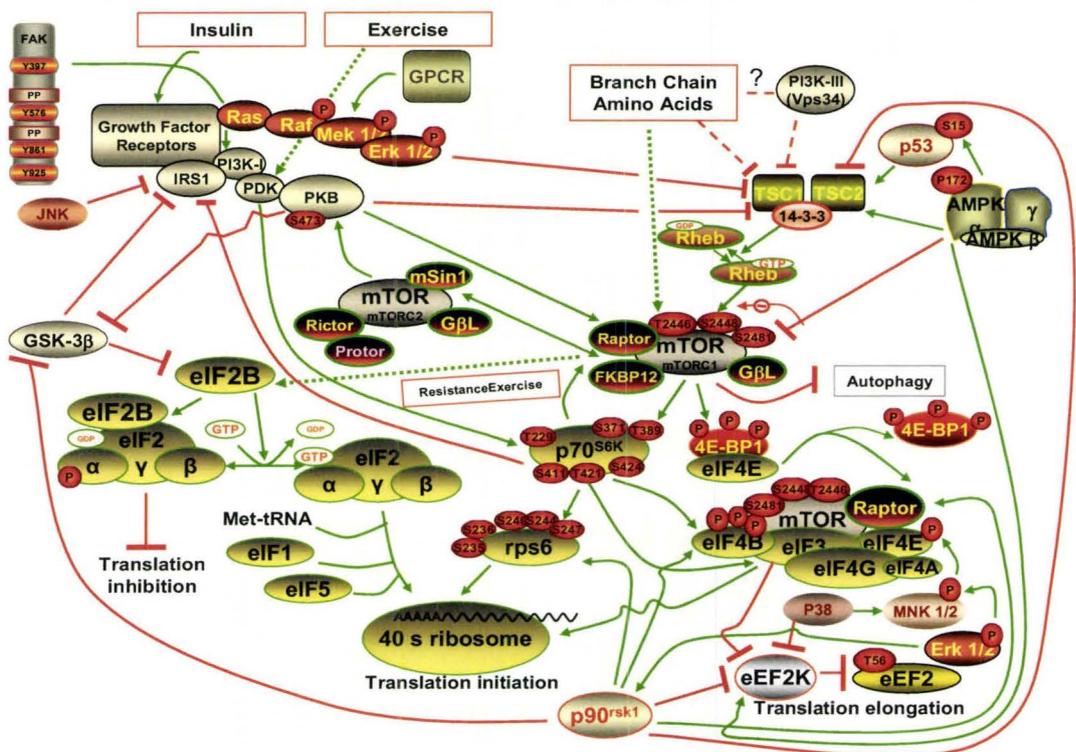


Figure 2. Regulation of translation. Red line: inhibitory effect. Green line: Stimulatory effect. Dashed line: indirect/unknown pathway through which effects are exerted. (Modified from Science Slides 2007, VisiScience, Chapel Hill, NC)

In response to activation of receptors associated with the plasma membrane, the enzyme phosphatidylinositol 3- kinase (PI3K) generates phosphatidylinositol-3,4,5-triphosphate from phosphatidylinositol-4,5-diphosphate at the plasma membrane, which in turn recruits and activates phosphatidylinositol-dependent protein kinase 1 (PDK1) and Akt/protein kinase B (Akt/PKB)(27). PDK1 phosphorylates Akt at Thr³⁰⁸, an initial step in activation (5). Akt/PKB targets multiple proteins and kinases, including glycogen synthase kinase (GSK) α/β (inhibitory phosphorylation at Ser^{21/9}), the serine/threonine kinase mTOR (stimulatory phosphorylation at Ser²⁴⁴⁸), and tuberous sclerosis complex 2 (TSC2) (inhibitory effects at Ser⁹³⁹, Ser¹¹³⁰, Thr¹⁴⁶²) (27). mTOR is inhibited by a complex containing TSC2 and TSC1, via TSC2-mediated activation of the GTPase function of the G protein “ras homologue enriched in brain” (Rheb) (27; 227). When GTP-bound to Rheb it complexes with mTOR to activate its kinase activity (227). Glycogen synthase kinase 3 (GSK3) suppresses activity of the guanine nucleotide exchange factor eukaryotic initiation factor 2B (eIF2B) by phosphorylation at Ser⁵⁴⁰ of the epsilon subunit (231), therefore Akt-mediated phosphorylation of GSK would contribute to releasing eIF2B from inhibition. Global upregulation of protein synthesis is promoted by eIF2B by recharging eIF2 with GTP, enabling it to bind to the 40S ribosomal subunit and initiator methionyl tRNA in a ternary complex (27). eIF2 itself can be regulated by phosphorylation on Ser⁵¹ of the α subunit by four kinases in response to conditions of cellular stress (166). Phosphorylation of eIF2 α transforms it into a competitive inhibitor of eIF2B (166), an additional means by which general rates of translation can be blunted.

A key node in this pathway, Akt, targets mTOR when in the regulatory associated protein of target of rapamycin (raptor)-G-protein beta-subunit-like (GβL) complex (mTORC1, rapamycin sensitive), but when mTOR is bound to the rapamycin-insensitive companion of mTOR (rictor)-GβL complex (mTORC2) *in vitro*, it is able to phosphorylate Akt at Ser⁴⁷³, a residue required for full activation (103; 184). The significance of this feedback loop *in vivo*, particularly in humans in response to stimuli such as exercise and nutrients, remains to be determined. Activation of mTOR in the raptor-GβL complex enables it to act on downstream targets such as the inhibitory eIF4E-binding protein (4E-BP1) and p70 ribosomal protein S6 kinase 1 (S6K1) (27; 227). Evidence has been presented for direct phosphorylation of mTOR at Ser²⁴⁴⁸ and Thr²⁴⁴⁶ by S6K1 establishing yet another potential feedback loop in the pathway (98). mTOR and S6K1 themselves bind to eIF3 (a multi-subunit complex that interacts with 4G and the 40S ribosome and assists in ribosome binding to mRNA and met-tRNA-eIF2B-GTP), alternating between an inactive state (when S6K1 is bound and unphosphorylated) and active state (when mTORC binds and S6K1 is released due to phosphorylation) (97).

Phosphorylation of 4E-BP1, mediated by mTOR, at Thr³⁷ and Thr⁴⁶ is a priming event for subsequent phosphorylation at Ser⁶⁵ and Thr⁷⁰ (78), which induces release from eukaryotic initiation factor 4E (eIF4E), enabling binding to eIF4G, together with 4A to form the eIF4F complex, involved in binding of mRNA to the ribosomal subunit (27). Formation of the 4F complex tends to promote translation of transcripts with extensive, highly structured 5' untranslated regions (227). Sequential phosphorylation of S6K1 at residues Thr³⁸⁹ (mTOR mediated) and Thr²²⁹ (PDK1 mediated) activates the enzyme,

enabling phosphorylation of the S6K1 target, ribosomal protein S6 (rps6) (Ser^{240/244}, Ser^{235/236}), a component of the 40S ribosomal subunit (27), as well as eIF4B (Ser⁴²²), an accessory protein of the eIF4F complex that interacts with RNA helicase eIF4A, which unwinds the 5' untranslated region of mRNAs (170; 227).

Recently it has been shown that, in addition to the ribosomal subunits, all elongation factors and the e, f, and h subunits of eIF3 are 5'-terminal oligopyrimidine (5'TOP) encoded mRNA, suggesting that they may be playing regulatory roles in translational control (100). The 5'top mRNAs have 4-15 pyrimidines at the 5' end and are regulated in a growth-associated manner (e.g. presence of amino acids). Originally rps6 was thought to play a role in promoting translation of this type of mRNA but has since been found not to be required for efficient translation (180). Thus, the physiological role(s) of rps6 are currently under debate.

Insulin acts on the PI3K-Akt-mTOR pathway through its tyrosine kinase receptor, which when activated phosphorylates insulin receptor substrates 1/2 (IRS 1/2), in turn activating PI3K by binding to its p85 subunit (27). A potential negative feedback mechanism exists at this level via GSK3- β , S6K1 and c-Jun NH2-terminal kinase (JNK) mediated inhibitory phosphorylation of IRS-1 at Ser³¹² (Ser³⁰⁷ in mouse, JNK and S6K1), Ser³³² (GSK3- β) and Ser²⁷⁰, Ser¹¹⁰¹ and Ser^{636/639} (S6K1), (4; 124; 215; 241) findings that may implicate dysregulated translational signaling in the etiology of insulin resistance.

Leucine appears to activate targets like S6K1 and 4E-BP1 without affecting phosphorylation of Akt, indirectly through mTOR via a protein recently implicated in autophagy, the type III PI3K, vacuolar protein sorting 34 (Vps34) (108; 227). Resistance

exercise appears to feed into this pathway at a branch below PI3K, although the PI3K inhibitor wortmannin blocks passive-stretch induced phosphorylation of Akt (182), and PDK1 has not been examined with resistance exercise (27). Resistance exercise in rats has been shown to increase the activity of eIF2B (65); a link between resistance exercise and mTOR and an increase in eIF2B epsilon subunit protein has also been demonstrated 16h post exercise, and interestingly administration of rapamycin prevented the increase in eIF2B translation without inhibiting post exercise phosphorylation of 4E-BP1 and S6K1 (119). *In vitro*, activation of mTOR was recently found to be sufficient to induce this increase in translation of eIF2B mRNA (120).

The so-called cellular energy sensor 5'-AMP dependent protein kinase (AMPK) (93), can downregulate the energy-requiring process of protein synthesis through its capacity to activate TSC2 (93), directly inhibit mTOR (108) and inhibit the mTORC1 complex member raptor (89). In addition, AMPK modulates fatty acid synthesis by inhibitory phosphorylation of acetyl-CoA-carboxylase β (ACC β) at Ser⁷⁹ (β is the major isoform in muscle) (90), thus ACC β phosphorylation provides a marker of AMPK activity. AMPK also activates eEF2kinase (eEF2K) (Ser³⁹⁸) (34), which inhibits eEF2 by phosphorylation at Thr⁵⁶ (227). However, S6K1 can counteract this by inhibiting eEF2K at Ser³⁶⁶ (226), and mTOR controls, directly or indirectly, additional inhibitory phosphorylation of eEF2K at Ser⁷⁸ and Ser³⁵⁹ (227).

The mitogen activated protein kinases (MAPK) act on various targets in the Akt-mTOR pathway and MAPK, particularly p38, are phosphorylated with exercise (42; 49; 75; 107; 117; 199; 234). p38 can inhibit eEF2K by phosphorylation at Ser³⁵⁹ in a

rapamycin-independent manner, suggesting a separate pathway from the mTOR-dependent phosphorylation discussed above (110). In addition, p38 phosphorylates MAPK-activated protein kinases 1 and 2 (MNK 1,2) (Thr¹⁹⁷ and Thr²⁰²) (228), thus enabling MNK 1/2 to regulate eIF4E activity through phosphorylation at Ser²⁰⁹ (229). Extracellular signal-regulated kinases (ERK) 1/2 also act on MNK1/2 (228), TSC2 directly (133), and p90 ribosomal s6 kinase (p90rsk) a kinase that, like S6K1, can phosphorylate rps6 (but only at Ser^{235/236}) (178), eEF2K, and eIF4B (9). In addition p90rsk can phosphorylate the mTOR complex protein raptor, and inhibit TSC2 and GSK3 (9). Finally, c-Jun NH2-terminal kinases/stress-activated protein kinases (JNK/SAPK) are also upregulated with exercise, particularly damaging exercise, but their role appears to be more of that of an activator of mitogenic transcription factors such as c-Jun and c-Fos (117).

It is not clear how the stimulus of muscular contraction is transduced to an intracellular response, but cell culture and animal work has implicated the integrin signaling protein, focal adhesion kinase (FAK), as a potential mechanosensor in skeletal muscle (61). Associated with sarcolemmal focal adhesion complexes (61), it is a tyrosine kinase that is phosphorylated at multiple residues. Phosphorylation occurs at site Tyr³⁹⁷, an autophosphorylation event that enables binding to the p85 unit of PI3K, Src-mediated phosphorylation at catalytic residues Tyr^{576/577}, and the C-terminal residues Tyr⁸⁶¹ and Tyr⁹²⁵ (141). Adding credence to its role as a potential mechanosensor, appropriate differences in FAK protein concentration and/or phosphorylation have been observed in association with states of loading and unloading in rodents by Fluck and colleagues (71;

83). Studies of load-dependent changes in skeletal muscle FAK in humans have been limited to date (see following sections), but FAK expression and phosphorylation have been found to be altered in cardiac tissue from humans with ischaemic cardiomyopathy (together with decreased beta(1)D-integrin) (160) and mitral regurgitation (129).

Phosphorylation of many of the aforementioned proteins has been used as markers of translation initiation or synthetic activation in exercise and feeding studies (Table 1). The degree to which phosphorylation reflects protein activation and ultimately regulation of protein synthesis, and the extent to which these interactions occur in human muscle is unknown (for example, pathways whereby insulin promotes MPS *in vitro* may not be pertinent to intact human muscle). Importantly, an appreciation of the potential complexity of the signaling cascades that regulate protein synthesis can be gained from a consideration of the possible ways in which these factors and pathways can interact. Given the potential feedback cycles and cross-talk between parallel pathways and signaling factors, it is likely that measurements of one or even a few of these factors is not sufficient to tease out the individual contributions of each input to the overall integrated *dynamic* response, especially for complex stimuli such as feeding and exercise in the intact organism. Thus dynamic measures of synthesis should, when possible, be made in conjunction with these snapshot measurements.

Proteolytic Systems in Skeletal Muscle

Skeletal muscle contains the three proteolytic systems, albeit their relative activities and importance differ from that in tissues of higher protein turnover (e.g. cathepsins are abundant in kidney, spleen, and liver (13)). In addition, the pro-apoptotic

protease, caspase-3, has been recently implicated in initiating myofibril cleavage under atrophic conditions. It should be emphasized that most of the work characterizing these proteolytic systems has been carried out in animal and cell culture systems. Given that net muscle protein balance is a reflection of the balance between protein synthesis and degradation a brief review of the major proteolytic systems in skeletal muscle follows.

The acid lysosomal system consists of membrane bound structures that contain the major cathepsins L,B,D and H as well as glycosidases, lipases, nucleases and phosphatases (13). Cathepsins cleave a variety of myofibrillar substrates *in vitro*. For example, cathepsin H has specificity for troponin T; cathepsin B targets MHC, troponin T, troponin I and tropomyosin; cathepsin L can degrade most myofibrillar proteins with the exception of troponin C and tropomyosin. Cathepsin expression, while relatively low in muscle, has been identified in adult muscle, cell lines and adult human muscle satellite cells (13). In work predating discovery of the ubiquitin-proteasome system as the main proteolytic system in muscle tissue, *ex vivo* studies using inhibitors of proteolytic systems led Furuno and Taillandier and colleagues to conclude that the lysosomal systems do not play a major role in proteolysis during denervation atrophy and hindlimb unloading (73; 196). In contrast, Tischler et al. observed that the more extreme model of denervation muscle did involve increased lysosomal proteolysis whereas the accelerated proteolysis of unweighted muscle involved calcium dependent and thiol protease based mechanisms (209).

The potential importance of lysosomal autophagy as a feeding- and muscle activity- regulated proteolytic process has been recently recognized with observations

that the atrophy-inducing transcription factor FOXO3 controls both autophagic and proteasomal gene expression in rodent fasting and denervation (137; 242). Inactivation of the DNA-binding protein, Runx1, induces autophagy and exacerbates wasting in denervated muscle (224) and activity of Vps34, implicated in mTOR activation (as discussed above) and macroautophagy, increases following resistance exercise in fasted rats (136), which an interesting observation since it provides a link between degradative and synthetic processes. These findings remain to be confirmed in human studies.

Skeletal muscle expresses the ubiquitous calpains -1 (mu-calpain), calpain-2 (micro-calpain), as well as calpain-3 (p94) (12), a muscle-specific form that binds to the giant sarcomeric protein connectin/titin (191) and when defectively expressed, results in limb girdle muscle dystrophy type IIA (176). *In vitro*, calpain 1 exhibits activity at micromolar concentrations of calcium (1-50), whereas calpain 2 requires higher concentrations (250-1000 μ M) (12) and calpain 3 submicromolar concentrations; in addition, autolysis of their propeptide enables prolonged activation under reduced calcium concentrations (33; 81). *In vivo*, it appears that calpains 1 and 3 have roughly similar sensitivities to calcium levels (148). Calpain 3 can cleave calpains and calpastatin (155), and calpain substrates include enzymes such as calcium/calmodulin-dependent kinase (CaM kinase II), protein kinase C (PKC-alpha, PKC-betaI, PKC-betaII, and PKC-gamma), and calcineurin (165), suggesting that calpains may exert a regulatory role via degradation of specific proteins rather than regulating bulk proteolysis. In rodents, overexpression of the endogenous calpain inhibitor calpastatin attenuated hindlimb unloading-induced atrophy by 30%, but also increased fibre number (204), therefore the

effects of manipulating calpain activity in this manner in mice may not reflect the true contribution in humans under varying conditions of loading. Calpain-mediated cleavage has been proposed to be the “upstream” event in bulk proteolysis allowing site-specific cleavage of myofibrillar proteins to release them from the sarcomere for further degradation (12; 81; 165; 233). Proteins such as titin and nebulin appear to be particularly susceptible to calpain-mediated cleavage (81). This mechanism would, it has been argued, be necessary as the proteasome cannot degrade intact sarcomeric myofibrillar proteins (12; 81; 190) and conversely, calpains do not degrade free actin, actinin or myosin heavy chain (12; 81). Instead, calpains are concentrated at the Z-disk (12; 81; 121), further suggesting that they may function in the initial stages of myofibrillar disassembly. Evidence obtained from pharmacological inhibitors and calpastatin supports the activation of calpains in sepsis (64; 230) but their contribution to disuse atrophy requires confirmation.

Caspase-3 is an enzyme classically associated with apoptosis, but has been reported to cleave acto-myosin complexes *in vitro*, generating a characteristic 14kDa actin fragment (59). Increased calpain activity can, via caspase 12, activate caspase-3 (39; 165), and caspase-3 also targets calpastatin (222). Thus ‘cross-talk’ between calpains and caspase-3 to promote myofibrillar release has been proposed to potentially mediate upstream release of myofilaments from the sarcomere during conditions such as disuse (165) (Figure 3). Increased caspase-3 activity is apparent in muscles of diabetic and uremic mice (59; 225) but the extent to which caspase-3 plays a role in disuse atrophy has not been established.

The ubiquitin (Ub) proteasome system is regarded as the primary system by which cellular proteins are degraded (171; 197). Targeting of proteins for the proteasome begins with conjugation of a single ubiquitin moiety to a protein by an E2 enzyme which has been previously 'charged' with ubiquitin that has been activated by the E1 enzyme in an ATP-dependent process (Figure 3). For efficient targeting for degradation, a chain of at least 4 Ub moieties must be attached to a protein and this is accomplished by E2 in partnership with the E3 ligases, which imparts specificity to the system because each E3 recognizes a limited number of protein substrates (171; 197). There are dozens of E2s and hundreds of potential E3s, thus the large number of possible E2 and E3 combinations provide an additional level of specificity (171; 197). Proteins can also be deubiquitinated (8; 152) and conversely the proteasome can process non ubiquitinated proteins in an ATP-independent manner (197). For example oxidized proteins can be proteolytically degraded via the 20S proteasome core subunit (187), provided they have not formed inhibitory aggregates (87). It should also be noted that, as a reversible post-translational modification like phosphorylation, ubiquitination can affect protein-protein interactions by alteration of substrate topology. Such a process allows modulation of diverse non-proteolytic cellular processes such as endocytosis, trafficking, transcriptional control and DNA repair (91; 109).

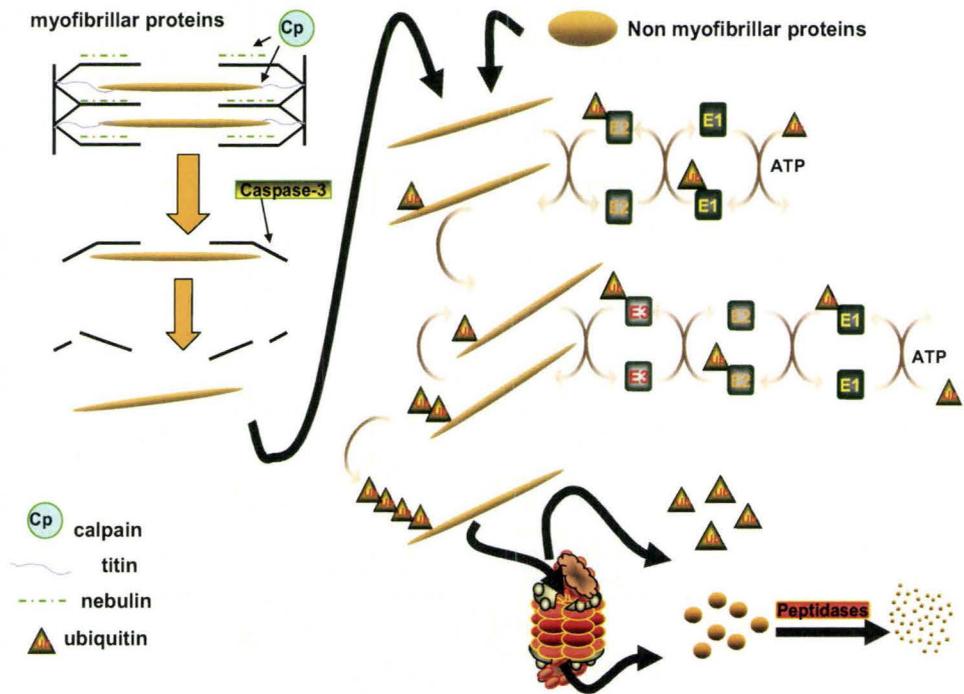


Figure 3. Putative proteolytic pathways of muscle proteins.

Two E3 Ub ligases have been identified that are transcriptionally upregulated in muscle under pro-atrophic conditions in animal and cell models (atrogin-1/muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1) (24; 82). Since these reports were published much attention has been focused on quantifying the expression of the mRNA that encodes these proteins as a markers of atrophy/increased proteolysis (149; 171; 197)). Regrettably, no link between the increased abundance of these mRNA and actual measured proteolytic rates or even *in vitro* proteasomal activity has been reported to date. Recently another E3 ligase, Nedd4, has been identified as upregulated specifically in conditions of reduced loading (hindlimb suspension, denervation) (111)

leading to speculation that this ligase is also a regulated step in proteolytic degradation during atrophy.

The intact proteasome is composed of the 20S core with non-catalytic alpha and catalytic beta subunits, and 19S complexes at each end, which possess subunits with ATPase activity (197). ATP is required to promote assembly of the full proteasome, gating and feeding of unfolded substrate into the core, proteolysis, and release of peptides (197). The peptides produced by the proteasome are further degraded by a tripeptidyl peptidase II and exopeptidases (197). The expression of mRNA encoding proteasomal components and proteasomal activity has also been used as a measure of increased capacity for degradation in many studies (101; 106; 139; 149; 171; 196; 197). However, E1 and 26S proteasome are abundant in cells, and are not thought to be the rate limiting steps of the pathway (171). In addition, neither the proportion of proteasome C9 subunit nor E2 enzyme mRNA associated with ribosomes were found to be increased in muscle from hindlimb suspended rats relative to controls (196); therefore, changes in mRNA levels for these components may mean little in terms of actual upregulation of proteasome-dependent proteolysis. Overall, information about protein level and catalytic activity changes for various components of the pathway, particularly the Ub ligases, is scant. Hence, there is a true lacuna in our understanding of how changes in mRNA coding for proteasomal components correspond to changes in protein levels and ultimately, catalytic activity. In addition, levels of ubiquitin protein and mRNA, as well as polyubiquitination of proteins, have been used as markers of increases in proteasomal system activity (149; 171; 197); however, care should be taken in the interpretation of

such an outcome as inhibition of the proteasome can also cause accumulation of Ub-protein conjugates and would not mean increased, but rather reduced proteolysis (200). Furthermore, the identities of targeted proteins in muscle remain largely unknown, but regulatory factors such as MyoD (1; 205) and S6K1 (223) have been identified as targets of E3 ligases like atrogin-1/MAFbx and/or ubiquitin mediated degradation, suggesting that one of the major reasons for increased ubiquitin dependent proteasomal activity during states of altered loading would be remodeling of the suite of metabolic and regulatory factors of the protein synthetic machinery consistent with what would be required for maintenance of the new steady state phenotype.

Resistance exercise and the acute response of muscle protein turnover

From work carried out over the last 15-20 years, we have developed a reasonable understanding of the regulation of the skeletal muscle mass in humans. The refinement of stable isotope techniques has enabled hour-by-hour measurements of protein synthesis and breakdown and how these variables change in response to the primary drivers of anabolism, muscular activity and nutrition. Hypertrophy is the phenotypic response to long term resistance training (134), but the acute synthetic response of human muscle to a bout of resistance exercise was first reported for the biceps brachii of resistance-trained males in the fed state by Chesley et al (41). Since then, the potent stimulation of mixed and myofibrillar MPS by heavy resistance exercise has been demonstrated to increase in vastus of young untrained subjects by 60 -180 minutes post exercise (55; 58; 122; 186) and remain elevated in biceps brachii of fed trained (135) and vastus of fasted untrained (163) subjects for 36-48 h, respectively. The effect of an acute bout of resistance exercise

on mitochondrial protein synthesis in the fasted state is currently unknown but would be expected to be similar to that seen in the fed state (232). Recent timecourse data for fasted state myofibrillar protein synthesis over the four hours succeeding exercise would suggest that in the absence of feeding synthesis is maximally stimulated at a minimal intensity of 60% of 1 repetition maximum (1RM) but does not remain elevated beyond 2 h post exercise; however, these findings may have been affected by the volume of the exercise stimulus (122). Furthermore, MPB is also stimulated post exercise (19; 162-164). In fact, a significant relationship was noted between increases in MPS and MPB (163), suggesting that the two processes are related at least in the 24 h period post exercise, although MPS remained elevated longer than MPB. While MPS is still robustly stimulated after 10 sessions of resistance training (239), the rises in synthesis and breakdown both appear to be attenuated with longer-term training (162; 164). The contractile mode also appears to make a difference, as maximal lengthening contractions stimulate MPS more rapidly than shortening (143). It is currently unknown if MPB is also stimulated more by maximal eccentric contractions, but submaximal concentric and eccentric did not differ in terms of mixed MPB (163).

While not as informative as direct incorporation measures, amino acid kinetics using arterial-venous balance methods offer some insight into amino acid flux and muscle protein turnover. After a bout of resistance exercise, hyperemia was reported several hours later (19), increasing arterial delivery of amino acids 80-100%, without a change in blood and intracellular concentrations. Post-exercise hyperemia has been measured recently in our lab in response to intense resistance exercise and found to be much more

transient, returning to baseline levels only 30 min post-exercise. Despite these differences Biolo and colleagues reported that inward transport of certain amino acids was increased in conjunction with increased intracellular appearance (due to breakdown/*de novo* synthesis), possibly driving the increase in post exercise protein synthesis in the fasted state (19). According to Biolo, the role of inward transport in determining synthetic rates is highlighted by the fact that in a catabolic condition such as severe burns, where breakdown is increased but inward transport impaired (18; 19), synthesis is not stimulated sufficiently to offset breakdown and net protein loss occurs.

Translational Signaling – Resistance Exercise

Human exercise and feeding studies have recently begun to include measures of the signaling proteins implicated in translational control as elucidated in cell and animal models, although only a few have reported both turnover (primarily synthesis) and signaling (Table 1). Acute resistance exercise studies have attempted to map out changes in the 0-24 h following exercise. For example, immediately after an exercise bout in the fasted state it appears that there is no major change in phosphorylation at activating phosphorylation sites on Akt, mTOR or S6K1, regardless of glycogen status (43). Indeed a few studies have reported a decrease in Akt phosphorylation (22; 49; 99). In line with this, Dreyer and colleagues (54; 55) reported a concomitant decrease in MPS over the period of resistance exercise and an increase in AMPK α 2 activity. Maximal eccentric contractions, however, were reported to increase S6K1 phosphorylation and rps6 at Ser^{235/236} immediately after exercise without an increase in phosphorylation of Akt or mTOR (63; 199). In the fasted state, AMPK phosphorylation was found not to change

post resistance exercise in some studies (42; 53; 99; 199), but others have demonstrated immediate or transient increases (42)(in endurance trained subjects)(58; 115). Dreyer et al. showed an increase in activity of AMPK at 2 h post exercise without an increase in phosphorylation (53). As AMPK activity is putatively inhibitory to protein synthesis via targeting of TSC2, mTOR, and eeF2K (see above section), it is difficult to reconcile this finding (53) with the increase in protein synthesis post resistance exercise demonstrated in other reports from this group (54; 55). Within the first couple of hours post exercise in the fasted state, Akt phosphorylation only appears to increase under conditions of high glycogen (43) or transiently (53-55), while phosphorylation of mTOR has only been reported to increase in some reports (55; 138). In fact, Mascher et al. reported an increase in mTOR phosphorylation at the Ser²⁴⁴⁸ site but not at Ser²⁴⁸ an autophosphorylation site (159) more closely associated with mTOR activity (LS Jefferson, personal communication). Phosphorylation of S6K1 at Ser⁴²⁴/Thr⁴²¹ has shown a consistent tendency to increase with exercise while phosphorylation at Thr³⁸⁹ seems to be more variable and perhaps dependent on the intensity or novelty of the exercise (42; 49; 55; 107; 115; 122; 138). Recent timecourse data to 4 h post exercise demonstrate that increases and falls in phosphorylation of S6K1 and its target 4E-BP1 precede changes in myofibrillar MPS, suggesting that the phosphorylation of S6K1 at Thr³⁸⁹ is related to MPS rates, at least in the fasted state (122).

Table I-B. Summary of human studies with translational signaling measures

Type	Study	Design	Insulin	FSR/MPS/MPB						Signalling (h, in case of ex, post ex)						protein
				0	1	2	3	4	5	6 h	0	1	2	3	4	
I	(96)	N=5, saline, N=6, insulin	120 min hyperinsulinemic (~8-9 fold) euglycemic clamp	No MPS						↑ ↔						P70 ^{66k} (β+γ/total) 4EBP-1 (upper band/lower)
I	(56)	N=6, [AA] ↓ (30-60%) (subjects from 14) N=4, [AA] ~ ↔ (↓ 9%)	hyperglycemic- hyperinsulinemic hyperlipidemic clamp elevate levels 7 fold (40 μU/ml) Local femoral insulin infusion to elevate levels ~4fold (48 μU/ml)	↓(P=0.08) MPS ↓ MPB ↑ MPS ↔MPB						↔ ↔ ↔ ↔ ↔ ↔ ↑ ↑ ↑						mTOR (Ser ²⁴⁴⁸) p70 ^{66k} (Thr ³⁸⁹) eEF2 (Thr ⁵⁶) eIF2α (Ser ⁵²) IRS-1 (Ser ³¹²) IRS-1 (Ser ^{636/639}) Akt (Ser ⁴⁷³) AS160 AMPK (Thr ¹⁷²) ACCCβ (Ser ⁷⁹) Akt (Ser ⁴⁷³) mTOR (Ser ²⁴⁴⁸) p70 ^{66k} (Thr ³⁸⁹)
E	(234)	N=8, 3 x 10 KE @ 70% 1RM		NO MPS						↑ ↑ ↑ ↔ ↔ ↔						ERK 1/2 (Thr ²⁰² /Tyr ²⁰⁴) p90 ^{sk} (Tyr ⁵⁷³) MNK1 (Thr ^{197/202}) eIF4E (Ser ²⁰⁹) p38 MAPK (Thr ¹⁸⁰ /Tyr ¹⁸²) JNK/SAPK (Thr ¹⁸³ /Tyr ¹⁸⁵)
E	(107)	N=7, 4 sets LP x 10reps @ 80% 1RM	↑ endog insulin post ex	NO MPS						↑ ↑ns ↑ ↔ ↔ ↔ ↔ ↔ ↔ ↑ ↔ ↔ ↑ ↔ ↔						p70 ^{66k} (Ser ⁴²⁴ /Thr ⁴²¹) p70 ^{66k} (Thr ³⁸⁹) rps6 (Ser ^{235/236}) ERK 1/2 (Thr ²⁰² /Tyr ²⁰⁴) p38 MAPK (Thr ¹⁸⁰ /Tyr ¹⁸²)
E	(43)	N=8, 3 x 10 KE @ 70% 1RM, Fasted but high (HCHO) and low glycogen (LCHO) states	No change	NO MPS						↔ ↑ (10 min) ↔ ↑ (10 min) ↔ ↔ (10 min) ↔ ↑ (10 min) ↔ ↔ (10 min)						ERK2 (Thr ¹⁸³ /Tyr ¹⁸⁷) H and L CHO p90 ^{sk} (Thr ⁵⁷³) H and L CHO Akt (Ser ⁴⁷³) L CHO Akt (Ser ⁴⁷³) H CHO mTOR (Ser ²⁴⁴⁸) L and H CHO
E	(22)	Same as (107) (EX only trial)	Not reported	NO MPS						↔ ↔ ↔ ↓ ↔ ↔ ↔ ↔ ↔						mTOR (Ser ²⁴⁴⁸) Akt (Ser ⁴⁷³) GSK-3β (Ser ⁹)
E	(63)	N=10, 4sets LP x 6 ECC or CON max (unilateral) N=6, 4sets LP x 6 max CON, submax ECC (unilateral)	Not reported	NO MPS						↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↔ ↑ ↑ ↑ ↔ ↔ ↔ ↑ ↑ ↑ ↔ ↔ ↔ ↑ ↑ ↑ ↔ ↔ ↔						Akt (Ser ⁴⁷³) mx ECC Akt (Ser ⁴⁷³) mx CON/smx ECC mTOR (Ser ²⁴⁴⁸ , Ser ²⁴⁸¹) mx ECC mTOR (Ser ²⁴⁴⁸) mx CON/smx ECC p70 ^{66k} (Ser ⁴²⁴ /Thr ⁴²¹) mx ECC p70 ^{66k} (Ser ⁴²⁴ /Thr ⁴²¹) mxCON/sm ECC p70 ^{66k} (Thr ³⁸⁹) mx ECC p70 ^{66k} (Thr ³⁸⁹) mx CON/smx ECC rps6 (Ser ^{235/236}) mx ECC rps6 (Ser ^{235/236}) mx CON/smx ECC

F: feeding; I: insulin; E: exercise; BCAA: branched chain amino acid, AA: amino acid; TAA: total amino acids; EAA: essential amino acids; Leu: leucine; CHO: carbohydrate; KE: knee extension; LP: leg press; FSR: fractional synthetic rate; MPS: muscle protein synthesis; NB: net balance; R_d: rate of disappearance; R_a: rate of appearance; ns: non significant; (P-)4EBP-1: (phospho)4E binding protein 1; (P-)p70^{66k}: (phospho-)p70 ribosomal s6 kinase; (P-)GSK3α/β: (phospho-)glycogen synthase kinase α/β; eIF2α: eukaryotic initiation factor 2α; eIF2B(ε): eukaryotic initiation factor 2ε; mTOR: mammalian target of rapamycin; TSC2: tuberous sclerosis 2; eEF2: eukaryotic elongation factor 2; AMPK: 5' adenosine monophosphate-activated protein kinase; IRS-1: insulin receptor substrate 1; rps6: ribosomal protein s6; ERK1/2: extracellular signal regulated (MAP) kinase; MNK: MAP interacting kinase; p38 MAPK: p38 mitogen activated protein kinase; eIF4E: eukaryotic initiation factor 4E; eIF4B: eukaryotic initiation factor 4B; eIF4G: eukaryotic initiation factor 4G; p90^{sk}: p90 ribosomal s6 kinase; JNK/SAPK: c-jun amino-terminal kinase/stress activated kinase; AS160: Akt substrate of 160 kDa; ACCβ: acetyl-CoA carboxylase β; IHC: immunohistochemistry; Ser: serine; Thr: threonine; Tyr: tyrosine; mx: maximal contraction; smx: submaximal contraction

Phosphorylation of the target protein of S6K1, rps6, only increases if S6K1 is phosphorylated at Thr³⁸⁹ (63; 107; 115; 138; 199). Evidence in support of mTOR-mediated signaling driving the increase in synthesis post exercise was recently obtained in a study in which rapamycin was administered to participants prior to exercise, resulting in an attenuated exercise-induced increase in MPS (58) and delayed increases in mTOR phosphorylation at Ser²⁴⁴⁸ as well as S6K1 at Thr³⁸⁹. While mTOR mediated Thr^{421/424} phosphorylation of S6K1 was abolished, however, association of mTOR with raptor did not differ from the control group and increased by 2h post exercise. Unfortunately MPS was not measured beyond 2 h post exercise, therefore it is unknown if MPS increased as the phosphorylation of the factors increased in the rapamycin group. The effects of exercise on signaling proteins in these various studies is also 'blurred' due to sampling from the vastus, a mixed fibre muscle, as phosphorylation of S6K1 and rps6 primarily appears to increase in type II fibres (115; 199). However, this may reflect the fact these fibres are recruited during higher intensity exercise such as resistance exercise.

Phosphorylation of GSK3 and eIF2B ϵ have not been explored very much in humans in the hours shortly after resistance exercise. In the few studies that have examined them, GSK phosphorylation did not change and 2B ϵ showed contradictory and non significant changes post exercise (22; 42; 58; 138). Given the relatively tight relationship between eIF2B activity and resistance exercise-induced increases in MPS in rodents (65), it would seem pertinent to further characterize these proteins in humans.

The phosphorylation of the MAPK, ERK and p38, have been reported to be increased following exercise in a number of studies (42; 43; 49; 58; 107; 234).

Interestingly, rapamycin administration in humans completely suppressed post exercise ERK phosphorylation, likely via an indirect mechanism (58). A target of several proteins in the pathway, TSC2 was also reported to be phosphorylated to a lesser degree at Ser¹⁴⁶² in the hours after exercise (42; 55); the relevance of this finding remains to be elucidated.

Overall, most studies have looked only within the first few hours post exercise, with one report 24 h post (49), but a clear time course study of events within the first hour and over several hours post exercise has not yet been conducted. Furthermore, an increase in synthesis is well known to occur with resistance exercise alone (i.e., in the absence of feeding), but the signaling does not show a consistent increase in the reports possibly due to differing exercise protocols and training states of the subjects. For example, only lengthening and not shortening contractions showed phosphorylation of key signaling protein amino acid residues (63). These findings are consonant with the findings of Moore et al. that lengthening contractions more rapidly raise MPS (143). In light of the fact that synthesis is known to be elevated for 24-48 post exercise, the lack of long term phosphorylation of translational regulators (49) would suggest that much more work remains to be performed in furthering our understanding of the underlying mechanism regulating MPS and also in identifying markers that are more reflective of changes in protein synthetic rates after resistance exercise. There are many instances, for example, when initiation factor phosphorylation levels parallel neither changes in enzymatic activity nor rates of MPS or MPB(54-56; 80; 84; 232). It may be that single markers do not exist for such complex processes, or that our measures are not currently sensitive enough to uncover the relationships between the factors and processes.

Nevertheless, the extent of phosphorylation of S6K1 at Thr³⁸⁹ after resistance exercise in humans has been significantly correlated with increases in MPS (122) and long term hypertrophy (201). Thus while it may not be a direct marker of MPS, it is currently the best candidate molecule for playing a mechanistic role in the regulation of MPS with feeding and exercise. In addition, the use of activity assays (or directly measuring synthetic rates) would be preferable to reliance on protein phosphorylation as an indicator of activity, since there does not always appear to be a direct relationship between phosphorylation and activity (for example, dissociation of AMPK phosphorylation from changes in activity (53)). Assays have been used to measure activity changes in putative regulatory factors such eIF2B (65) and S6K1 and Vps34 (136) in response to contraction in rats. Activity assays are more difficult to perform in tissue from humans, however, due to the requirement for fresh and relatively large amounts of tissue. Nevertheless, future studies must include dynamic measures of these regulatory factors in order to establish relationships between enzyme activities and rates of protein synthesis.

Markers of Breakdown Post Exercise

No study to date has attempted to link post-exercise rises in MPB with changes in proteolytic markers or more specifically proteolytic enzyme activity. Several studies have documented the effects of resistance exercise on various static measures of breakdown. On the transcript level, heavy or lengthening resistance exercise generally appears to increase MuRF1 early and transiently (132; 138; 169; 238) but suppress atrogenin-1/MAFbx expression over the later period (3 – 48h post) (50; 116; 132; 138; 238), although an increase in atrogenin-1/MAFbx in young males has been observed

immediately after exercise (50). No changes in calpain mRNA (132; 238) or regulatory subunit protein (193) were observed 4 or 24 h post exercise, although a delayed increase in calpain 1 mRNA (72h) has been reported (50). Increases in the autolyzed, presumably active, form of calpain 3 have been recently reported to increase 24 h after 30 min of lengthening contractions (147), but not cycling exercise (148). Interestingly caspase-3 increases at the mRNA, protein and activity levels have been reported 4 and 24 h post exercise in untrained subjects (235; 238).

Lengthening contractions have been reported to increase ubiquitin mRNA and protein (203; 235), ubiquitinated proteins (193; 203), and E2 and 20S proteasome protein levels (235) within the first 2 d post exercise. In contrast, proteasome subunit C2 mRNA has been reported not to change (50). Interestingly, a decrease in myofibrillar protein concentration was detected 24 h after an eccentric bout by Willoughby et al (235); however, somewhat surprisingly, myofibrillar breakdown, as estimated by interstitial levels of 3-methylhistidine (3-MH) and chemical arterio-venous (a-v) balance, was not reported to increase at any point over the 24h following performance of heavy resistance exercise in novice volunteers (212). This is an unusual finding in light of the fact that MPB is well known to increase after damaging/heavy resistance exercise.

Protein turnover with feeding

Feeding was shown 26 y ago to increase MPS, indeed the rise in MPS accounts for much of the rise in whole body synthesis with feeding (173), despite the slow turnover of muscle protein. Whether by intravenous infusion (15; 16; 20; 25; 26; 84; 126; 153) or oral consumption of various doses in varying patterns (i.e., bolus, small feeds) of

crystalline amino acids (AA) (44; 157; 221) or intact/hydrolysed protein (146), protein/AA feeding stimulates muscle protein synthesis with only a small time lag of ~30 minutes after the onset of hyperaminoacidemia (26) and reverses negative muscle net protein balance. In the interest of maintaining focus, a detailed discussion of intact/hydrolysed protein consumption will be omitted except when signaling information was obtained in the study. To date, this delay has not been correlated with changes in translational signaling proteins, as samples prior to an hour post feeding have not been taken in humans, therefore we do not know the mechanistic basis for the delay in MPS (26). In the presence of sustained hyperaminoacidemia, MPS has been reported to become refractory to the continued availability of AA and return to baseline after 2 h (26), although another infusion study reported elevated MPS rates even over the final 3 h of a 6 h infusion (126). The physiological relevance of this induced unresponsiveness is unclear as prolonged hyperaminoacidemia is not encountered with typical feeding patterns.

Only the essential amino acids (EAA) are required to stimulate synthesis (44; 72; 157). Ingestion or infusion of non-essential AA do not increase synthesis (189; 208) and leucine alone appears sufficient to enough to stimulate synthesis (188). Infusion of branched chain amino acids (BCAAs), improves net muscle protein balance but did not elevate MPS in the absence of an ongoing supply of AA (plasma concentrations of some AA fell) (125); it is possible that a substantial and sustained measured increase in synthesis could not be supported due to lack of substrate. Inclusion of carbohydrate (CHO) appears to further increase AA-induced stimulation of MPS likely via an increase

in insulin ((221) compared with (220)), as co-infusion of AA with insulin showed a greater increase in MPS than AA alone (153), although CHO alone does not stimulate MPS at rest (31). Provided alone, AA have a suppressive effect on whole body protein breakdown (16; 38; 79; 130; 131), the effects on muscle protein breakdown are less clear. Some studies have found decreases in muscle protein breakdown (130; 131; 151) while others have not (15; 20; 72; 84; 84; 126) (amino acid infusion) (157; 221) (oral feeding), even with (modest) increases in endogenous insulin (20; 72; 157; 221). Inclusion of CHO with AA clearly suppresses MPB (220), likely due to the greater increase in insulin, as co-infusion of AA with insulin shows similar effects on MPB (84; 153). Infusion of crystalline AA increased synthesis and decreased degradation of mixed leg proteins in one study, but estimates of myofibrillar protein breakdown by 3-methylhistidine (3-MH) release across the limb showed no change with feeding (194). The authors concluded that amino acids mainly inhibit proteolysis of non-myofibrillar proteins. Caution should be exercised when interpreting chemical A-V balance of 3-MH, as a net positive uptake has been calculated when measured by HPLC (219), in contrast to consistent net release when using a tracer approach (219). In sum, amino acids clearly stimulate whole-body and muscle protein synthesis and do so through mechanisms related to amino acids themselves, likely predominantly via leucine, and not through an effect mediated by insulin. While amino acids can suppress proteolysis the mechanism may be dependent on insulin and yet can also act independently if the concentrations of amino acids are high enough.

Rates of MPS have been shown to be correlated with changes in extracellular (plasma) AA concentrations in a dose-responsive manner rather than intracellular concentrations (25; 44). In fact, intracellular concentrations decreased when rates were increasing (25). Significant correlations were noted between amino acid delivery (i.e., flow multiplied by amino acid concentration), inward amino acid transport, and muscle protein synthesis (20) suggesting that the rate of AA transport into cells may be a better indicator of the availability of free amino acids for synthesis rather than intracellular concentrations. Leg blood flow has been reported to increase with continuous IV feeding (20; 84) but not with oral feeding (157; 221); this is likely an effect mediated by insulin, which can induce increases in flow via nitric oxide. The role of blood flow *per se* needs to be clarified in a study where AA concentrations are kept constant while blood flow is manipulated (20), perhaps by ischemia or administering agents such as nitroglycerin (a substrate for nitric oxide synthase) to increase flow or N (G)-nitro-L- arginine methyl ester (L-NAME) to reduce flow; either way the concentration of AA needs to be clamped in the face of changing flows.

Provision of AA appears to have a global effect on MPS, stimulating all myocellular protein fractions to increase their synthetic rates simultaneously (myofibrillar, sarcoplasmic and mitochondrial) (25; 26; 146). Some studies have shown differential effects, i.e. somewhat lower responses of the sarcoplasmic fraction (44; 142) in response to AA provision. The timecourse of MPS following oral consumption of a bolus of intact dietary protein (25 g whey) was recently delineated for the sarcoplasmic and myofibrillar fractions, with maximal and similar stimulation of both fractions by 3 h

post exercise and a fall to baseline by 5 h post exercise (146). The timecourse of the response of the mitochondrial fraction to oral consumption of dietary protein remains to be confirmed; however, since it is small portion of the sarcoplasmic fraction it is would likely closely follow a similar response. How feeding stimulates the synthesis of individual muscle proteins remains to be determined, but recent advances have been made in identification of specific mitochondrial proteins (105).

Translational Signaling - Amino Acid feeding

Beyond the role of AA as building blocks of protein, they (particularly leucine) are now recognized to be independent stimulators of the signaling pathways that activate MPS; this work is beginning to be confirmed in humans. For example, leucine (86) increased S6K1 phosphorylation, whereas BCAA (125) or complete AA infusion (126) increase S6K1 and 4E-BP1 phosphorylation, even without an increase in synthesis (125). In contrast, an increase in MPS was reported with a three hour amino acid infusion in both vastus lateralis and soleus with no increase in S6K1 or 4E-BP1 phosphorylation (37). Phosphorylation of AMPK has not been extensively examined with feeding but phosphorylation at Thr¹⁷² has been reported to decrease modestly 1 h after consuming EAA and CHO (72).

Phosphorylation of Akt and its downstream target GSK3, however, appear to require an increase in insulin with (3; 72; 84; 88; 128), or without (56) provision of AA.

Interestingly, eIF2B phosphorylation (44) or exchange activity (127) does not seem to be affected by feeding or insulin, despite being a target of GSK3. Phosphorylation of S6K1 has been reported to increase in response to a hyperinsulinemic, euglycemic clamp (84;

96) but this appears to be a physiologically irrelevant effect mediated by Akt as they have been observed to both increased in a dose dependent manner with insulin infusion while 4E-BP1 phosphorylation, and more importantly MPS, did not (84).

There are many lines of evidence that suggest feeding can activate putative regulatory signaling proteins to activate protein synthesis; however, not all findings are congruent, especially in humans. In addition, we still remain largely unclear about which signaling proteins are required to be active, even if transiently, to stimulate a rise in MPS. Also, we do not know whether phosphorylation of these proteins is via a direct amino acid-mediated mechanism or via insulin, or possibly both. Recent work from Greenhaff *et al* (84) suggests that insulin is mildly stimulatory for protein synthesis at lower concentrations (*i.e.*, less than 10IU/ml) although above that level it is not stimulatory for protein synthesis but is inhibitory for proteolysis. At the same time, changes in signaling protein phosphorylation were disproportionate to changes in protein synthesis leading to confusion over what changes in signaling protein phosphorylation mean in terms of activation of protein synthesis.

Insulin, Amino acids – Proteolytic markers

Very little work has been performed in humans to elucidate changes in mediators/markers of breakdown with acute insulin and/or AA feeding. Greenhaff *et al.* (84) recently reported dose-related changes in protein-level expression of atrogen-1/MAFbx, decreases in proteasome C2 subunit that became significant at the highest infusion rate, but no change in MuRF-1 across the range of insulin rates and in fact an increase at the highest dose. In contrast, MPB decreased at the second infusion rate and

did not show further increases with higher rates, suggesting that these markers are not closely reflective of the dynamic nature of MPB. Adegoke et al. (3) have recently reported a decrease in ubiquitin-conjugated proteins after a 2h hyperinsulinemic-hyperaminoacidemic clamp, but no change in ubiquitin, MAFbx and MuRF-1 transcript levels over that time. This is obviously an understudied area and one that is ripe for future experiments. It seems though that static measures of markers of proteolytic components may mean far less than one might suspect since they are not temporally nor quantitatively related to measured rates of MPB.

Feeding-Exercise Interactions

While an acute bout of resistance exercise reduces a negative net balance by stimulating MPS, AA intake makes it positive, and both stimuli combined produce the greatest effect on mixed or myofibrillar MPS (20; 30; 32; 54; 114; 140; 145; 198; 206; 207), mitochondrial MPS (232) but not sarcoplasmic MPS, which is responsive to feeding but not exercise (146). Post exercise, MPS is enhanced by AA in a dose responsive manner and has been determined to be maximally stimulated at an oral intake of 20 g dietary protein (~8.6 g EAAs) (144). Carbohydrate alone does not enhance post exercise MPS (31), although the combination of AA and carbohydrate may have an additive effect particularly if doses of AA/protein are less than what are required to maximally stimulate MPS (140; 168; 207). In contrast, a recent report found no additional benefit to consuming carbohydrate in conjunction with protein hydrolysate (112). Differing findings may be due to use of a-v balance methods in the earlier studies and ingestion of intact protein in Koopman et al (112). Carbohydrate alone can reduce

post exercise MPB (31; 140; 179), but AA intake post exercise, with or without carbohydrate, has generally been reported not to affect post exercise MPB (30; 32; 140; 168), with the exception of one report (20), in which the lack of rise in MPB post exercise may possibly be due to use of an AA infusion and increased endogenous insulin.

Translational Signaling- Feeding and Exercise

The combination of exercise and AA consumption (45; 51; 54; 113; 232), EAA (57) and BCAA (22; 107) feeding clearly increases phosphorylation of proteins comprising the Akt/mTOR/S6K1 pathway and their downstream targets rps6, 4E-BP1 and eEF2 just about anytime during the 6 h period following exercise, and this aligns with increases in synthesis (45; 54; 57; 232). Increases in mTOR phosphorylation in humans are actually quite variable exhibiting sustained increases in some studies (54; 57) but transient or no increases in others (22; 232; 236). Two studies have directly compared and shown enhanced or sustained phosphorylation of components of the Akt/mTOR pathway for exercise and protein/AA feeding compared to the exercise only (54) or exercise and CHO condition (113), with one report also showing enhanced synthesis (54). In addition to the synergistic effect on the acute (1-3 h) rise in post-exercise MPS, feeding in the post-exercise period appears to induce a sustained elevation (24 h) in phosphorylation of factors, particularly Akt (45).

Consumption of only carbohydrate after exercise can increase phosphorylation of 4E-BP1, rps6 and S6K1 (Ser⁴²⁴/Thr⁴²¹), likely via an increase in insulin (113). This would suggest that while protein synthesis is not stimulated by carbohydrate ingestion post exercise, signaling molecules are phosphorylated, likely via the rise in insulin, but in the

absence of protein no synthetic response occurs. Post-exercise consumption of differing doses of protein, however, did not show differential phosphorylation of signaling proteins, despite a dose response in post exercise MPS (145)

An inhibitory mechanism, Akt-mediated phosphorylation of GSK, despite concomitant increases in phosphorylation of Akt, is not always observed. A transient increase immediately post exercise has been demonstrated (232) although others have shown no change (22) or a delayed decrease (57). Wilkinson et al. (232) also reported a delayed increase in 4E phosphorylation which may be due to solely to feeding as the only other resistance exercise study to report 4E phosphorylation showed no change immediately after exercise, but in the fasted state (234). A transient decrease in eIF2 α phosphorylation at 1h was reported by Drummond et al. (57). A similar finding has been observed at a later time point (6h) in the resting state with a mixed amino acid infusion (127). Changes in AMPK with exercise in the fed state have not been studied extensively but a transient increase has been reported immediately post exercise (232) while another study reported no difference between exercised and non exercised legs following exercise performed 1 h after consuming a standardized meal (236). In the study by Witard et al., however, biopsies were taken ~2 h post prandially and changes in AMPK phosphorylation may have been missed. AMPK phosphorylation has been reported to decrease with feeding (72) therefore it is interesting that Wilkinson et al. demonstrated that exercise appears to override, at least temporarily, the inhibitory effect of feeding on AMPK phosphorylation, suggesting that MPS is likely suppressed during exercise even in the fed state (Wilkinson maintained bolus feeding throughout the exercise protocol).

Differences between studies of post exercise fed-state signaling are likely largely due to differences in the timing of feeding (for example feeding was started prior to exercise in some studies, amounts provided, and the source [intact protein versus crystalline amino acids]).

Protein Turnover during Disuse

In this context, the term “disuse” refers to an extreme form of inactivity, a near-complete cessation of the daily voluntary movement generated by skeletal muscle. The bed rest model, which does not involve restricting limb movement with a brace or sling, reduces loading on weight bearing muscles but also redistributes body fluids and induces cardiovascular deconditioning, therefore it can be considered a systemic model of inactivity. Local models of disuse, such as cast/brace immobilization and lower limb suspension with a sling or thick soled shoe, avoid the changes seen in bed rest while only inducing atrophy in the unloaded/fixed limb. Immobilization by a brace or cast limits range of motion, while the target muscles may still be loaded depending on the degree of joint angle fixation and target limb (leg vs.arm). Unweighting by a sling (a model termed unilateral lower limb suspension or ULLS) restricts range of motion somewhat, while the thick-soled shoe model of limb suspension allows for complete range of motion but no weight bearing (2). All local models effectively induce atrophy, suggesting that unloading of the limb or restricting range of motion are sufficient to reduce muscular contraction and initiate the atrophic process. Bed rest and the local models of disuse are considered non-inflammatory models of atrophy, in contrast to cachectic disease states such as cancer and sepsis, in which elevated pro-inflammatory cytokines and catabolic

hormones play a contributing role to the atrophic process (149). A decrease in thigh muscle volume of 3 % is detectable over as little a period of unloading as 7 d of bed rest indicating a rate of change of 0.4 %/d (68). Similarly, de Boer et al (46) reported a reduction in muscle CSA of 5% after 10d of unilateral limb suspension via a raised shoe, which equates to a rate of loss of 0.5%/d. Post-absorptive mixed muscle (67; 76; 77) and myofibrillar (46; 80) protein synthesis falls 25-50% and remains suppressed with local (47; 76; 77; 80) or whole body (bed rest) unloading (67). Surface electrical stimulation (77) or periodic resistance exercise (69) prevents the fall in MPS and accompanying disuse atrophy. Collectively, these findings (47; 67; 76; 77; 80) suggest that maintenance of MPS is the primary mechanism by which loss of muscle mass can be prevented. A decrease in fasted and fed state protein synthesis with unloading remains to be confirmed for the sarcoplasmic and mitochondrial fractions, but since mixed MPS declines parallel those of myofibrillar protein synthesis it seems reasonable to speculate that declines in the synthetic rate of these fractions would decrease also.

One conspicuous difference between the results from human and animal studies is that human muscle shows much smaller, if any, differences between fibre types in the degree of disuse atrophy (11; 95; 210; 211; 240). Rodents lose greater quantities (up to 30% of the total muscle mass within 2 wk) and also show muscle-specific differences. Thus, in predominantly fast fiber dominated muscles (extensor digitorum longus and tibialis anterior) losses are $\sim 3. \text{ mg} \cdot \text{ d}^{-1}$ or $\sim 1.6 \% \cdot \text{ d}^{-1}$, whereas they are $\sim 6.2 \text{ mg} \cdot \text{ d}^{-1}$ or $\sim 2.7 \% \cdot \text{ d}^{-1}$ in slow fiber dominated muscles (usually soleus) (29; 60; 92; 102; 118; 158; 181; 218). In the normal (i.e., fully loaded) state, human muscles of different fibre

compositions demonstrate remarkably consistent synthetic rates and respond similarly to feeding (37; 142). Soleus muscle has, however, been reported to display a blunted rise in MPS with resistance exercise (214), but this may relate to the exercise protocol and the fact that some people cannot fully recruit the soleus muscle even during a maximal contraction. The muscles of the triceps surae muscle group also appears to be more prone to atrophy, even with exercise counter-measures under unloaded conditions (6; 7; 213). Given the large role that the soleus plays in locomotion and static posture it would therefore be of interest to determine the fasted- and fed-state responses of MPS in soleus in response to unloading.

Measures of MPB have not yet been made in an immobilization or lower limb suspension model but reports from 14-28d bed rest studies have shown no change in post-absorptive whole body breakdown (17; 67; 185; 192) or MPB in the post-absorptive state (67; 195) and over a period that included feeding (156). In addition, the whole body synthetic response to an amino acid infusion is blunted following 14 d bedrest (17). Interestingly, Biolo reported that bed rest did not change AA infusion-induced suppression of whole body Ra, suggesting that the capacity of amino acids to suppress whole body proteolysis is not lost with inactivity, although insulin levels were not reported. After 14 d bed rest Ferrando et al (67) noted a significant relationship between intracellular rate of AA appearance, in which there was a tendency to decrease, and the decline in MPS. A speculation was that decreased synthesis during bed rest may be due to loss of AA from the IC pool due to net outward transport (there was a trend for an increase in net outward transport after bed rest). These observations are in contrast to the

opposing situation post-resistance exercise when inward AA transport is increased (Biolo AJP 268:E514, 1995). Intracellular AA concentrations were not reported by Ferrando et al (67) but Gamrin (74) reported a 48% increase in free BCAA in muscle with immobilization, and increased intracellular phenylalanine had been reported during bed rest, albeit with induced hypercortisolemia (Paddon-Jones 2006). In light of the observation that extracellular amino acid concentrations have a closer relationship to MPS rates than intracellular concentrations (25), it appears unlikely that suppressed rates of MPS during disuse are a consequence of decreased intracellular concentrations. Moreover, given the dynamic nature of the intracellular AA pool, changes in concentration in any condition are difficult to determine especially since MPS and MPB may be changing as well as intermediary metabolism and *de novo* synthetic rates.

Disuse and Translational Signaling

To date, little work has been done to characterize anabolic signaling in muscle atrophy due to disuse. Short-term immobilization (48 h with a cast that allowed for ambulation) showed a decrease in phosphorylation of members of the PI3K-Akt signaling pathway, with a 21-25% reductions in phosphorylation of Akt (Ser⁴⁷³) and GSK-3 β at (Ser⁹) (217), and recently we have reported a transient 20% drop in mTOR phosphorylation after 2 d of immobilization (Abadi et al., PLoS ONE, *In Press*). Ten to twenty-one days of lower-limb suspension, however, did not change the total or phosphorylated levels, at rest, of the anabolic signaling molecules Akt, TSC2, mTOR, S6K1, 4E-BP1 and eEF2 in the post-absorptive state (47), but phosphorylated FAK was 30% decreased at 10 days. Moreover, 2 wk of immobilization did not change AMPK

Thr¹⁷² phosphorylation or $\alpha 1$, $\alpha 2$ and $\beta 2$ subunit protein levels (62). Together, these findings indicate that, while there may be an early and transient fall in signaling factor phosphorylation, disuse does not markedly affect the total content of proteins of the Akt-mTOR pathway.

Disuse and Catabolic Markers

Far less is known about the regulation of MPB than MPS and our knowledge is mainly confined to measurement of changes in gene expression (mRNA) and, in some instances, protein content. Most studies have focused on changes in components of the pathways thought to degrade protein in muscle. Until recently, despite numerous published reports of changes in gene and protein abundance for components of the pathways, we have lacked concomitant *kinetic* measures of protein degradation in human muscle. In a recent 21d bed rest study, however, increases in atrogen-1 (albeit mild) (35) were not accompanied by increases in MPB (195).

In contrast to the striking and consistent changes in proteolytic components observed in animal models of disuse such as denervation and hindlimb unloading, human responses to disuse via immobilization have proven to be muted and far more variable. Immobilization has been associated with modest (1-2 fold) increases in mRNA for the muscle specific E3 ligases atrogen-1/MAFbx and MuRF1 (40; 106), while spinal cord injury appears to induce more extreme transcriptional changes for these putative regulators of atrophy (~6-12 fold) (216). Furthermore, increased immunohistochemical staining for atrogen (48; 216) and the proteasome core subunit PSMD11 as well as the endogenous protease inhibitor SLP1 (216) at the periphery of fibres has been reported in

SCI and elderly patients (48; 216). Urso and colleagues (216) proposed that the peripheral localization of these proteins may reflect early targeting of the membrane/extracellular matrix in spinal cord injury, but it remains to be determined if a similar process is occurring during disuse atrophy. These situations may be representative of pathological processes only evident under conditions of marked hormonal or cytokine-mediated mechanisms in injury or illness. Interestingly, MuRF1 has been found to target myosin heavy chain (MHC), but subsarcolemmal accumulation of MHC is induced when it is deficient, suggesting that MuRF1 is important in maintaining the normal turnover of contractile proteins (70). In young women, MuRF1 protein selectively increased in soleus after 60d bed rest, but appeared to increase in the cytosol only of ‘subpopulations’ of atrophic fibres in both vastus lateralis and soleus when examined immunohistochemically (183).

Notably, the mRNA contents of the ubiquitin conjugating enzymes (E2D3 and E2N) were actually reported to fall with immobilization (40) and increases in transcripts for deubiquitinating and anti-proteasomal enzymes were reported in a short-term (48 h) model of immobilization (217) and with spinal cord injury (216). Transcript levels of the calpain system does not appear to increase with disuse, as no change or decreases in calpain3/p94, calpastatin, and calpains 1 and 2 have been reported with immobilization (40; 106). Future studies should measure autolysis of the calpains to gain a more accurate understanding of their activation during disuse.

Interestingly, dynamic resistance exercise performed immediately on remobilization following 14 d of casting resulted in a 33-39% decrease in MAFbx and

MuRF1 and increases in calpain 1, calpain 2 and calpastatin mRNA, measured 24h post-exercise (106). Unfortunately in the absence of a control group conclusions cannot be made about the extent to which this response is specific to recovery from immobilization rather than the bout of exercise. However, knowing that exercise increases MPB, the reversal in expression patterns of the two systems would suggest that calpains, at least post-immobilization, may play a greater role in post exercise breakdown than the ubiquitin proteasome system.

To date, the only report of changes in ubiquitinated proteins with disuse in humans is a 20 d bed rest study of young men (154) in which atrophy was accompanied by a fourfold accumulation of ubiquitinated proteins and transcriptional increases in atrogenin (but not MuRF1) and the ubiquitin ligase cbl-6. The accumulation of ubiquitinated proteins would serve as a more direct reflection of a commitment to proteolysis than indirect markers, such as mRNA levels of ubiquitin-proteasome system components (149). Nevertheless it is not a specific marker of protein degradation as the ubiquitin tag can be removed (152). Short-term (72 h) unilateral lower limb suspension was reported to increase post-absorptive interstitial 3-MH concentrations 44% (202), an elevation similar to that which was reported for resting concentrations of 3-MH in the elderly compared to the young (212), despite no other evidence for elevated MPB in the elderly. This is an interesting finding, but one that has recently been criticized for the robustness of the measurement (174) (see also the discussion above in section on use of 3-MH to assess post-exercise MPB). Moreover, the increase in 3-MH concentration with disuse is a finding that is incongruent with the observation that over time the relative concentrations

of mixed and myofibrillar and sarcoplasmic fractions are maintained in the vastus with disuse, as are specific contractile/connective proteins such as actin, myosin and collagen (36; 94; 123), which argues that myofibrillar-specific protein degradation is unlikely.

One hypothesized pathway that is proposed to lead exclusively to myofibrillar protein degradation is the initial cleavage of actin and myosin by activated caspase-3. In this scenario, caspase-3 ‘snips’ myofibrillar proteins to create targets for proteasomal degradation (see section on proteolytic systems). To date, no data exist regarding changes in levels of the caspase-3 mediated actin 14kDa cleavage fragment with simple disuse in humans, but increased levels have been reported in hip arthroplasty, hemodialysis and burn patients, with a significant correlation between the 14kDa fragment concentration and MPB in hip surgery patients, and a decrease in dialysis patients with endurance training (237). However, the catabolic states studied likely involve many confounding factors (ageing, disease, drug, hormonal and inflammatory effects) that would preclude extension of these findings to situations of simple unloading.

In summary, unlike animal models of disuse atrophy, human wasting is not accompanied by substantial increases in ‘catabolic markers’ i.e. components of the ubiquitin-proteasomal system or other proteolytic systems. In spite of increases in ubiquitination and ubiquitin ligases, medium-term disuse (bed rest) does not elevate MPB. More work is required to determine the changes in turnover in the early phase (24-72 h) of disuse, and how they relate to levels of pro-anabolic and pro-catabolic factors.

Study Objectives

The interactive and synergistic effects of feeding and resistance exercise on human MPS are well known (167). The translational signaling response, however, is less well characterized. In the unloaded state, it remains to be determined if the feeding induced rise in muscle protein synthesis is preserved, along with the associated signaling response (72; 126). Finally, very little information is available regarding markers of human muscle protein breakdown or oxidative damage in response to immobilization. Therefore, the overall aim of the studies was to investigate the interaction between changes in activity (acute bout of resistance exercise or 14 d of knee-brace mediated immobilization) and nutritional status (feeding v. fasted) in terms of protein turnover and/or anabolic and catabolic markers. Stable isotope methodology was employed to measure protein turnover (Study 2), immunoblotting to assess changes in translational signaling and proteolytic pathways, and HPLC to measure changes in blood and muscle amino acids.

Specific Studies and Accompanying Hypotheses

Study 1

Aims: To determine the independent and combined effects of feeding and acute resistance exercise on the phosphorylation status of key translational signaling pathway members (mTOR, S6K1, rpS6, Akt, GSK, eIF2B ϵ , FAK) in young trained males.

Hypotheses: We hypothesized that, in line with the known synergistic effect of feeding and resistance exercise on muscle protein synthesis (MPS), phosphorylation of the signaling molecules would also be most enhanced (or in the case of inhibitory

phosphorylation, diminished) in the fed and exercised state. Feeding or exercise alone would have intermediate effects.

Study 2

Aims: To confirm the depression of fasted MPS and determine the effect of disuse on fed state MPS using a 14 d knee brace mediated immobilization model. In addition, to measure the phosphorylation status of key translational signaling proteins in order to determine potential mechanisms that may underlie the synthetic differences between the immobilized and non immobilized legs.

Hypotheses: We hypothesized that while there would still be a rise in MPS in response to amino acid feeding, it would be blunted in magnitude, and possibly also delayed, in the immobilized leg as compared to the non immobilized leg. We expected that this would be reflected in the phosphorylation of translational signaling proteins.

Study 3

Aims: To determine the effect of 2 and 14 d of knee-brace mediated immobilization on selected markers of muscle protein proteolysis (polyubiquinated proteins, 14 kDa actin fragment) and oxidative modification (4-HNE, protein carbonyls) in the pellet fraction of muscle homogenates (males N=13, females N=8).

Hypotheses: Given the lack of increase in MPB with bed rest, we did not expect to see an increase in polyubiquinated proteins or the 14kDa actin fragment, or increases in oxidatively modified proteins.

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Chapter 2: Study 1

Resistance exercise decreases eIF2Bε phosphorylation and potentiates the feeding-induced stimulation of p70^{s6k1} and rpS6 in young men

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Resistance exercise decreases eIF2 β phosphorylation and potentiates the feeding-induced stimulation of p70^{S6K1} and rpS6 in young men

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Glover EI, Oates BR, Tang JE, Moore DR, Tarnopolsky MA, Phillips SM. Resistance exercise decreases eIF2 β phosphorylation and potentiates the feeding-induced stimulation of p70^{S6K1} and rpS6 in young men. *Am J Physiol Regul Integr Comp Physiol* 295: R604–R610, 2008. First published June 18, 2008; doi:10.1152/ajpregu.00097.2008.—We investigated the effect of resistance exercise and feeding on the activation of signaling proteins involved in translation initiation. Nine young men (23.7 ± 0.41 yr; BMI = 25.5 ± 1.0 kg/m²; means \pm SE) were tested twice after they performed a strenuous bout of unilateral resistance exercise, such that their contralateral leg acted as a nonexercised comparator, in either the fasted and fed [1,000 kJ, each 90 min (3 doses; 10 g protein, 41 g carbohydrate, 4 g fat)] states. Muscle biopsies were obtained 6 h postexercise from both legs, resulting in four experimental conditions: rest-fasted, rest-fed, exercise-fasted, and exercise-fed. Feeding increased PKB/Akt (Ser473) phosphorylation ($P < 0.05$), while exercise increased the phosphorylation of Akt and the downstream 70 kDa S6 protein kinase (p70^{S6K1}, Thr389) and ribosomal protein S6 (rpS6, Ser235/236, Ser240/244; all $P < 0.05$). The combination of resistance exercise and feeding increased the phosphorylation of p70^{S6K1} (Thr389) and rpS6 (Ser240/244) above exercise alone ($P < 0.05$). Exercise also reduced phosphorylation of the catalytic epsilon subunit of eukaryotic initiation factor 2B (eIF2 β , Ser540; $P < 0.05$). Mammalian target of rapamycin (mTOR, Ser2448), glycogen synthase kinase-3 β (GSK-3 β , Ser9), and focal adhesion kinase (FAK, Tyr^{576/577}) phosphorylation were unaffected by either feeding or resistance exercise (all $P > 0.14$). In summary, feeding resulted in phosphorylation of Akt, while resistance exercise stimulated phosphorylation of Akt, p70^{S6K1}, rpS6, and dephosphorylation eIF2 β with a synergistic effect of feeding and exercise on p70^{S6K1} and its downstream target rpS6. We conclude that resistance exercise potentiates the effect of feeding on the phosphorylation and presumably activation of critical proteins involved in the regulation of muscle protein synthesis in young men.

hypertrophy; lean body mass; protein accretion; weightlifting

MUSCLE PROTEIN SYNTHESIS (MPS) is synergistically increased by resistance exercise (3, 12, 17, 18, 48, 61) and feeding (4, 25, 51, 57). Although there are resistance exercise-induced changes in muscle protein breakdown (3, 48, 49), they are small by comparison to changes in MPS, which varies 3- to 4-fold between the fed and postabsorptive states and also with performance of resistive exercise (47, 52, 53). These observations point to the regulation of MPS as the primary locus of control in determining resistance exercise-induced changes in muscle protein mass. Moreover, while resistance exercise obviously stimulates transcription of genes relevant to adaptation and ultimately hypertrophy (for review, see Ref. 29), in the

absence of a translational response (i.e., protein synthesis), transcriptional changes will not affect protein content (9, 34).

A number of studies in rodents (21, 22) and humans (5, 14, 17, 18, 20, 32, 36, 37) have characterized signaling proteins that are activated (i.e., dephosphorylated) in skeletal muscle with resistance exercise (18, 20, 37) and in combination with feeding branched-chain amino acids (5, 19, 32), or a mixture of essential amino acids/protein and carbohydrate (14, 17, 36). During recovery from resistance exercise, energy charge of the muscle is restored and phosphorylation of signaling proteins such as mTOR, the 70-KDa ribosomal protein kinase (p70^{S6K1}), and ribosomal protein S6 (rpS6) are increased. Phosphorylation of these proteins can occur in response to contractile activity (18, 20, 37) but also occur with feeding (14, 17, 32, 36). In fact, feeding (i.e., amino acids and/or insulin) per se can increase phosphorylation of many signaling proteins involved in activation of MPS (25, 39–41).

We examined how resistance exercise independently, and superimposed against the background of mixed meal feeding, impacted phosphorylation of proteins thought to be relevant in translation initiation (Akt/PKB, mTOR, p70^{S6K1}, rpS6), ribosomal recycling (eIF2 β , GSK-3 β), and mechanotransduction (FAK). Several studies have demonstrated that the resistance exercise-induced increase in MPS persists for several hours (e.g., 8–48 h) (33, 43, 46, 48, 55). As feeding synergistically interacts with resistance exercise to further increase MPS (4, 46, 51, 57), it is likely that feeding at any time after acute resistance exercise would increase MPS to a greater degree than feeding alone (47). For this reason, a biopsy sampling time point of 6 h following resistance exercise was selected to examine how muscle contractile activity (or skeletal muscle loading) affects the anabolic response to feeding late into recovery. This muscle sampling time allows for a time that we know from previous work when MPS is still elevated (46, 48); however, the anabolic response of feeding alone may be reduced (7). Previous studies have demonstrated that muscle is able to respond to repeated boluses of amino acids, even when plasma levels remain elevated above resting levels (11), presumably because protein synthesis is modulated by changes in extracellular amino acid availability (6). Of note, however, is that MPS becomes refractory to provision of amino acids, when supplied by continuous infusion, after \sim 2 h (7). Thus, in contrast to a single large bolus (17, 19) or intravenous feeding (4), we chose to provide aliquots of a mixed meal supplement every 90 min in an attempt to mimic a more realistic pattern of

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food consumption after exercise. Subjects were studied at rest and after resistance exercise in both the fed and fasted states. Because MPS is synergistically stimulated by feeding and resistance exercise (4, 46, 51, 57), we hypothesized that this response may be underpinned by a feeding and exercise-mediated synergistic stimulation of key signaling proteins involved in supporting enhanced MPS.

METHODS

Subjects. Subjects ($n = 9$ males; age = 23.7 ± 0.4 yr; mass = 80.8 ± 4.0 kg; height = 178 ± 2 cm; BMI = 25.5 ± 1.0 kg/m²; means \pm SE) were recruited locally from the McMaster University campus via poster advertisements. All subjects completed a standard health questionnaire and reported being nonsmokers, were not taking any prescription medication, and were free from any medical conditions that would preclude their participation in a study of exercise and metabolic responses. Subjects were all habitually active and actively resistance trained (average 3 or 4 days/wk) at the time of the study. All subjects gave their written and informed consent prior to any participation in the research study. All procedures were approved by the Hamilton Health Sciences and local McMaster University Research Ethics Board and conformed to the Helsinki Declaration of 1983 on the use of human subjects in research.

Study design. A week before any experimental trials, all subjects participated in a familiarization session where their unilateral 10 repetition maximum (RM) was determined for each leg for both leg press and knee extension exercises. Subjects reported, after having consumed no food after 2200 the evening prior, to the Exercise Metabolism Research Laboratory at 0700 on two different occasions separated by at least 2 wk. Each subject completed the fasted or fed condition trials in a randomized and counter-balanced manner. Subjects began each trial by performing a bout of unilateral leg press and knee extension exercise (4 sets of each at a workload equivalent to their previously determined 10 RM), which was again randomly assigned but counterbalanced based on voluntary strength. In this way, within two trials, we obtained legs that represented one of four conditions: rest-fasted (Rest-Fast), exercise-fasted (Ex-Fast), rest-fed (Rest-Fed), and exercise-fed (Ex-Fed).

Following the bout of exercise, subjects had a 20-gauge catheter inserted into a prominent dorsal hand vein; the catheter was kept patent by means of a 0.9% saline drip. Arterialized blood samples were obtained by warming the hand with a heating blanket ($\sim 50^\circ\text{C}$)

with all blood samples being drawn into heparinized tubes every 30 min postexercise (Fig. 1). During the fed trial, subjects consumed a mixed-meal drink (Boost, Novartis Nutrition, Mississauga, ON, Canada) containing 1,000 kJ and 10 g protein (as casein proteins), 41 g carbohydrate (as maltodextrin and sucrose), and 4 g of fat (as soy oil) at $t = 90, 180,$ and 270 min postexercise. Muscle samples were obtained from the vastus lateralis of both legs within 10 min of each other at $t = 360$ min postexercise. Biopsies were taken under local anaesthetic (2% lidocaine) using a 5-mm Bergstrom needle modified for manual suction. Upon excision, samples were blotted free of blood and immediately snap frozen in liquid N₂. Samples were stored at -80°C until analysis.

Blood analyses. Whole blood was precipitated in 500 μl of 0.6 M perchloric acid (PCA) and neutralized by the addition of 1.25 M KHCO₃ as previously described (45, 59). Plasma was obtained from the remaining whole blood by centrifugation (4500 g for 10 min at 4°C). All blood samples were stored at -20°C until analysis. Plasma was extracted in PCA, as described above and analyzed for amino acid content using HPLC methods that have been described previously (59). Whole blood glucose concentrations were determined using fluorometric methods (42). Plasma insulin was analyzed using a commercially available radioimmunoassay kit (Diagnostic Products, Los Angeles, CA). Inter-run and intra-run CVs for these assays were all less than 5%.

Western Blot analyses. A small piece of wet muscle (~ 20 mg) was homogenized by hand on ice in a 20 mM Tris (pH 7.2) buffer containing 1 mM Na₂VO₄, 50 mM NaF, 40 mM β -glycerolphosphate, 20 mM sodium pyrophosphate, 0.5% vol/vol Triton-X 100, and Complete Protease Inhibitor Mini-Tabs (Roche, Indianapolis, IN). Protein content of the homogenates was determined by the Bradford assay.

Samples (50 μg of protein) were loaded on 7.5 or 10% SDS-polyacrylamide gels and then transferred to a PVDF membrane. Membranes were blocked with 5% BSA (wt/vol) in Tris-buffered saline with 0.1% Tween (vol/vol) (TBST), and then incubated overnight in primary antibody overnight at 4°C : FAK Tyr^{576/577} (Santa Cruz Biotechnology, Santa Cruz, CA; no. 21831R, 1:1,000); total FAK (Santa Cruz Biotechnology, no. 558, 1:1,000); p70^{S6K1} Thr³⁸⁹ (Santa Cruz Biotechnology; no. 11759, 1:1,000); total p70^{S6K1} (Santa Cruz Biotechnology, no. 230, 1:1,000); GSK3 β Ser⁹ (Cell Signaling Technology, Danvers, MA; 9336; 1:2,000); total GSK3 β (Cell Signaling Technology, no. 9332; 1:2,000); rpS6 Ser^{235/236} (Cell Signaling Technology, no. 2211; 1:2,000); total rpS6 (Cell Signaling Tech-

Exercise: Unilateral leg press and knee extension (4 sets x 10 reps)

Drink (FED condition only): Boost (1 can)

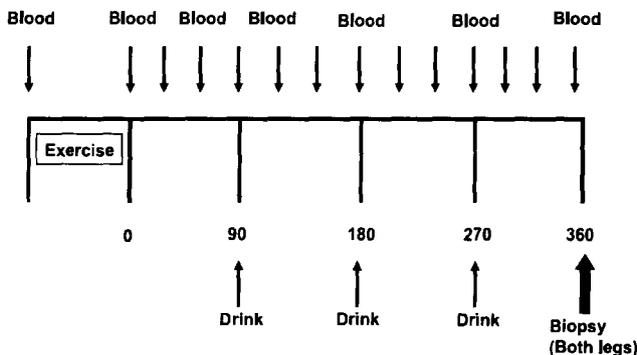


Fig. 1 Schematic representation of the study protocol

R606

SIGNALING PROTEINS WITH RESISTANCE EXERCISE

nology; no. 2217; 1:1,000); mTOR Ser²⁴⁸ (Cell Signaling Technology; no. 2971; 1:1,000); total mTOR (Cell Signaling Technology; no. 2972; 1:1,000); PKB/Akt Ser⁴⁷³ (Cell Signaling Technology; no. 9271; 1:1,000); total PKB/Akt (Cell Signaling Technology; no. 9272; 1:1,000); eIF2 β Ser⁵⁴⁰ (Genetex, San Antonio, TX; no. GTX24775; 1:8,000); total eIF2 β (Abcam, Cambridge, MA; no. ab32713; 1:1,500). After washing in TBST, membranes were incubated in HRP-linked anti-rabbit IgG secondary antibody (Amersham Biosciences, Piscataway, NJ; no. NA934V; 1:6,000), washed with TBST, developed using ECL (Amersham Biosciences; model no. RPN2106), and wrapped in Saran Wrap. Membranes were exposed to X-ray film (Kodak, Rochester, NY). Films were scanned with a CanoScan N1220U scanner, and bands were quantified with AlphaEase Fluorchem SP software (Alpha Innotech, San Leandro, CA). Membranes were probed with antibodies against the phosphorylated forms first, then incubated with stripping buffer (25 mM glycine-HCl pH 2.0, 1% SDS), and washed before incubation with primary antibody against total protein, with the exception of rpS6, for which the total form was probed first.

Statistical analyses. Amino acid, glucose, and insulin data were analyzed using a two-factor (feeding vs. time) repeated-measures ANOVA. Data were analyzed using a 2-way ANOVA (feeding and exercise as factors). Significant effects were analyzed with post hoc preplanned comparisons using a Student's *t*-test. Significance was accepted at $P < 0.05$. All data are expressed as means \pm SE.

RESULTS

Insulin and glucose. Subjects ingested a total of 30 g of protein, 123 g of carbohydrate, and 12 g of fat (3,000 kJ or 37 kJ/kg) over the course of the protocol. Ingestion of the supplement drink every 90 min resulted in increments in blood glucose and plasma insulin above those seen in the fasted trial that coincided with meal consumption. Elevations in blood glucose and plasma insulin are shown in Fig. 2, A and B. The area under the insulin concentration by time curve in the fed state was 269% greater than in the fasted trial (which represents the response of insulin to exercise alone).

Amino acids. Increases in whole blood amino acid concentration were seen following each bolus drink. Changes in essential amino acid (EAA) were greater in the fed than in the fasted trial (Fig. 3). Changes in leucine, branched chain, and total amino acids followed the same pattern (data not shown).

Signaling proteins. Phosphorylation (Ser⁴⁷³) of PKB/Akt was increased in the rested condition due to feeding (Fig. 4A) and was also elevated by exercise in the fasted state. mTOR itself was not phosphorylated (Ser²⁴⁸) to any significant degree due to either feeding or exercise (Fig. 5B). Downstream of mTOR, the phosphorylation of p70^{S6K1} (Thr³⁸⁹) was robustly increased by exercise, with a further increase seen with feeding (Fig. 4C). The p70^{S6K1} target protein rpS6 was also phosphorylated (Ser^{235/236}) as a result of exercise, with no additional effect of nutritional provision on the Ser^{235/236} site; however, as with p70^{S6K1}, we observed that nutritional provision did result in increased phosphorylation of rpS6 Ser^{240/244} beyond the activation induced by exercise alone (Fig. 4D). Phosphorylation of eIF2 β at Ser⁵⁴⁰ strongly decreased in response to exercise regardless of nutritional state, with no effect of feeding at rest (Fig. 5A). Glycogen synthase kinase-3 β phosphorylation (Ser⁹) was also measured and found not to be affected by either nutrition or exercise or the combination of the two interventions (Fig. 5C). Similarly, we did not see any change in the phosphorylation status of focal adhesion kinase (FAK, Tyr^{576/577}; Fig. 5D).

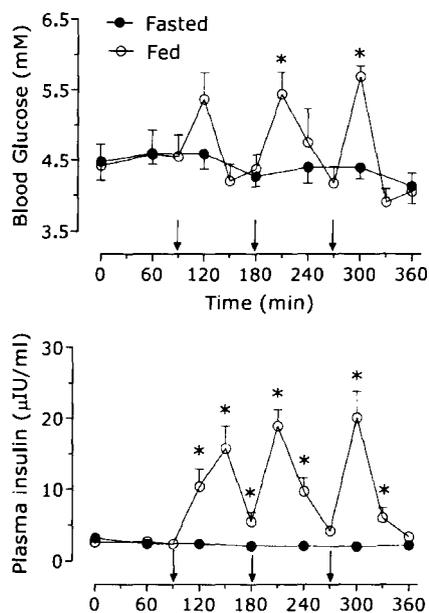


Fig. 2. Plasma glucose (A) and plasma insulin (B) during the protocol. Values are expressed as means \pm SE. *Significantly different ($P < 0.05$) from the same time point during the fasted-state trial. Time-dependent changes are omitted for clarity.

DISCUSSION

We report here for the first time that resistance exercise is a potent stimulus for modifying the phosphorylation of eIF2 β in humans. PKB/Akt, p70^{S6K1}, and rpS6, known regulators of MPS, are activated in response to exercise, while a sustained (insulin mediated) feeding effect is seen for PKB/Akt. Amino acid provision following resistance exercise synergistically stimulates MPS (4, 17, 46), and this combined effect was observed for the phosphorylation of p70^{S6K1} and rpS6, even 6 h after exercise and 1.5 h after feeding.

In the first stage of translation initiation, eIF2 recruits the initiator methionyl-tRNA to the 40S ribosomal subunit, a process that requires GTP. eIF2B catalyzes the exchange of GDP for GTP on eIF2, renewing eIF2's tRNA, binding capacity (34). Recent data (35, 38) have suggested that "global" protein synthesis, which would appear to be stimulated by both feeding and resistance exercise (13, 14, 44), is primarily regulated by the activation of eIF2B. For instance, Farrell et al. (21, 22) has reported, in a rat model of plyometric resistance (approximating resistance exercise), that eIF2B activity was increased, whereas eIF4E-4G complex formation was not and thus eIF2B appeared to be more important than 4E binding 4G in regulating peptide chain initiation after resistance exercise. As such, we sought to examine the GSK-3 β -mediated phosphorylation of eIF2B's catalytic ϵ subunit at Ser⁵⁴⁰ (rat Ser⁵³⁵) (58). Our results show for the first time in humans that resistance exercise results in a striking dephosphorylation of eIF2 β at Ser, regardless of feeding status. Reduced phosphorylation at residue Ser⁵⁴⁰ releases eIF2 β from an inhibitory state and would likely contribute to an increase in its guanine

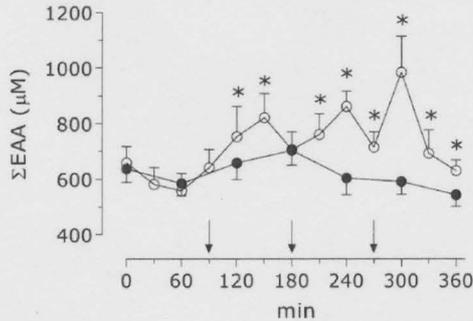


Fig. 3. Plasma concentration of sum of essential amino acids during the protocol (leucine, isoleucine, valine, lysine, histidine, methionine, threonine, and phenylalanine). Values are presented as means \pm SE. *Significantly different from the same time point during the fasted-state trial, $P < 0.05$. Time-dependent changes are omitted for clarity.

nucleotide exchange activity (58). Considering our measure of phosphorylation was made at a time when muscle protein synthesis would be markedly elevated (46, 48), these results suggest that, similar to rats (21, 22), eIF2B also plays an important role in the regulation of protein synthesis in response to resistance exercise in humans.

Interestingly, neither feeding nor resistance exercise impacted GSK3 β 's phosphorylation status. It may not be overly surprising that feeding did not affect the phosphorylation of this protein, since Liu et al. (40) did not see any effect of infused amino acids or low-dose insulin on GSK-3 β phosphorylation. Blomstrand et al. (5) also reported little effect of exercise on GSK3 β , either in the absence or presence of branched chain amino acids. It is possible that our failure to observe reciprocal changes in GSK3 β and eIF2B phosphorylation is the result of the timing of our biopsy sample, whereby Akt-mediated changes in GSK3 β may have returned to baseline with their effects still manifested in the phosphorylation state of eIF2B. Drummond et al. (19) recently reported a reduction in GSK3 β phosphorylation 5 h after an EAA drink consumed 1 h postexercise, a finding that is difficult to interpret given that Akt phosphorylation transiently increased in that study, and a reduction in GSK3 β 's phosphorylation would predictably increase its activity. Inactivation of eIF2B by GSK3 β first requires the phosphorylation of its epsilon subunit at a number of different residues (58). It is possible that the phosphorylation of these priming sites is reduced by resistance exercise, thereby interfering with the ability of GSK to repress the activity of eIF2B through phosphorylation at residue Ser⁵⁴⁰. Alternatively, Kimball et al. (34) has suggested that exercise may modulate eIF2B activity through dephosphor-

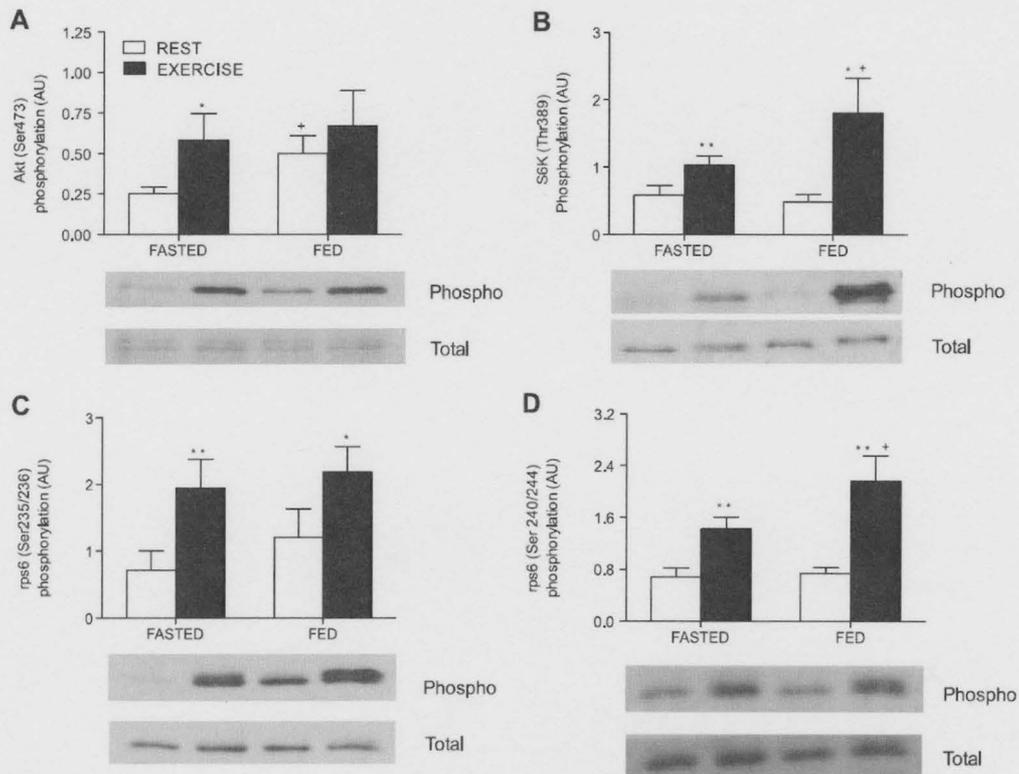


Fig. 4. Phosphorylated/total of PKB/Akt (Ser⁴⁷³) (A), p70^{S6K1} (Thr³⁸⁹) (B), rps6 (Ser^{235/236}) (C), and rps6 (Ser^{240/244}) (D). Values are expressed as means \pm SE. Significantly different from REST in the same nutritional condition, * $P < 0.05$, ** $P < 0.01$; + significantly different from FAST in the same activity state, $P < 0.05$.

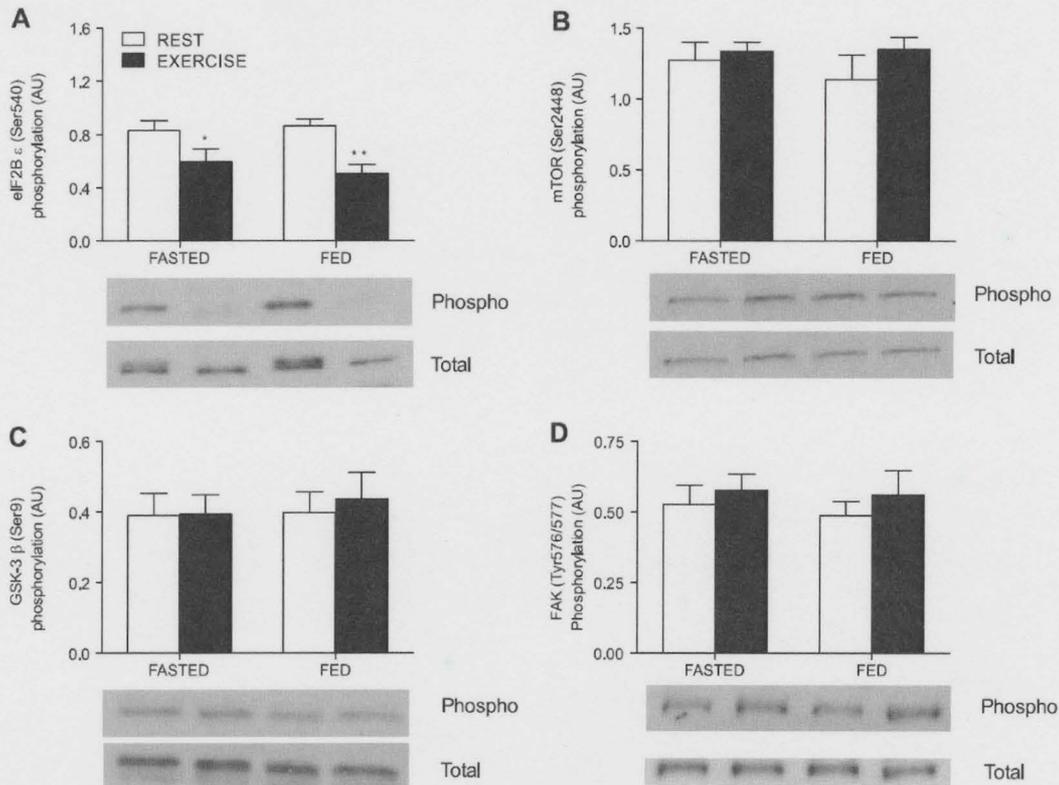


Fig. 5. Phosphorylated/total of eIF2B ϵ (Ser⁵⁴⁰) (A), mTOR (Ser²⁴⁴⁸) (B), GSK-3 β (Ser⁹) (C), and FAK (Tyr^{576/577}) (D). Values are expressed as means \pm SE. Significantly different from REST in the same nutritional condition. * $P < 0.05$, ** $P < 0.01$.

ylation of the epsilon subunit. IGF-1-induced activation of eIF2Bepsilon in neuronal cells is mediated by dephosphorylation by protein phosphatase 1 (50). Future studies should investigate whether exercise-induced decreases in phosphorylation of eIF2B ϵ are mediated by a pathway independent of GSK3 β .

Several studies in humans have recently examined the effect of resistive-type exercise on the phosphorylation of a number of proteins believed to regulate MPS, either in the fasted state (5, 17, 18, 20, 32, 37) or in conjunction with amino acids/protein provision with (16, 17, 36) and without (5, 19, 32) carbohydrate. Data from these studies indicate that mTOR signaling is elevated early (1–2 h) after a bout of resistance exercise (17, 18), with additional enhancement from amino acid and carbohydrate feeding (5, 17). In agreement with these studies, we found that the phosphorylation of p70^{S6K1} was robustly stimulated by exercise, with or without feeding. In light of the strong relationship between p70^{S6K1} phosphorylation and hypertrophy in rodents (2) and humans (56), our observation is perhaps not surprising. Rodent data suggest that leucine alone is stimulatory for the phosphorylation of p70^{S6K1} and its downstream target rpS6 (1) but that the phosphorylation of rpS6 is more persistent and present long after the phosphorylation of p70^{S6K1} has returned to baseline. This suggests that other pathways likely also affect the phosphorylation state of rpS6. Altered phosphatase activity, as may be the case for

eIF2Bepsilon, could promote the prolonged hyperphosphorylated state of rpS6 postexercise. We found that resistance exercise enhanced the phosphorylation of rpS6 at Ser^{240/244} in response to feeding. Moreover, our data also show that phosphorylation at Ser^{235/236} is increased in response to exercise alone. This effect could be mediated through p90rsk, which exclusively phosphorylates rpS6 at Ser^{235/236} in an mTOR-independent manner (54), thereby providing a link to the upstream Ras/ERK signaling pathway, which is known to be activated by exercise (19, 32, 60).

We had hypothesized that the synergistic stimulation of MPS would be reflected in greater phosphorylation/dephosphorylation of the signaling proteins that are required to activate protein synthesis (8, 9, 17, 36, 38). In the present study, the effect of feeding was only evident at the level of PKB/Akt, suggesting that this sustained response was insulin mediated. Increased Akt phosphorylation after amino acid feeding has only been shown in studies when insulin increased or was elevated [compare (25, 28) with (27, 39)]. Our data for p70^{S6K1} and rpS6 (Ser^{240/244}) support the concept that resistance exercise plus nutritional provision results in an additive phosphorylation (i.e., greater than exercise or feeding alone). However, upstream members of the pathway (i.e., PKB/Akt and mTOR) did not display enhanced phosphorylation with the combined stimulus. That is not to say that the combination of resistance exercise and feeding do not synergistically activate PKB/Akt

and mTOR, but rather, we acknowledge that our decision to obtain biopsies 6 h postexercise may have resulted in our failure to observe changes in phosphorylation status of these proteins (10). However, several studies have shown increased p70^{S6K1} Thr³⁸⁹ phosphorylation in the absence of elevated Ser²⁴⁸ phosphorylation of mTOR (5, 20, 30, 32).

Focal adhesion kinase (FAK) has been shown to be responsive to loading and unloading in rodent models (23, 24, 26) and cyclic mechanical stretch in cell culture (62). As such, the acute regulation (i.e., phosphorylation) of FAK following intense resistance exercise could be a potential link in the mechanotransduction of a loading stimulus to induce increases in MPS. Such a possibility is predicted by the cellular "tensegrity" model, in which focal adhesion complexes anchor cytoskeletal proteins to the extracellular matrix of the cell to transmit forces and activate relevant signaling cascades, reviewed in Ref. 31. Our data showed that at 6 h postexercise, we could detect no changes in FAK phosphorylation (Tyr^{576/577}). In the absence of any other human data examining this protein, we are unable to speculate why we observed this result since overload in animal models robustly increases not only FAK content but also FAK tyrosine phosphorylation, whereas unloading rapidly reduces FAK content and phosphorylation (15, 23, 26). It may be that increases in FAK activation occur in close temporal proximity to contraction itself or in response to chronic loading; thus, our stimulus may have been insufficient in magnitude or duration to elicit a response at 6 h postexercise.

Perspectives and Significance

Our data show, for the first time in humans, that resistance exercise reduces the phosphorylation of eIF2 β , which is a potential mechanism that underlies the rise in muscle protein synthesis that occurs with this stimulus. Moreover, we have confirmed that resistance exercise is a potent activator of several key regulatory signaling proteins known to be critical in the regulation of MPS. Furthermore, resistance exercise potentiates the effect of feeding on the activation of p70^{S6K1} and rps6, up to 6 h postexercise; this may explain the resistance exercise-induced increase in sensitivity to amino acid feeding. Clearly, future studies would benefit from delineating the earlier time-dependent changes in phosphorylation of these proteins in addition to characterizing changes in MPS.

GRANTS

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Chapter 3: Study 2

Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion

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We tested the hypothesis that increasing blood amino acid (AA) availability would counter the physical inactivity-induced reduction in muscle protein synthesis. We determined how 14 days of unilateral knee immobilization affected quadriceps myofibrillar protein synthesis (MPS) in young healthy subjects (10 men, 2 women, 21 ± 1 years; 80.2 ± 4.0 kg, mean \pm s.e.m.) in the post-absorptive state and after infusing AA (10% Primene) at low or high doses (43 and 261 mg kg⁻¹ h⁻¹). Muscle cross-sectional area (MRI) and peak isometric torque declined in the immobilized leg ($-5.0 \pm 1.2\%$ and $-25 \pm 3\%$, respectively, both $P < 0.005$), but were unchanged (all $P > 0.6$) in the non-immobilized leg. Immobilization induced a 27% decline in the rate of post-absorptive MPS (immobilized, 0.027 ± 0.003 ; non-immobilized, $0.037 \pm 0.003\%$ h⁻¹; $P < 0.001$). Regardless of dose, AA infusion stimulated a greater rise in MPS in the non-immobilized legs; at 4 h MPS was greater by $+54 \pm 12\%$ with low dose and $+68 \pm 17\%$ with high dose AA infusion (both $P < 0.001$). There was some evidence of delayed responsiveness of phosphorylation of Akt to high doses of AA and p70S6k at both doses but no marked differences in that of mTOR, GSK3 β or eEF2. Phosphorylation of focal adhesion kinase (Tyr^{576/577}) was reduced ($P < 0.05$) with immobilization. We observed no change in polyubiquitinated protein content after immobilization. We confirm that 14 days of immobilization reduces MPS in the post-absorptive state and this diminution is reduced but not abolished by increased provision of AA, even at high rates. The immobilization-induced decline in post-absorptive MPS with the 'anabolic resistance' to amino acids can account for much of immobilization-induced muscle atrophy.

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Disuse atrophy is characterized by a reduction in muscle fibre cross sectional area. The consequences of inactivity-induced muscle wasting are reductions in strength and muscle quality (for review see Adams *et al.* 2003), with deleterious effects on quality of life and independence. Furthermore, this reduction in metabolically active lean tissue results in decreases in the capacities of whole-body glucose storage and metabolism (Stein & Wade, 2005; Wolfe, 2006), which contribute to insulin resistance, and a lower whole-body metabolic rate (Johnstone *et al.* 2005). Disuse of human muscle has been studied after a variety of interventions including bed rest,

casting, limb suspension and spaceflight (Adams *et al.* 2003). Whenever human muscle protein synthesis has been measured after inactivity, a marked decline has been observed in fasted-state muscle protein synthesis (MPS) (Gibson *et al.* 1987; Ferrando *et al.* 1996; Paddon-Jones *et al.* 2006; de Boer *et al.* 2007). The reduced fasted-state MPS occurs relatively early in immobilization (10 days) and does not decline further (de Boer *et al.* 2007). However, the possible effect of immobilization on the stimulation of MPS to essential amino acids, the primary drivers of anabolism (Bohe *et al.* 2001, 2003; Fujita *et al.* 2007), remains unstudied. The expected increase in whole-body protein synthesis in response to amino acid feeding is impaired after bed rest (Biolo *et al.* 2004), which was interpreted as being due to a reduction in muscle

This paper has online supplemental material.

protein synthesis, but this hypothesis was not tested by those authors.

Regulation of MPS on an hour-to-hour basis is predominantly at the translational level with changes in phosphorylation of the Akt–mTOR–p70S6 pathway proteins playing a critical role (for reviews see Kimball *et al.* 2002; Wang & Proud, 2006; Proud, 2007). Such signalling proteins in human muscle are normally responsive to feeding (Fujita *et al.* 2007), high-intensity exercise (Dreyer *et al.* 2006; Eliasson *et al.* 2006) and combinations of the two (Karlsson *et al.* 2004; Cuthbertson *et al.* 2006; Dreyer *et al.* 2008). However, 10 or 21 days of immobilization of human muscle are reported to have no effects on the states of phosphorylation of components of this pathway (Akt/PKB, TSC-2, p70S6 and 4EBP1) in the post-absorptive state in humans (de Boer *et al.* 2007) or rats (Vargas & Lang, 2008).

Previous work on disuse atrophy of human muscle (de Boer *et al.* 2007) showed that focal adhesion kinase (FAK) phosphorylation was reduced with immobilization. As a mechanically sensitive transduction protein (Fluck *et al.* 1999; Gordon *et al.* 2001), we wished to measure FAK phosphorylation to possibly demonstrate a functional link between reduced FAK phosphorylation and dampened responsiveness of the Akt–mTOR–p70S6 pathway to feeding.

We aimed to test the hypothesis that during immobilization, amino acid-induced stimulation of human myofibrillar protein synthesis (MPS) and anabolic signalling would be preserved, tending to counter the immobilization-induced deficit in the rate of post-absorptive MPS, but that the effects would not restore the rate to that observed in the non-immobilized leg after amino acid feeding.

Methods

Subjects

Twelve recreationally active (i.e. exercise ≤ 2 days week⁻¹) men ($n=10$, 21 years, 81 kg, 24.7 kg m⁻²) and women ($n=2$, 21 years, 82 kg, 27.3 kg m⁻²) participated in the study. Subjects were screened to exclude smokers, females taking oral contraceptives, any person with lower-limb injury within 1 year prior to the start of the study, or family history of thrombosis. All subjects underwent 14 days of unilateral knee-brace-mediated immobilization; see Yasuda *et al.* (2005) for details. Subjects were divided into two groups of six (5 men and 1 woman in each) to receive either a low or a high dose (43 or 261 mg kg⁻¹ h⁻¹) amino acid infusion after 14 days of immobilization, so the groups were equivalent until they were infused with amino acids. The groups were similar in terms of mean lean mass (low dose: 57.9 \pm 2.9 kg; high dose: 58.0 \pm 4.5 kg), total body mass (low dose: 79.2 \pm 5.3 kg; high dose:

80.3 \pm 6.5 kg) and BMI (low dose: 24.3 \pm 1.3 kg m⁻²; high dose: 24.8 \pm 1.2 kg m⁻²) (all $P > 0.78$). The study was approved by the McMaster University and the Hamilton Health Sciences Research Ethics Boards according to the *Declaration of Helsinki* and informed written consent was obtained from each participant before each study.

Muscle size and function tests

All subjects were familiarized with the muscle strength testing procedure at least 1 week before beginning. Each subject's voluntary single-repetition maximum (1-RM) for isometric knee extension was determined on the morning of immobilization (day 1) and after the infusion trial on day 15 using the Biodex System 3 (Shirley, NY, USA). Prior to and on day 14 of immobilization, measurements were made using dual-energy X-ray absorptiometry (DXA; Hologic 4500A, Bedford, MA, USA) for leg lean mass, and magnetic resonance imaging (MRI) for mid-thigh cross sectional area (CSA). MRI was performed in a 3 Tesla HD scanner (Sigma MRI system, GE Medical, Milwaukee, WI, USA) at the Brain–Body Institute, Imaging Research Centre, St Joseph's Healthcare (Hamilton, ON, Canada). Image acquisition was carried out using a T1 flair in the axial plane with the following parameters: repetition time/echo time, 2574 ms/6.7 ms; field of view, 25–30 cm; matrix size, range from 320/320 to 512/512 phase/frequency; inversion time, 958 ms; slice thickness, 5 mm. Thigh image acquisition utilized an 8-channel torso coil with two excitations (EX). During the pre-immobilization scan, the distance from a bony landmark to the first axial scan was recorded. This distance was used in the post-immobilization scan to ensure identical positioning. The MRI image analysis was performed using Medical Image Processing, Analysis and Visualization (MIPAV) software (downloaded with permission from the National Institutes of Health; <http://mipav.cit.nih.gov/>).

Experimental protocol

We chose to use 14 days of unilateral knee immobilization. The knee chosen to be immobilized was identified randomly, counter-balanced for dominance based on strength in each leg such that six subjects each had their stronger and thus six had their weaker legs immobilized. Testing was performed after immobilization using the non-immobilized leg as a control.

On the first day of immobilization, subjects arrived in the laboratory by 08.00 h and after strength testing, were fitted with a knee immobilization brace (Donjoy IROM; Vista, CA, USA) and instructed in the use of the provided set of crutches. The Velcro straps of the brace

were bound with plastic adhesive tape over which the investigators' signatures were inscribed. Breaking the tape seal, so the brace could be adjusted or removed, would thus render the tape irreplaceable without damaging the signature, ensuring compliance with the immobilization procedures. Subjects returned to the lab daily throughout the immobilization period, when they were permitted to remove the brace, under supervision, for approximately 15 min to allow visual inspection of the immobilized leg and knee brace. Any signs of chaffing or swelling were noted, the brace was adjusted as necessary, re-applied and secured as described above. All subjects found the brace tolerable, and none reported any adverse events such as tightness or swelling.

Infusion and sampling protocol (Fig. 1)

Participants reported to the laboratory at 06.30 h after an overnight fast. The immobilizing knee brace was removed and subjects remained supine for the remainder of the infusion protocol. One catheter was inserted in the medial vein of one arm for tracer infusion and the other was inserted in a dorsal hand vein for arterialized blood sampling. Arterialized blood samples were obtained by wrapping the hand and forearm in a heating blanket warmed to 45 °C for the duration of the infusion. Baseline blood samples were drawn and then participants received priming doses of L-[ring- $^{13}\text{C}_6$]phenylalanine ($2 \mu\text{mol kg}^{-1}$, 99 atom %; Cambridge Isotopes) prior to beginning a constant infusion of L-[ring- $^{13}\text{C}_6$]phenylalanine ($0.05 \mu\text{mol kg}^{-1}$,

99 atom %; Fig. 1). L-[ring- $^{13}\text{C}_6$]phenylalanine was added to the amino acid mixture (Primene) to maintain plasma enrichments. Arterialized blood samples were collected every 0.5–1 h into evacuated heparinized tubes (Fig. 1) and chilled on ice. Blood was processed to yield perchloric acid extracts and plasma as previously described (Tang *et al.* 2007).

Muscle biopsies were taken from the vastus lateralis using a 5 mm Bergström needle (custom modified for manual suction) under 2% xylocaine local anaesthesia. Muscle biopsies were freed from any visible blood, fat and connective tissue and rapidly frozen in liquid nitrogen for further analysis. Four muscle biopsies were taken from each leg, but at a different incision site at least 4–5 cm proximal to the previous incision, at 150, 210, 270 and 390 min after start of the L-[ring- $^{13}\text{C}_6$] phenylalanine infusion for a given trial.

Blood sample analysis

The PCA blood extract was analysed for amino acid concentrations as described (Wilkinson *et al.* 2007). Blood L-[ring- $^{13}\text{C}_6$] phenylalanine enrichments were determined by heptafluorobutyric acid (HFBA) derivatization of cation exchange resin-purified PCA plasma extracts and subsequent quantification using capillary gas chromatography–electron impact ionization–quadrupole mass spectrometry (GC/MS; GC Hewlett Packard 6890, Palo Alto, CA, USA; MS Agilent 5973, Palo Alto) by monitoring ions at m/z (mass/charge) 316 and 322. Insulin levels were determined using a

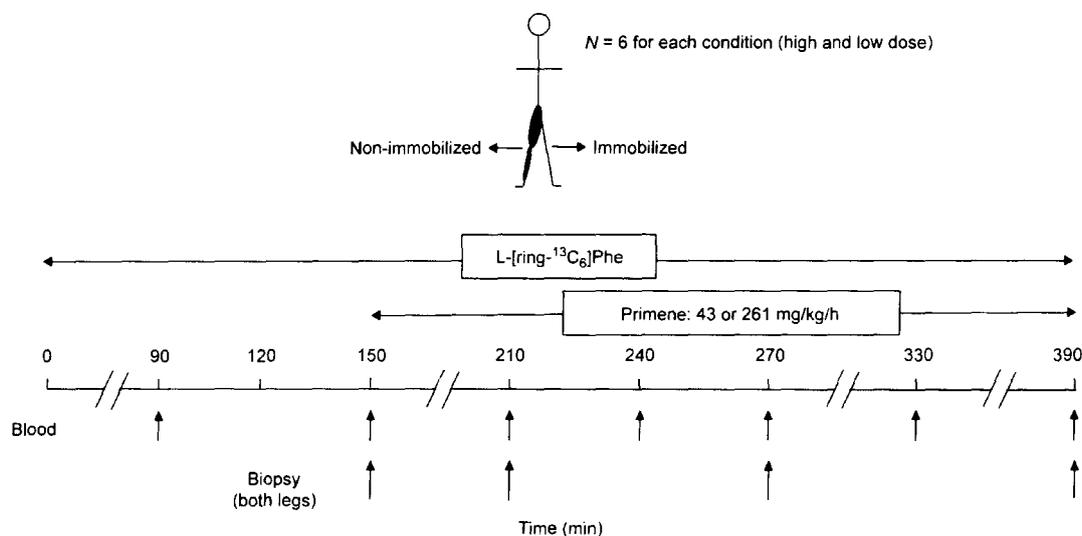


Figure 1. Schematic representation of the infusion protocol

commercially available radioimmunoassay kit from Diagnostic Products Corporation (Los Angeles, CA, USA). Coefficients of variation (CV) for this assay did not exceed 7% for between-sample duplicates. Blood glucose was measured using a standard assay as previously described (Moore *et al.* 2005; Wilkinson *et al.* 2007), with duplicate sample CV of less than 4%.

Muscle tissue analysis

Sarcoplasmic and myofibrillar muscle proteins were isolated from biopsy specimens (~40–50 mg wet weight) by homogenizing in 7.5 $\mu\text{l mg}^{-1}$ homogenization buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.1% 2-mercaptoethanol, protease inhibitor tablet per 10 ml (Roche, Indianapolis, IN, USA), 10 mM β -glycerophosphate, 0.5 mM Na_2VO_4) on ice. The samples were vortexed and centrifuged (950 g, 10 min, 4 °C). The supernatant was removed and the pellet re-spun (6550 g, 3 min, 4 °C) to remove remaining supernatant. Supernatant concentrations were determined by Bradford assay (Sigma). Aliquots were diluted to 3 $\mu\text{g } \mu\text{l}^{-1}$ with homogenization buffer and 5 \times Laemmli buffer, boiled at 100 °C for 5 min, then stored at –80 °C until Western blot analysis (see below). The remaining pellet was washed with homogenization buffer and centrifuged (6550 g, 3 min, 4 °C). The supernatant was discarded and the pellet incubated with 0.3 M NaOH (30 min, 37 °C) and centrifuged (950 g, 10 min, 4 °C). This was repeated and the supernatants were pooled. PCA (1 M) was added to precipitate protein, samples vortexed and centrifuged as above, then the pellet was washed twice with 70% ethanol and allowed to dry. Myofibrillar pellets were hydrolysed overnight in a slurry of 0.1 M HCl and Dowex 50WX8–200 resin (110 °C; Sigma, St Louis, MO, USA). Free amino acids were purified using cation exchange chromatography (Dowex 50WX8–200 resin) and converted to their *N*-acetyl-*n*-propyl ester derivatives for analysis by gas chromatography combustion–isotope ratio mass spectrometry (Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnigan). To estimate ^{13}C background enrichment in body phenylalanine, plasma protein from the baseline blood sample was isolated by precipitation with ice-cold ethanol, centrifuged, and washed with 70% ethanol. The pellet was then processed as described for the myofibrillar pellet.

Intracellular amino acids were extracted from a separate ~20 mg piece of muscle by incubation in ice-cold (12.5 $\mu\text{l mg}^{-1}$) 0.6 M PCA and 62.5 ng mg^{-1} norleucine with agitation for 10 min at 4 °C. Samples were centrifuged (15000 g, 2 min, 4 °C) and extraction was repeated with (7.5 $\mu\text{l mg}^{-1}$) 0.6 M PCA. The pooled extract was neutralized with 1.25 M KHCO_3 , centrifuged and transferred to fresh tubes. Aliquots (30 μl) were set aside for

determination of intracellular amino acid concentrations as previously described (Wilkinson *et al.* 2006). The remainder of the extract was purified by cation exchange chromatography and derivatized with HFBA as above to obtain intracellular enrichments.

Samples of protein homogenate (30–60 μg) were loaded on to 7.5% (mTOR, FAK, eEF, p70s6 k) or 10% (Akt, GSK3 β) SDS–polyacrylamide gels prior to being transferred to a PVDF membrane for blotting. Membranes were blocked with 5% BSA (w/v) in Tris-buffered saline with 0.1% Tween (v/v) (TBST) and then incubated overnight in primary antibody at 4 °C: Akt Ser⁴⁷³ (Cell Signalling Technology, Danvers, MA, USA, no. 4058, 1: 4000); total Akt (Cell Signalling, no. 9272; 1: 4000); mTOR Ser²⁴⁴⁸ (Cell Signalling, no. 2971; 1: 1000); total mTOR (Cell Signalling, no. 2972; 1: 1000); p70S6K1 Thr³⁸⁰ (Santa Cruz Biotechnology, no. 11759; 1: 5000); total p70S6K1 (Santa Cruz, no. 230, 1: 4000); GSK-3 β Ser⁹ (Cell Signalling, no. 9336; 1: 2000); total GSK-3 β (Cell Signalling, no. 9332; 1: 6000); eukaryotic elongation factor-2 (eEF2) Thr⁵⁶ (Cell Signalling, no. 2331; 1: 3000); total eEF2 (Cell Signalling, no. 2332; 1: 3000); FAK Tyr^{557/577} (Santa Cruz, no. 21831, 1: 16000), total FAK (Santa Cruz, no. 558; 1: 8000). After washing in TBST, membranes were incubated in HRP-linked anti-rabbit IgG secondary antibody (Amersham Biosciences, Piscataway, NJ, USA, product NA934V; 1: 10000), washed with TBST, and developed using SuperSignal West Dura substrate (Pierce) and wrapped in Saran wrap. Signals were digitally captured on a Fluorochem SP imaging system and quantified using AlphaEase Fluorochem SP software (Alpha Innotech, San Leandro, CA, USA). Membranes were also probed for actin (BD Biosciences, Mississauga, ON, Canada; no. 612656; 1: 10000, anti-mouse secondary (Amersham Biosciences, Piscataway, NJ, USA; NA931, 1: 10000). Phosphorylated proteins are expressed relative to actin, except for FAK, which is expressed relative to total protein. Actin concentrations of the vastus lateralis have not been found to change with unilateral lower limb suspension (Haus *et al.* 2007), nor did we find a change in actin levels with Western blotting (data not shown). Ubiquitin–protein conjugates were quantified by probing blots (15 μg protein load) with a rabbit polyclonal antibody against ubiquitin protein conjugates (Biomol International, Plymouth Meeting, PA, USA; no. UG-9510, 1: 4000) and corrected for loading with Ponceau S (Sigma) staining of sample lanes.

Calculations

The rates of myofibrillar protein synthesis were calculated using the standard precursor–product method:

$$\text{Fractional protein synthesis (FSR}(\% \text{ h}^{-1})) = \frac{\Delta E_{\text{p}}/E_{\text{p}}}{t} \times 100$$

where ΔE_b is the change in bound protein enrichment between two times, E_k is the mean enrichment over time of the muscle intracellular pool, and t is the time between biopsies. E_k was a weighted average calculated as the area under the intracellular enrichment by time curve divided by time.

Statistics

As the present study used a within subject design, changes in MPS and the phosphorylation status of signalling proteins were analysed using a two-factor ANOVA with conditions for leg (immobilized and non-immobilized) and time (during the infusion protocol) where appropriate. Differences in ANOVA means were determined using only relevant pre-planned pair-wise comparisons and a Holm–Sidak *post hoc* test. For all analyses, statistical significance was set at $P \leq 0.05$. Values are expressed as means \pm s.e.m.

Results

Muscle cross sectional area (CSA), mass and leg extension strength

Muscle CSA measured by MRI at mid-thigh was reduced as a result of immobilization ($-5.0 \pm 1.2\%$, $P < 0.005$), whereas CSA of the non-immobilized leg remained unchanged (-0.9 ± 0.5 , $P = 0.51$) (Fig. 2). Muscle mass assessed using dual-energy X-ray absorptiometry yielded revealed similar, albeit more variable, results to those of the MRI-based CSA analysis: mean leg muscle mass in the immobilized leg declined from 10251 ± 420 g to 10003 ± 432 g ($-2.5 \pm 1.1\%$, $P < 0.005$), whereas it remained unchanged in the non-immobilized leg (10117 ± 362 g and 10106 ± 383 g ($-0.2 \pm 0.9\%$, $P = 0.77$). There were no significant declines in total lean or body mass for either group, suggesting that subjects maintained adequate energy and protein intakes over the immobilization period. Isometric strength declined by $-25 \pm 3\%$ (range -9 to -42% , $P < 0.001$) in the immobilized leg but was unchanged in the non-immobilized leg ($3 \pm 3\%$; range -3 to $+17\%$, $P = 0.67$).

Blood amino acids and insulin

Patterns of aminoacidaemia were broadly similar for total, essential (EAA) and branched chain amino acids; we have presented data only for the EAA (Fig. 3A), which are more likely to be important for driving MPS. At the low dose ($43 \text{ mg kg}^{-1} \text{ h}^{-1}$), EAA concentration was significantly elevated above the post-absorptive basal level (by about 50%) only at 270 and 330 min of

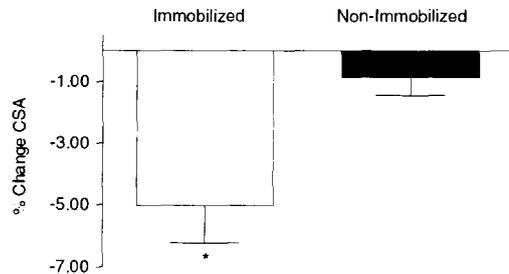


Figure 2. Quadriceps femoris cross-sectional area
Values are means \pm s.e.m. *Significantly different from pre-immobilization in immobilized ($P < 0.005$) and non-immobilized ($P = 0.47$).

infusion (both $P < 0.05$ versus basal; Fig. 3A). During the high dose ($261 \text{ mg kg}^{-1} \text{ h}^{-1}$), EAA concentrations were rapidly elevated, reaching a plateau of ~ 3 times post-absorptive values between 330 and 390 min (Fig. 3A,

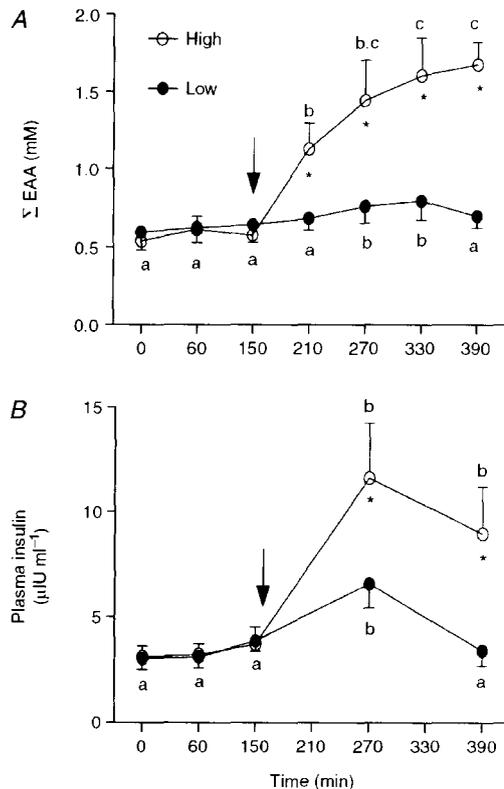


Figure 3. Summed concentration of essential amino acids (EAA; A) and plasma insulin (B)
Values are means \pm s.e.m. Values associated with different letters are significantly different from each other ($P < 0.05$). *Significantly different from low dose condition ($P < 0.01$).

Table 1. Muscle intracellular essential amino acid concentrations (pmol mg wet muscle⁻¹)

	Low dose							
	Immobilized				Non-immobilized			
	Fasted	1 h	2 h	4 h	Fasted	1 h	2 h	4 h
Ile	93 ± 11*	96 ± 9*	84 ± 11	83 ± 11	61 ± 3	61 ± 5	63 ± 8	64 ± 8
Leu	169 ± 23*	159 ± 14*	135 ± 20	145 ± 18	120 ± 7	121 ± 5	106 ± 9	110 ± 6
Val	206 ± 22	203 ± 13*	183 ± 17	174 ± 18	172 ± 5	168 ± 7	152 ± 8	148 ± 6
∑BCAA	469 ± 54*	457 ± 35*	403 ± 45	402 ± 44	353 ± 12	350 ± 14	321 ± 23	322 ± 17
Lys	152 ± 19 ⁺	132 ± 15 ⁺	154 ± 16	163 ± 32	256 ± 25	242 ± 28	230 ± 32	248 ± 36
Met	29 ± 7	31 ± 6	21 ± 4	23 ± 2	18 ± 4	18 ± 3	18 ± 4	19 ± 2
Phe	81 ± 11	80 ± 4 ⁺	83 ± 5	73 ± 6	58 ± 4	60 ± 4	71 ± 8	60 ± 5
Thr	605 ± 80	422 ± 43	409 ± 55	524 ± 50	545 ± 53	522 ± 90	543 ± 35	428 ± 33
∑EAA	1336 ± 145	1122 ± 47	1070 ± 66	1185 ± 38*	1329 ± 23	1193 ± 114	1184 ± 68	1076 ± 29

	High dose							
	Immobilized				Non-immobilized			
	Fasted	1 h	2 h	4 h	Fasted	1 h	2 h	4 h
Ile	74 ± 7 ⁺ ^a	120 ± 9 ^b	136 ± 8 ⁺ ^{bc}	148 ± 13 ^c	49 ± 3 ^a	95 ± 6 ^b	102 ± 6 ^b	120 ± 7 ^c
Leu	147 ± 16 ^{#a}	230 ± 17 ⁺ ^b	246 ± 13 ^{#bc}	288 ± 29 ^c	107 ± 14 ^a	174 ± 17 ^b	198 ± 14 ^b	256 ± 15 ^c
Val	176 ± 9 ⁺ ^a	257 ± 16 ^b	296 ± 13 ^{#bc}	346 ± 30 ^c	148 ± 11 ^a	226 ± 16 ^b	248 ± 16 ^b	338 ± 22 ^c
∑BCAA	397 ± 30 ^{#a}	607 ± 41 ⁺ ^b	678 ± 32 ^{#bc}	782 ± 70 ^c	304 ± 26 ^a	495 ± 26 ^b	548 ± 35 ^b	704 ± 42 ^c
Lys	120 ± 17 ⁺ ^a	186 ± 30 ^{ab}	220 ± 42 ⁺ ^{bc}	282 ± 50 ^c	211 ± 23 ^a	271 ± 40 ^{ab}	284 ± 39 ^{ab}	399 ± 57 ^b
Met	25 ± 5	21 ± 5	19 ± 5	28 ± 4	20 ± 2	22 ± 3	17 ± 4	23 ± 5
Phe	70 ± 5 ⁺ ^a	89 ± 6 ⁺ ^b	102 ± 3 ^{#b}	95 ± 5 ^b	56 ± 4 ^a	66 ± 7 ^{ab}	78 ± 3 ^b	78 ± 7 ^b
Thr	530 ± 58	609 ± 76	617 ± 51	642 ± 92	486 ± 64	602 ± 58	606 ± 104	568 ± 54
∑EAA	1141 ± 55 ^a	1511 ± 76 ^b	1636 ± 58 ^{bc}	1830 ± 140 ^c	1077 ± 69 ^a	1456 ± 83 ^b	1543 ± 151 ^{bc}	1792 ± 72 ^c

Significant differences: **P* < 0.05, ⁺*P* < 0.01, [#]*P* < 0.005 between legs (immobilized versus non-immobilized) at the same time point. Means with different letters are significantly different (*P* < 0.05) across time, within the same leg.

P < 0.01 versus 210 min). High dose EAA concentrations were significantly greater than those achieved during the low dose infusion at all times (*P* < 0.001, Fig. 3A).

Plasma insulin increased moderately from 3 to 10 μIU ml⁻¹ during the low dose amino acid infusion (Fig. 3B, *P* < 0.05), but then subsequently declined to baseline levels. At the high dose, insulin was increased by 270 min versus basal but thereafter remained elevated

(Fig. 3B). Blood glucose remained stable throughout both low and high dose amino acid infusions at 4.5 ± 0.4 mM (data not shown).

Muscle intracellular essential amino acid content

In general immobilization resulted in an elevation in some muscle intracellular EAA and their summed content (Table 1). A rise in intracellular EAA content was seen in the high dose AA infusion in both the immobilized and non-immobilized leg (Table 1).

Myofibrillar protein synthesis

There were no differences between the fasted-state MPS rates in the muscles of individuals treated similarly with immobilization and the low or high dose infusion; we therefore pooled the data for all 12 participants (Fig. 4). Immobilization caused a reduction in post-absorptive MPS (27 ± 7%; Fig. 4, *P* < 0.001).

Infusion of low dose amino acids stimulated MPS in the non-immobilized leg at 2 h and induced a further rise at 4 h (Fig. 5A). The rise in MPS was blunted at 2 h in the immobilized leg but was significantly elevated at 4 h versus

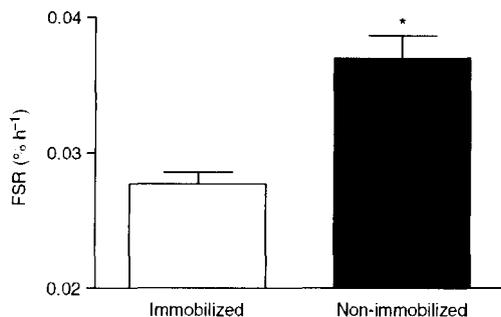


Figure 4. Pooled (from both low and high infused groups) resting fasted myofibrillar protein fractional synthetic rate (FSR)
*Significantly different from immobilized (*P* < 0.001).

fasted values. However, at 4 h MPS remained significantly less in the muscle of the immobilized compared to the non-immobilized leg (Fig. 5A).

At the high dose amino acid infusion, the results resembled those at the low dose, but were exacerbated. At both 2 h and 4 h MPS in the non-immobilized leg was significantly higher than that in the immobilized leg (Fig. 5C). As a measure of the cumulative incorporation over the 4 h of infusion, we calculated the area under the FSR-time curves for both the immobilized and non-immobilized legs. The areas under the curve were significantly lower in the immobilized than in the non-immobilized leg at both doses (Fig. 5B and D).

Signalling proteins

Focal adhesion kinase phosphorylation was found to be reduced ($-23 \pm 6\%$, $P < 0.05$; Fig. 6) in the immobilized state compared to the non-immobilized leg; these results were unaffected by feeding (data not shown).

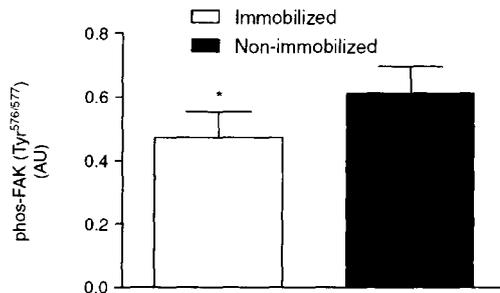


Figure 6. Phosphorylation (Tyr^{576/577}) of focal adhesion kinase (FAK) pooled from rested fasted immobilized (N = 12) and non-immobilized (N = 12) legs
*Significantly different ($P < 0.05$) from immobilized.

Proteins of the Akt-mTOR-p70S6k pathway were examined and found to remain unchanged in content between immobilized and non-immobilized legs (see online Fig. 2 Supplementary data). At the high dose

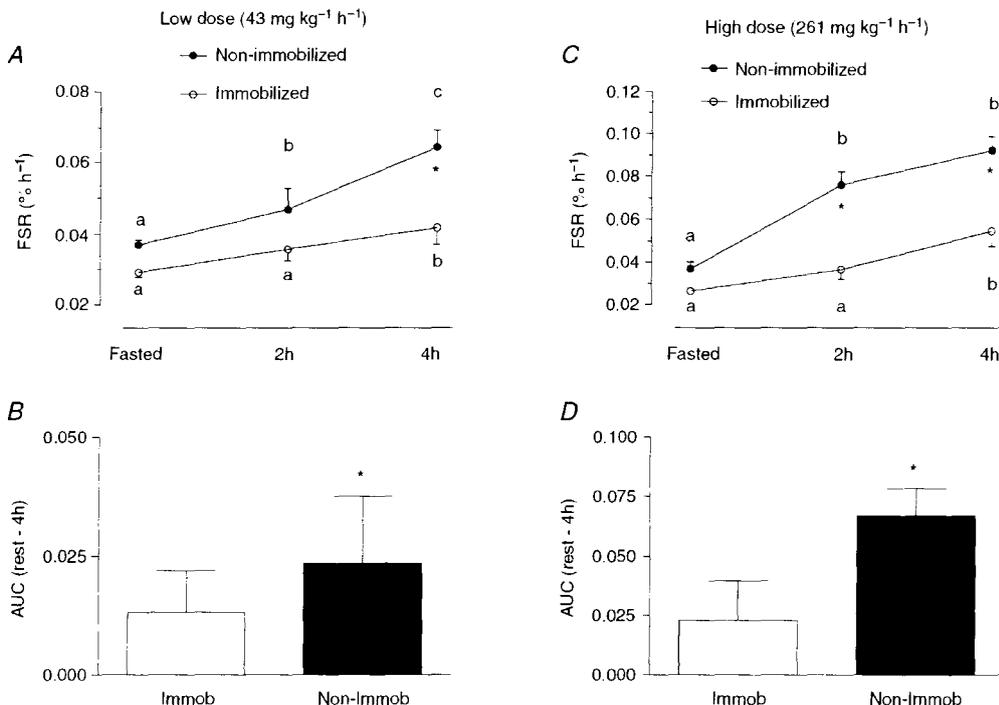


Figure 5. Myofibrillar fractional synthetic rate (FSR) and aggregate response of FSR indicated by area under the curve (AUC)

Fasted and fed state (2 h and 4 h) myofibrillar fractional synthetic rate (FSR) at low (A) and high (C) dose infusions. *Significantly different from non-immobilized ($P < 0.001$). Aggregate response of FSR indicated by area under the curve (AUC) for the low (B) and high (D) dose infusions. Means with different letters are significantly different from each other ($P < 0.05$). *Significantly different from immobilized ($P < 0.001$).

amino acid infusion, changes in phosphorylation of Akt were greater ($P < 0.05$) 1 h into the infusion in the non-immobilized leg (Fig. 7D; $P < 0.05$). Low dose amino acid infusion induced little notable change in Akt or mTOR phosphorylation (Fig. 7A and E). Low dose AA infusion did result in differential phosphorylation of p70S6k, however, with increases in phosphorylation

on this protein being greater ($P < 0.05$) at 1 h in the non-immobilized leg than the immobilized, a trend that was reversed at 2 h of infusion (Fig. 7C).

Phosphorylation of eEF2 showed little change with either feeding or immobilization (see Fig. 1 Supplementary data). Akt-mediated inhibitory phosphorylation of GSK3- β at serine 9 increased in

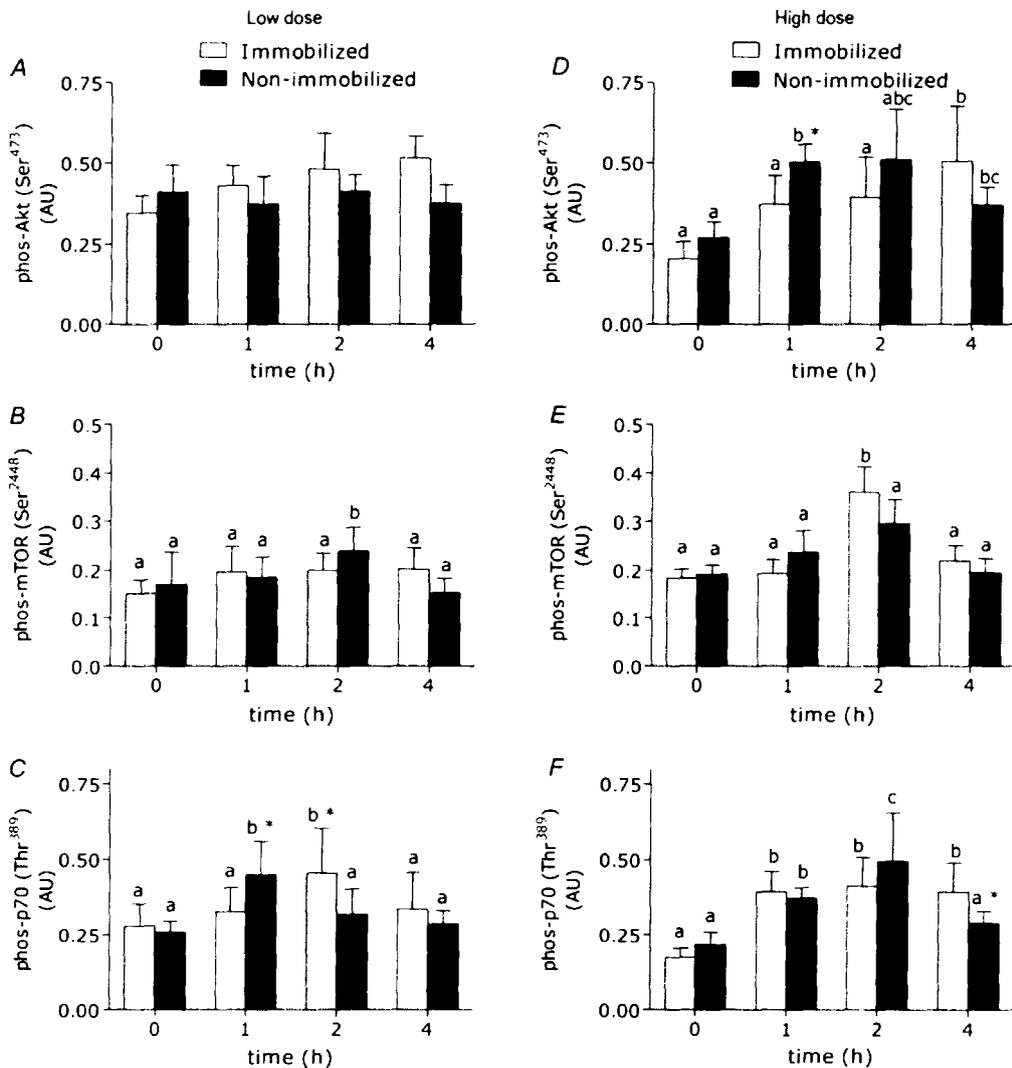


Figure 7. Phosphorylation (phos) of Akt, mTOR and p70S6k (normalized to α -actin) in immobilized and non-immobilized legs at a low dose (43 mg kg⁻¹ h⁻¹; panels on left) and a high dose (261 mg kg⁻¹ h⁻¹; panels on right)

*Significantly different from the opposite condition at the same time point ($P < 0.05$), means with different letters are significantly different across time ($P < 0.05$). Values are means + S.E.M.

the non-immobilized leg by 4 h of feeding at the high dose (see Fig. 1 Supplementary data). Neither immobilization ($P=0.3$) nor amino acid infusion ($P=0.09$) changed the muscle content of polyubiquitinated proteins (see Fig. 3 Supplementary data).

Discussion

We have discovered, for the first time in human subjects, that immobilization appears to cause a deficit in the protein synthetic response of muscle to increased availability of amino acids. We have also confirmed the results of others (Gibson *et al.* 1987; Ferrando *et al.* 1996; Paddon-Jones *et al.* 2006; de Boer *et al.* 2007), that immobilization reduces post-absorptive muscle protein synthesis (in our case by 27%). Provision of amino acids at both low and high doses resulted in a delayed and lower net response (i.e. area under the curve) of MPS in the immobilized compared to the non-immobilized leg. Thus, the effect of feeding is not simply an extension of the lower fasted-state synthetic rate but is an example of what has been previously described as anabolic resistance (Cuthbertson *et al.* 2005), which is observable in a wide variety of clinical and subclinical states of slow muscle wasting (Rennie & Wilkes, 2005). In anabolic resistance, the normal anabolic response to amino acids is less sensitive and has a lower capacity with the probable result that fed-state gains in protein, which normally balance fasted-state losses (Rennie *et al.* 2004; Phillips, 2004), are less. Over time, such a state would result in declines in muscle mass, but as a result of a lower post-absorptive and fed-state protein accretion and not elevated proteolysis. Calculations presented elsewhere (de Boer *et al.* 2007) indicate that the observed reductions in post-absorptive muscle protein synthesis (Gibson *et al.* 1987; Ferrando *et al.* 1996; Paddon-Jones *et al.* 2006; de Boer *et al.* 2007) in periods over 10 days in combination with predicted estimates, confirmed here, of the extent of reduction in fed-state MPS would be enough to account for most, if not all, of the immobilization-induced decline in muscle protein mass. Indeed, Biolo *et al.* (2004) noted that the reduction in fed-state net protein deposition in subjects after 14 days bed rest could be completely accounted for by a suppression in amino acid-induced stimulation of whole-body protein synthesis whereas post-absorptive and fed-state whole-body proteolysis were not elevated. Our results showing a lack of elevation in polyubiquitinated muscle protein suggest no increased protein flux through the ATP-dependent polyubiquitin proteasome pathway. Thus, it appears that, in contrast to data obtained from studies in small animals (Gomes *et al.* 2001; Lecker *et al.* 2004, 2006; Sacheck *et al.* 2007), elevated proteolysis probably cannot be the primary mechanism underpinning the reduction in human muscle protein mass resulting from disuse. In fact, given the reported

declines in post-absorptive (Gibson *et al.* 1987; Ferrando *et al.* 1996; Paddon-Jones *et al.* 2006; de Boer *et al.* 2007) and fed-state (current results) MPS, if proteolysis were even moderately elevated, then muscle mass would decline by more than 65% with ~40 days of disuse. Adams *et al.* (2003) showed, using multiple models of disuse, however, that the decline in muscle CSA with unweighting eventually reaches a nadir.

The situation may be different early in immobilization. There is some evidence of increased muscle expression of mRNA and protein for proteolytic enzymes early on after limb immobilization and spinal cord injury (Urso *et al.* 2006, 2007). Increased (~3-fold) mRNA expression of MuRF-1 (but no change in MAFbx or tripeptidyl peptidase II mRNA) has also been observed (de Boer *et al.* 2007). The lack of robust measurements of muscle protein breakdown by gold standard methods (e.g. stable isotope dilution across the immobilized leg of tracer amino acids) means, however, that we are still uncertain as to what happens in the early time period following immobilization. Recently indirect evidence for increased protein breakdown has been inferred from a comparison of measures of interstitial 3-methyl histidine derived from dialysis procedures in muscles from immobilized and non-immobilized legs (Tesch *et al.* 2008). Although there are a number of methodological difficulties with this approach, these data have been interpreted to suggest that in the short term (72 h), immobilization may induce a rise in interstitial 3-methyl histidine. Nevertheless, a disproportionate loss of the myofibrillar fraction or specific myofibrillar proteins (myosin and actin) from the vastus lateralis was not observed in a recent study with 35 day unilateral lower limb suspension (Haus *et al.* 2007). We and others (Paddon-Jones *et al.* 2001) did note increased intracellular concentrations of some amino acids (Table 1) with immobilization or bed rest, which could reflect a decreased demand for substrates to support synthesis. Intracellular amino acid concentrations increased in both legs during the high rate infusion, however, suggesting that uptake into the muscle is not impaired with immobilization.

Based on our work we propose that interventions to counter disuse atrophy over the medium to longer term would be most effective if they targeted the decline in protein synthesis rather than trying to offset increased proteolysis. An obvious intervention that has been repeatedly shown to be successful in countering disuse atrophy is resistance-based exercise (Carrithers *et al.* 2002; Alkner & Tesch, 2004; Haus *et al.* 2007) or combinations of resistance and aerobic-based exercise (Trappe *et al.* 2007, 2008). Our data indicate that amino acid or protein provision alone, without activity, would not ameliorate loss of muscle mass with disuse, and indeed several chronic bed rest studies have confirmed this proposition (Trappe *et al.* 2007, 2008; Brooks *et al.* 2008).

By contrast, Paddon-Jones found that 16.5 g per day of crystalline essential amino acids (equivalent to ~40 g of high quality protein) and glucose (30 g) consumed thrice daily was able to attenuate the decline in leg lean mass (Paddon-Jones *et al.* 2004) even with hypercortisolaemia (Paddon-Jones *et al.* 2005). However, given the failure of extra nutrition to maintain muscle mass during bed rest (Trappe *et al.* 2007, 2008; Brooks *et al.* 2008), the blunting of the whole-body protein synthetic response to hyperaminoacidaemia after 14 days of bed rest (Biolo *et al.* 2004), and our findings of anabolic resistance after acutely infusing quantities of amino acids greater than those used by Paddon-Jones *et al.* (2004, 2005), it is difficult to understand how an essential amino acid supplement was able to retain its capacity to repeatedly trigger a robust anabolic response throughout a 4 week period of bed rest. Whereas protein/amino acid supplementation has not been tested in an immobilization model, it is hard to imagine that an effect would be observed in a model of local atrophy when the majority of bed rest studies have failed to detect a benefit. Interestingly, net balance data indicated that the control group in Paddon-Jones *et al.* (2004) was in a mild catabolic state from the onset of bed rest, bringing into question its suitability. On the other hand, our subjects maintained their typical diets. Total body and lean mass did not change; therefore it is unlikely that they were in a general catabolic state, and yet they lost mass locally. We acknowledge that continuous amino acid infusion is not representative of the typical 'bolus' plasma response of oral feeding, and this difference may be raised as a reason for the divergent findings. We also noted, in contrast to Bohe *et al.* (2001), that MPS did not become refractory to high aminoacidaemia. In all likelihood this is due to the fact we did not achieve plateau in amino acid concentration until 3–4 h into the infusion *versus* a plateau at ~30 min (J. Bohe and M. J. Rennie, unpublished data). However, hyperaminoacidaemia by direct infusion is normally a robust stimulator of synthesis, and yet it failed to elicit an equivalent response in the disused leg.

Phosphorylation of FAK had been reported to be reduced with hindlimb suspension in rodents and increased with synergist ablation (Gordon *et al.* 2001) or chronic overload in avian muscle (Fluck *et al.* 1999). Surprisingly, there are few reports of FAK or other proteins of the focal adhesion complex (paxillin, serum response factor) in differing loading states in human skeletal muscle. FAK phosphorylation was reported to be reduced with immobilization (de Boer *et al.* 2007). FAK has been proposed to be a mechanically sensitive protein (Fluck *et al.* 1999; Gordon *et al.* 2001), and thus may be a functional link between the loading of the muscle and a dampened protein synthetic response. We did not note, as we reasoned we might, a strong link between the responsiveness of the Akt–mTOR–p70S6 pathway to feeding. Other mechanically responsive integrins such as

α 7A/B-integrin, β 1D-integrin and muscle agrin all appear to be altered with inactivity (Anastasi *et al.* 2006) and are perhaps worthy of further study.

In an attempt to understand the expected differences in MPS between the immobilized and non-immobilized limbs, we measured the phosphorylation of candidate signalling molecules known to be involved with the activation of protein synthesis in humans in response to feeding and/or altered loading (Kimball *et al.* 2002; Karlsson *et al.* 2004; Kimball & Jefferson, 2006; Dreyer *et al.* 2006; Eliasson *et al.* 2006; Fujita *et al.* 2007). We had hypothesized that the reduction in sensitivity of MPS to AA provision with immobilization would be accompanied by a broad reduction in phosphorylation (presumably as an indication of activity) of sites responsible for activation/deactivation of proteins that 'turn on' protein synthesis. Regrettably, little of the data we obtained can provide much insight into a potential mechanism of how immobilization might reduce the response of MPS to amino acid provision. We did see some evidence of a delayed phosphorylation of Akt at a high dose AA infusion and a similar phenomenon with p70S6k at the low dose, but the significance of these findings is unclear. Insulin increased during the high dose infusion, so the delay in Akt responsiveness may reflect some degree of inactivity-induced insulin resistance. Short-term (7 day) immobilization is sufficient to reduce insulin action on leg glucose uptake (Richter *et al.* 1989). Given that many of the signalling proteins are modulators of translational initiation, it is possible that we missed an early and transient divergence in the signalling response. Muscle protein synthesis is not up-regulated in response to amino acids until an hour after the onset of hyperaminoacidaemia (Bohe *et al.* 2001), but we are not aware of any information on the signalling response in humans within the first hour of feeding. It may be that there is no 'master switch' or regulator of protein synthesis but instead substantial redundancy and synergism (Proud *et al.* 2001; Averous & Proud, 2006). Furthermore, the suitability of using translational signalling factor phosphorylation as a surrogate of changes in human MPS is questionable. For example, the effects of amino acids and insulin on signalling and protein turnover have revealed a disconnect between insulin-induced increases in phosphorylation of Akt and p70S6k and MPS rates (Greenhaff *et al.* 2008). Nonetheless, several recent reports indicate that other signalling proteins may play greater regulatory roles in influencing protein synthesis than those we measured. For example, a recently identified site on eIF2B ϵ , Ser²⁵, has been shown to be critical for the activation of this protein in response to AA (Wang & Proud, 2008). Similarly, sites on 4E-BP1 (Wang *et al.* 2005) as well as eukaryotic elongation factor-2 (eEF2) (Smith & Proud, 2008) also appear to be critically important for regulation of protein synthesis in response to provision of AA. It will be important to define

the 'crosstalk' between mechanically responsive proteins and regulatory sites on critically important signalling proteins.

In summary, our data indicate that immobilized skeletal muscle exhibits a decrease in responsiveness of MPS to amino acids across a wide range of amino acid concentrations. Provision of very high concentrations of AA failed to return MPS to that seen in the non-immobilized muscle. This was associated with a marked reduction in the phosphorylation of the putative tension-sensing protein FAK in the immobilized leg, but with no striking evidence of decreased signalling in the Akt-mTOR-p70S6 pathway. Further work is clearly needed to confirm the possible interactions of FAK and the molecular regulators of muscle protein turnover. In particular, the elements which reduce the activation of protein synthesis in the immobilized state need to be identified.

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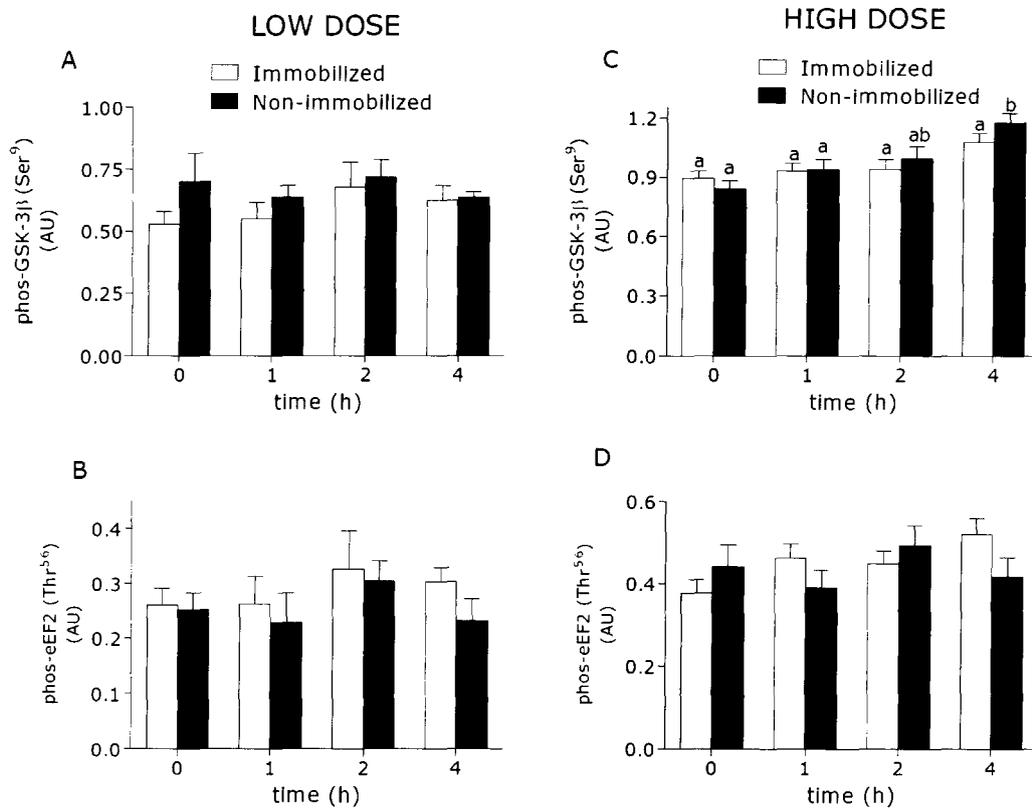
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Supplemental material

Online supplemental material for this paper can be accessed at: <http://jp.physoc.org/cgi/content/full/jphysiol.2008.160333/DC1>

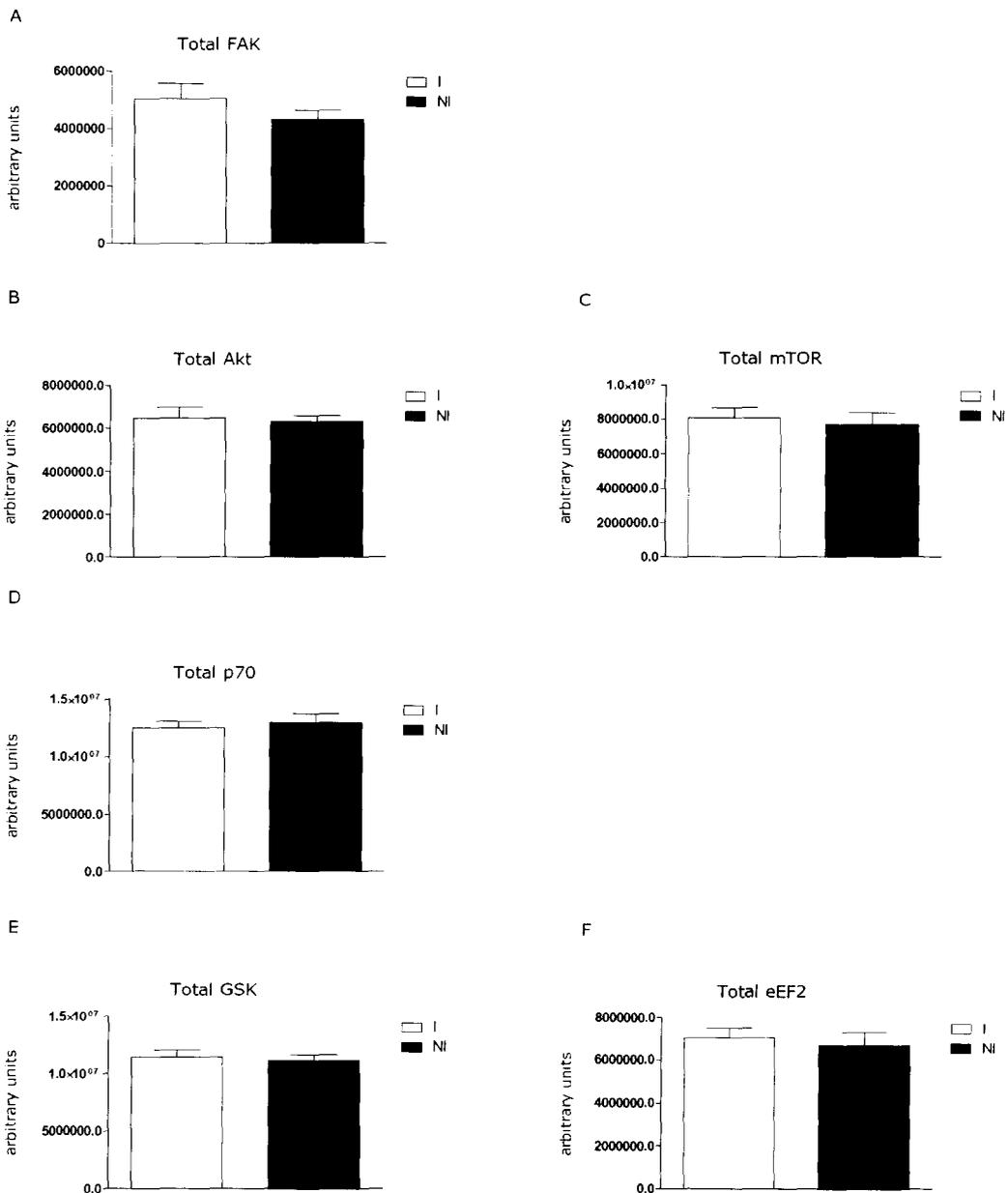
Glover et al. “Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion” Supplementary data.

Supplementary figure 1 (Glover et al). Phosphorylation (phos) of GSK-3 β and eEF2 (normalized to α -actin in immobilized and non-immobilized legs at a low dose ($43\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; panels on left A and B) and a high dose ($261\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; panels on right C and D). * Significantly different from the opposite condition at the same time point ($P < 0.05$), means with different letters are significantly different across time ($P < 0.05$). Values are means \pm SEM.



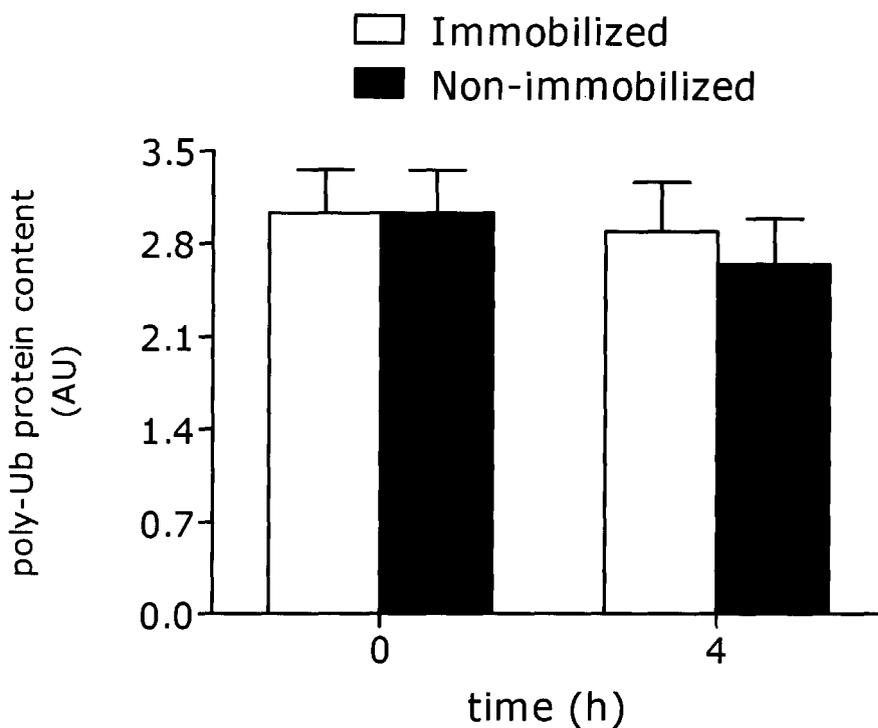
Glover et al. “Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion” Supplementary data.

Supplementary figure 2 (Glover et al). Total levels of FAK (A), Akt (B), mTOR (C), p70 (D), GSK-3B (E) and eEF2 (F) in immobilized and non-immobilized legs (pooled fasted values, N=12). Values are means±SEM.



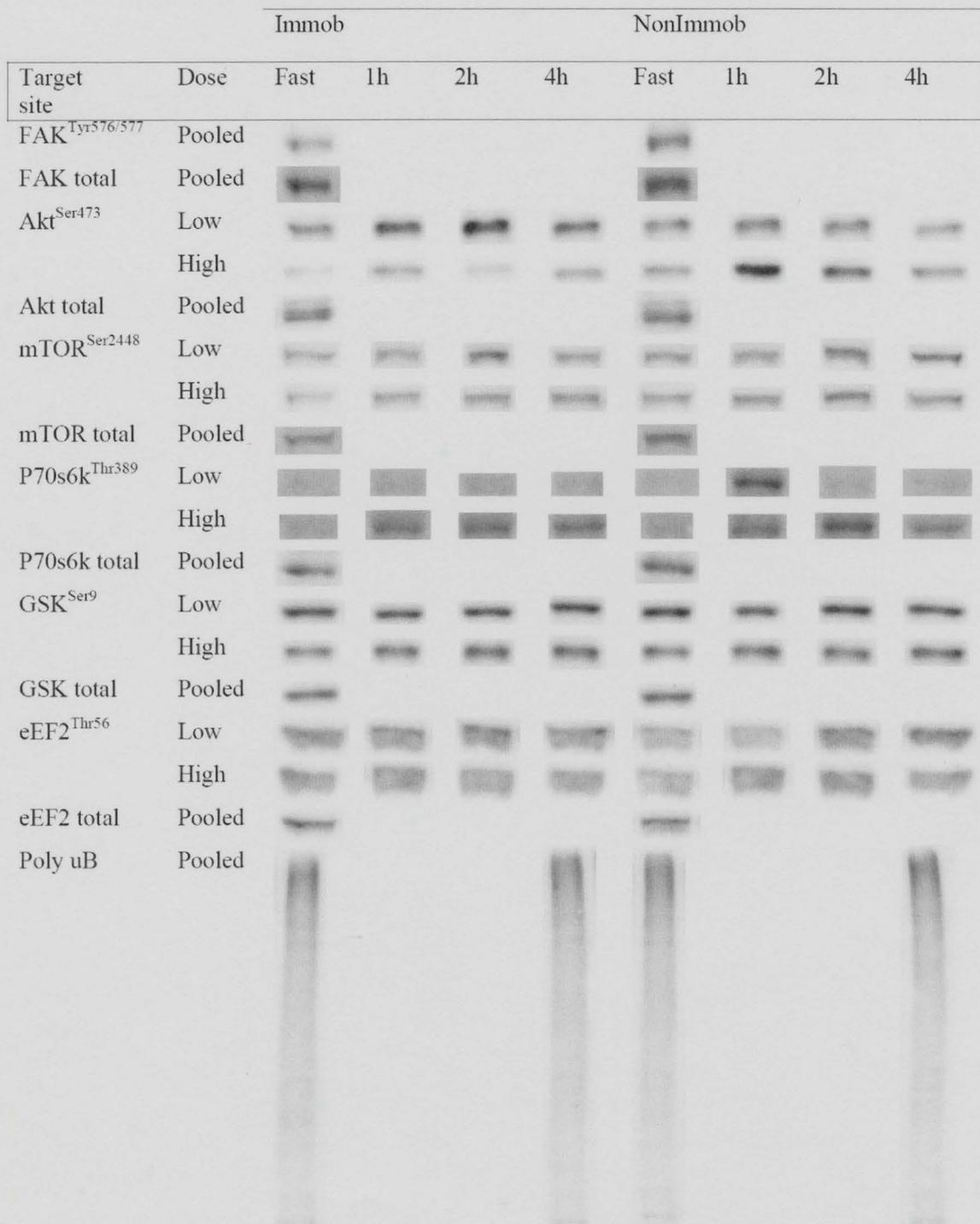
Glover et al. “Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion” Supplementary data.

Supplementary figure 3 (Glover et al). Polyubiquitinated (poly-Ub) protein content from immobilized and non-immobilized muscle (normalized with Ponceau red staining). Samples are for the high and low dose AA infusion, which were not different (not shown) and so results are pooled. Values are means±SE.



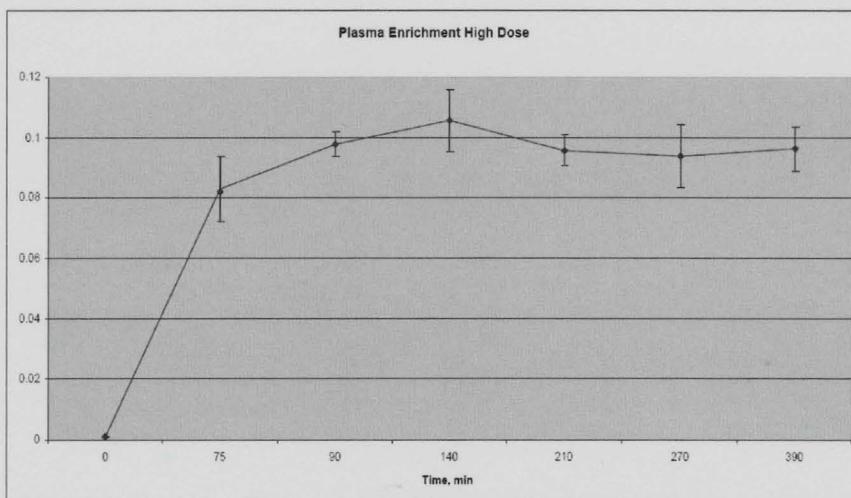
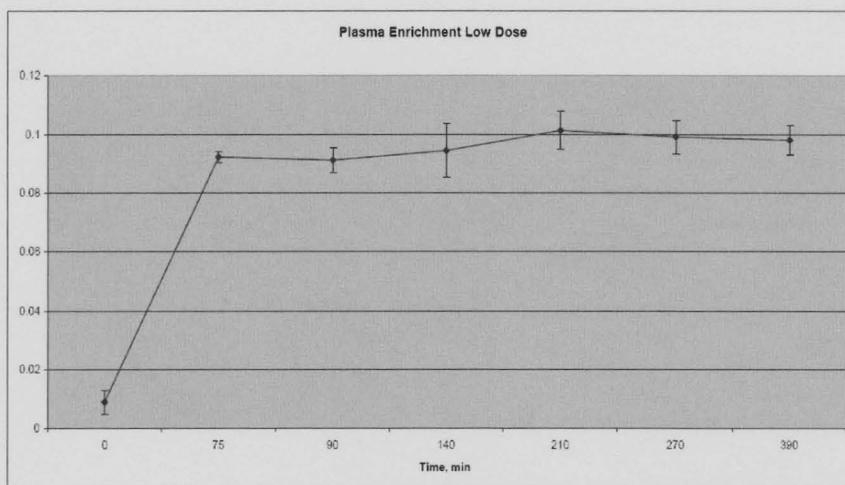
Glover et al. "Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion" Supplementary data.

Supplementary figure 4 (Glover et al). Examples of bands from blots. All bands for a particular antibody were obtained from a single Western blot.



Glover et al. "Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion" Supplementary data.

Supplemental data.



There was no significant drop in intracellular enrichments over the trial (see below)

	Immob				Non immob			
	Fasted	1h fed	2h fed	4h fed	Fasted	1h fed	2h fed	4h fed
42.5 mg	0.039±.002	0.061±.004	0.062±.004	0.070±.007	0.048±.005	0.065±.008	0.064±.007	0.066±.007
261 mg	0.043±.006	0.064±.003	0.063±.003	0.067±.003	0.043±.004	0.071±.005	0.075±.002	0.071±.002

Chapter 4: Study 3

Little change in markers of protein breakdown and oxidative stress in humans in immobilization-induced skeletal muscle atrophy

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In Review

Little change in markers of protein breakdown and oxidative stress in humans in immobilization-induced skeletal muscle atrophy

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Abstract

A number of studies in rodents suggest that disuse atrophy results from a large increase in proteolysis affected by, or accompanying, increased oxidative stress. Little information is available, however, about the effects of immobilization on markers of muscle protein breakdown and oxidative stress in humans. Therefore the purpose of this investigation was to measure markers of breakdown or oxidative stress in subjects who underwent 14 d of knee brace-mediated immobilization. *Vastus lateralis* samples taken from 21 young subjects before, 2 d and 14 d after single leg immobilization were measured for ubiquitin-protein conjugates, caspase 3/7 activity, the 14-kDa caspase-3 cleaved actin fragment, 4-hydroxy-2-nonenal adducts (4-HNE), and protein carbonyls. Quadriceps cross sectional area decreased by $5.7 \pm 1.1\%$ ($p < 0.0001$) following immobilization. Ubiquitin protein conjugates were elevated at 2 d of immobilization (12%, $P < 0.05$), but were not different from baseline at 14 d. Levels of the 14kDa actin fragment and caspase 3/7 activity did not change over the immobilization period. The oxidative stress markers, 4-HNE adducts and protein carbonyls, did not change at any time point. These static measures of breakdown and oxidative modification suggest that a small increase in protein ubiquitination occurs early (2 d), but elevations in ubiquitinated or oxidatively modified proteins are not sustained during the later phase (+14 d) of uncomplicated disuse atrophy in humans, suggesting that these pathways are not playing a major role in simple disuse-induced atrophic loss of protein mass.

Keywords: human disuse atrophy, protein turnover, proteolysis, oxidative stress, ubiquitination

Introduction

Muscle mass is maintained when the opposing processes of muscle protein synthesis (MPS) and breakdown (MPB) are in balance. Over the long term and with high intensity resistance exercise a shift in favour of MPS can result in hypertrophy. Conversely, conditions in which MPB predominates lead to atrophy, particularly when accompanied by increased catabolic hormones (i.e., sepsis, AIDS, cancer cachexia) and cytokines. We currently have a reasonable, and expanding, understanding of the effects of feeding, muscular activity and anabolic hormones on muscle protein synthesis in humans (Rennie et al. 2004). Our knowledge of the effects of these variables on the process of muscle protein breakdown is much more limited. This is primarily due to the technical challenges of making measurements *in vivo* of muscle protein breakdown in humans.

To date, we have little information about the modulation of human muscle protein breakdown during states of reduced loading or disuse. The bulk of our knowledge in this area is based on animal work, and the overall picture that emerges from these studies indicates that proteolysis is significantly elevated and is considered to be primarily responsible for the lean tissue loss that occurs with unweighting (Goldspink et al. 1986; Jackman et al. 2004; Kandarian et al. 2006; Loughna et al. 1987; Medina et al. 1995; Satchek et al. 2007; Tawa et al. 1997; Thomason et al. 1990; Wing et al. 1995). In contrast, studies in humans during immobilization have reported decreases in fasted and fed state muscle protein synthesis which, when considered in conjunction with reports of no change or possible decreases in rates of whole body (Biolo et al. 2004; Ferrando et al. 1996; Shangraw et al. 1988; Stuart et al. 1990) and muscle protein (Ferrando et al. 1997;

Paddon-Jones et al. 2004; Symons et al. 2009) breakdown after bed rest, are sufficient to account for the observed atrophy (de Boer et al. 2007; Gibson et al. 1987; Gibson et al. 1988; Glover et al. 2008). Whether these findings contrast with results from human studies due to the specifics of rodent physiology or the extreme conditions often employed in rodent models of atrophy remains to be clarified.

It has been speculated that in humans, as observed in rodent models (Kondo et al. 1993; Lawler et al. 2003), elevated oxidative stress may play a role in initiating the increased proteolysis driving disuse atrophy (Barker et al. 2007; Powers et al. 2005; Powers et al. 2007). However, definitive evidence for increased oxidative stress and proteolysis is lacking for human models of disuse atrophy in skeletal muscle. Hence, we assessed markers of protein breakdown (polyubiquitinated proteins and a caspase-3 cleavage product of actin which was reported to be elevated in catabolic conditions (Workeneh et al. 2006)) and oxidative stress (carbonyls, 4-hydroxy-2-nonenal adducts) in samples taken from participants who underwent 14 d of immobilization. Given the lack of observable increases in muscle protein breakdown with bed rest, we hypothesized that we would not see an elevation in ubiquitination, the 14kDa fragment, or oxidatively modified proteins at either 2 d or 14 d of immobilization.

Materials and Methods

Subjects and procedures

This paper reports the characterization of the changes in high molecular weight proteins from muscle samples taken from the *vastus lateralis* from 21 subjects (males N = 13, females N = 8), that took part in a previously reported immobilization study (Yasuda et

al. 2005). Details of subject screening, the immobilization protocol, testing procedures and subject monitoring have been described (Yasuda et al. 2005). This study, as well as this analysis, was approved by the McMaster University and Hamilton Health Sciences Research Ethics Board and all subjects provided informed written consent. All testing procedures conformed to those outlined in the Declaration of Helsinki (1964) on the use of human subjects in research. These subjects were included in the current analysis because they had complete sample sets available. Briefly, subjects had one leg immobilized by random assignment using a standard knee brace at 60° from full extension (epX Knee Control Plus, Smith Orthopedics, Topeka, KS) with a compression cotton stockinette and were provided with walking crutches. A unique identifier was applied to a piece of commercially available standard tape wrapped around to the brace to ensure that the brace could not be removed. Subjects reported to the lab on a daily basis, during which time the brace was removed and the leg was inspected. It is true that without careful attention edema can result during limb immobilization. Subjects' legs and feet were checked daily for signs of edema by observing leg colour and looking for unusual vein patterns. Using palpation we checked for warmth, tenderness, and cords, and gently squeezed the calf muscle against the tibia to check for deep pain. We also dorsiflexed the subjects' feet to look for Homans' sign. Subjects were allowed to take a shower in the laboratory without bearing weight on the treatment leg. Scans (magnetic resonance imaging – MRI) to determine muscle cross-sectional area were taken on the evenings prior to sample collection as previously described (Yasuda et al. 2005). Samples were obtained 5 days prior to immobilization (pre), 48h after beginning immobilization

(2 d) and after 14 days of immobilization (14 d). After removal of visible fat, a portion of each biopsy was submerged in liquid nitrogen and stored at -80°C until analysis, while additional portions of the pre and 14 d biopsies were mounted transversely in optimal cutting temperature compound (OCT).

Quadriceps and fibre CSA

MRI scans and 10 µm OCT-mounted sections were analysed as described in Yasuda and co-authors (Yasuda et al. 2005). MRI and fibre data (pooled across sex) are reported here for 21 and 15 subjects, respectively.

Caspase 3/7 activity

Caspase 3/7 activity was assessed in 600g supernatants for participants for which a complete sample set was available (N=14) using the Caspase-Glo 3/7 assay (Promega, Madison, WI). One hundred µl of the Caspase-Glo 3/7 reagent was added to 50 µg of sample diluted to 100 µl with doubly-distilled water in a 96-well plate. Following 30s of agitation (300-500 rpm), samples were incubated at room temperature for 2 h.

Luminescence was measured using the EnVision plate reader at 700 nm (Perkin-Elmer, Waltham, MA). Samples were run in duplicate on along with blank, caspase inhibitor (Sigma, A0835) and 0.001-1 ng of recombinant purified caspase (BD Pharmingen, #556472) from which a standard curve was generated. The intrassay coefficient of variation was 6.6%

SDS-PAGE and Western blotting

Frozen wet muscle (20-30 mg) was homogenized using a glass homogenizer with ice-cold buffer (50 mM Potassium phosphate, pH 7.4, 5 mM EDTA, 0.5 mM DTT, 1.15%

KCL (w/v)), then centrifuged (600g, 10 min, 4°C). After removal of the supernatant the pellets were stored at -80°C. Pellets were resuspended in 200 µl of the same buffer with the addition of 0.5% Triton-X and protease inhibitors (complete mini, EDTA-free, Roche, Laval, QC) and sonicated for 20 minutes at 30°C. Protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA). Proteins (15 µg for polyubiquitinated proteins, 5 µg for 14kDa fragment, 10 µg for 4-HNE) were separated by SDS-PAGE (7.5%, polyubiquitinated proteins; 15%, 14kDa fragment; 10%, 4-HNE; 100V) and transferred onto PVDF. After transfer, membranes were blocked for 1h-overnight in 5% BSA-Tris buffered saline with 0.1% Tween (TBS-T). Membranes were then incubated in primary antibody diluted in 5% BSA-TBST (1:2000 overnight, anti-ub-protein conjugates (#UG-9510, Biomol International, Plymouth Meeting, PA); 1:6000 overnight, anti-actin (#A2066, Sigma, St. Louis, MO); 1:3000 1 h, anti-4HNE, #ab48506, Abcam, Cambridge, MA). After washing in TBS-T, membranes were incubated in HRP-conjugated secondary antibody (1:10000 donkey anti-rabbit, Amersham, Piscataway, NJ, #NA-934; 1:10000 sheep anti-mouse for 4-HNE, Amersham, NJ, #NA-931). Membranes were washed again. Protein carbonyls were assessed by derivatization with dinitrophenyl hydrazine (DNPH) according to the manufacturer's instructions (Oxy-blot kit, Millipore, Billerica, MA), with the modification that secondary antibody was incubated overnight at 4°C with agitation. Blots were developed with ECLplus (Amersham, Piscataway, NJ) and exposed to X-ray film (Kodak, Rochester, NY). Films were scanned and signals quantified with AlphaEase software (AlphaInnotech, San Leandro, CA). Membranes were subsequently stained with Ponceau Red (Sigma, Oakville, ON) to correct for loading.

Statistics

One-way repeated measures analysis of variance (ANOVA) was performed to test for differences between the pre, 2 d and 14 d timepoints. Where a significant difference was detected, the Tukey *post hoc* test was performed (Prism 5, Graphpad Software Inc., La Jolla, CA). Significance was set at $P < 0.05$.

Results

Quadriceps and fibre atrophy

Quadriceps area of the subjects decreased an average of $5.7 \pm 1.1\%$ ($p < 0.0001$), while fibre area decreased 5.6-11.8%, depending on fibre type (all $p < 0.01$, Table 1).

Ubiquitin-protein conjugates

There was a significant ($\sim 12\%$, $P < 0.05$) elevation in overall ubiquitin-protein conjugates by 2 d of immobilization, but levels were not significantly different from the pre timepoint by 14 d of immobilization ($P = 0.113$; Figure 1a). Quantification of a single distinct band at ~ 55 kDa did not display differential ubiquitination over time with immobilization ($P = 0.30$) (Figure 1b). Accumulation of ubiquitinated proteins seemed to primarily occur at or above 200 kDa (see Fig. 1a inset).

Caspase 3/7 activity

Caspase 3/7 activity in the supernatants did not significantly change over the immobilization period ($P = 0.62$, Fig. 2a).

14kDa fragment

There was no significant change in levels of the 14kDa actin fragment over the immobilization period (Fig. 2b), although the ANOVA showed a trend towards a decrease at 2 d ($P=0.053$).

4-hydroxy-2-nonenal (4HNE)

Total levels of protein modified by the lipid peroxidation product, 4HNE (Fig. 3a) were not significantly elevated with immobilization at any timepoint ($P=0.50$), nor were the levels of a prominent 55kDa band significantly different ($P=0.32$; Fig. 3b).

Protein carbonyls

Total protein carbonyls as assessed by derivatization with DNPH (Fig. 4a) did not change significantly over the immobilization period ($P=0.55$). A single distinct band at ~125 kDa (Fig. 4b) did not show any change in intensity over the immobilization period ($P=0.69$).

Discussion

Immobilization-induced atrophy resulted in an early (2 d) but transient increase in ubiquitinated proteins, with no elevations in caspase 3/7 activity, the 14 kDa caspase-3 mediated cleavage product of actin, or two commonly measured markers of oxidatively modified macromolecules in the myofibrillar muscle protein fraction. Our model of immobilization was effective and induced a loss in mid-thigh muscle CSA of ~5.7% as well global reductions in fibre cross-sectional area, which is consonant with other unilateral models of disuse in humans (de Boer et al. 2007; Jones et al. 2004).

In the absence of breakdown information, it is difficult to comment on the significance of the early rise in ubiquitinated proteins in our study. Increased ubiquitinated

proteins have been observed in a 20 d bed rest study (Ogawa et al. 2006), despite reports of no increases in muscle protein breakdown in human-based bed rest studies of similar duration (Ferrando et al. 1996; Paddon-Jones et al. 2004; Symons et al. 2009). Inhibition, and not stimulation, of the proteasome would also result in accumulation of muscle ubiquitin conjugates (Tawa et al. 1997). Moreover, in humans a number of discrepancies between direct measurements of protein breakdown measurements and the expression of the ubiquitin ligases MuRF1 and atrogin and C2 proteasome subunits have been recently observed (Brodsky et al. 2004; Caiozzo et al. 2009; Greenhaff et al. 2008; Symons et al. 2009). Therefore, the relationship between changes in levels of ubiquitin ligase, proteosomal subunit transcripts, protein ubiquitination, and bulk proteolysis during disuse atrophy in humans may be more complex than currently theorized. Our data aligns with other reports in humans of upregulation of ubiquitin ligases at early stages of disuse (Urso et al. 2006; Urso et al. 2007). We also observed, in the same subjects, of a small but significant rise in MuRF1 and MAFbx/atrogin-1 mRNA abundance at 2 d of immobilization (Abadi et al. 2009). This finding is, however, perplexing in conjunction with the lack of increase in the 14kDa actin fragment which is considered a ‘biomarker’ of an upstream cleavage event that is necessary for release of actin from the myofibril and subsequent accessibility of actin to ubiquitin ligases (Du et al. 2004; Workeneh et al. 2006). It is possible that our model is not sufficiently catabolic to observe a measurable increase in the actin fragment. Previously, increased levels of the 14 kDa actin fragment seen in humans have, to date, only been reported in middle-aged patients with arthritis and poor mobility, haemodialysis patients and burn patients (Workeneh et al. 2006).

We have previously hypothesized (Rennie et al. 2008) that proteolysis of myofibrillar proteins is not occurring at an accelerated rate during disuse atrophy in humans. The myofibrillar fraction, and indeed actin and myosin themselves, were not found to disproportionately decrease compared to other proteins in the quadriceps with 35-90 days of unilateral lower limb suspension (ULLS) or bedrest (Carrithers et al. 2002; Haus et al. 2007; Lemoine et al. 2009). However, Tesch et al. (Tesch et al. 2008) recently reported increased intramuscular interstitial levels of the myofibrillar breakdown product 3-methylhistidine (3-MH) after 72 h of ULLS. Whether this is representative of a coordinated increase in protein breakdown in all pools or a preferential but transient dismantling of the myofibrillar apparatus, or merely methodological artifact is not clear; however, over the long term atrophying muscle appears to maintain a constant ratio of protein fractions.

The early increase in ubiquitinated proteins may instead reflect an increased targeting of regulatory factors crucial to metabolic control. Increases in ligases like MAFbx/atrogen-1 have been suggested to suppress protein synthetic capacity by enhanced degradation of growth promoting proteins (Ogawa et al. 2006). Recently, modulation of the “translational enhancer” eukaryotic initiation factor-3 subunit f (eIF3-f) by MAFbx has been reported under proatrophic conditions, providing a possible link between increases in ubiquitin ligase expression and the downregulation of muscle protein synthesis (Lagrand-Cantaloube et al. 2008), which we and others (Ferrando et al. 1996; Gibson et al. 1987; Gibson et al. 1988; Glover et al. 2008; Rennie et al. 2008), have proposed is the primary reason for muscle protein loss in human disuse atrophy.

We did not find evidence of elevated protein carbonyls or lipid peroxidation adducts at 2 d or 14 d of immobilization. In our hands, using the same methods we have detected significant decreases in older adults following resistance training (Safdar et al. 2008) and increased carbonyl content in the G93A mouse model of ALS (D'Sa et al. 2007). Furthermore, caspase 3/7 activity, which can be increased by reactive oxygen species (ROS) (Powers et al. 2005; Powers et al. 2007), was not significantly elevated over the course of the immobilization. Elevations in protein carbonyls or 4-hydroxy-2-nonenal adducts have been detected in human tissues during various pathologies (Aksenov et al. 2001; Calabrese et al. 2007; Negre-Salvayre et al. 2008; Poli et al. 2008; Ramlawi et al. 2007; Ryttila et al. 2006; Tsimikas 2008), but to our knowledge there is no report demonstrating a clear connection between disuse-induced dysregulation of skeletal muscle ROS generation, subsequent elevations in proteolysis, and consequent wasting in human muscle. In fact, our findings suggest that the pellet fraction of healthy young muscle, the majority of which is comprised of myofibrillar protein, is protected from and is not in fact susceptible to oxidative damage during disuse atrophy.

In summary, we observed a transient (2 d) increase in ubiquitinated proteins and no increase in oxidatively modified proteins during a 14 d immobilization intervention with a large sample of 21 subjects. We acknowledge that in the absence of concurrent measures of muscle protein breakdown, these ‘snapshots’ can only provide a limited amount of insight into the role that muscle proteolysis plays in disuse atrophy in humans. Thus, it will be important to characterize the early (2-4 d) response to unloading with direct measures of muscle protein breakdown, as well as the susceptibility of individual

proteins to oxidative damage and proteolysis, particularly those that play a role in the regulation of protein turnover. Indeed, specific skeletal muscle proteins may well be the targets of reactive oxygen species or ubiquitination under conditions of unloading, but we suggest that large scale increases in such modifications as a driver of atrophy are improbable.

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Tables

Table 1 Quadriceps CSA and fibre area data at Pre and 14 d. Pooled data from (Yasuda et al. 2005).

	Pre	14 d	% change	P
Quadriceps CSA, cm ²	72.9±3.4	68.8±3.3	5.7±1.1	<0.0001
Type I fibre area, µm ²	4258±227	4029±235	5.6±1.1	0.0002
Type IIa fibre area, µm ²	5836±383	5268±334	9.2±2.3	0.0056
Type IIx fibre area, µm ²	4748±316	4137±271	11.8±2.9	0.0027

Mean±SE. N=21 for quadriceps CSA and N=15 (N=9 males, N=6 females) for fibre area.

Figures

Fig. 1 Ubiquitin conjugated proteins prior to (PRE), 2 d and 14 d after immobilization from (a) full length blot or (b) 55 kDa band. Representative blot is shown in (a). Western blot data are corrected to Ponceau and expressed as means \pm SEM. * Significantly different from pre $P < 0.05$ by 1-way ANOVA .

Fig. 2 Caspase 3/7 activity (a) and 14 kDa actin fragment (b) prior to (PRE), 2d and 14d after immobilization. Representative blot is shown in (b). Western blot data are corrected to Ponceau. Values expressed as means \pm SEM. No significant differences by 1-way ANOVA.

Fig. 3 4-hydroxy-2-nonenal (4-HNE)-conjugated proteins prior to (PRE), 2 d and 14 d after immobilization from (a) full length blot or (b) 55 kDa band. Western blot data are corrected to Ponceau and expressed as means \pm SEM. No significant differences by 1-way ANOVA.

Fig. 4 Carbonylated proteins prior to (pre), 2d and 14d after immobilization from (a), full length blot and (b) 125 kDa band. Western blot data are corrected to Ponceau and expressed as means \pm SEM. No significant differences by 1-way ANOVA. Values are means \pm SEM.

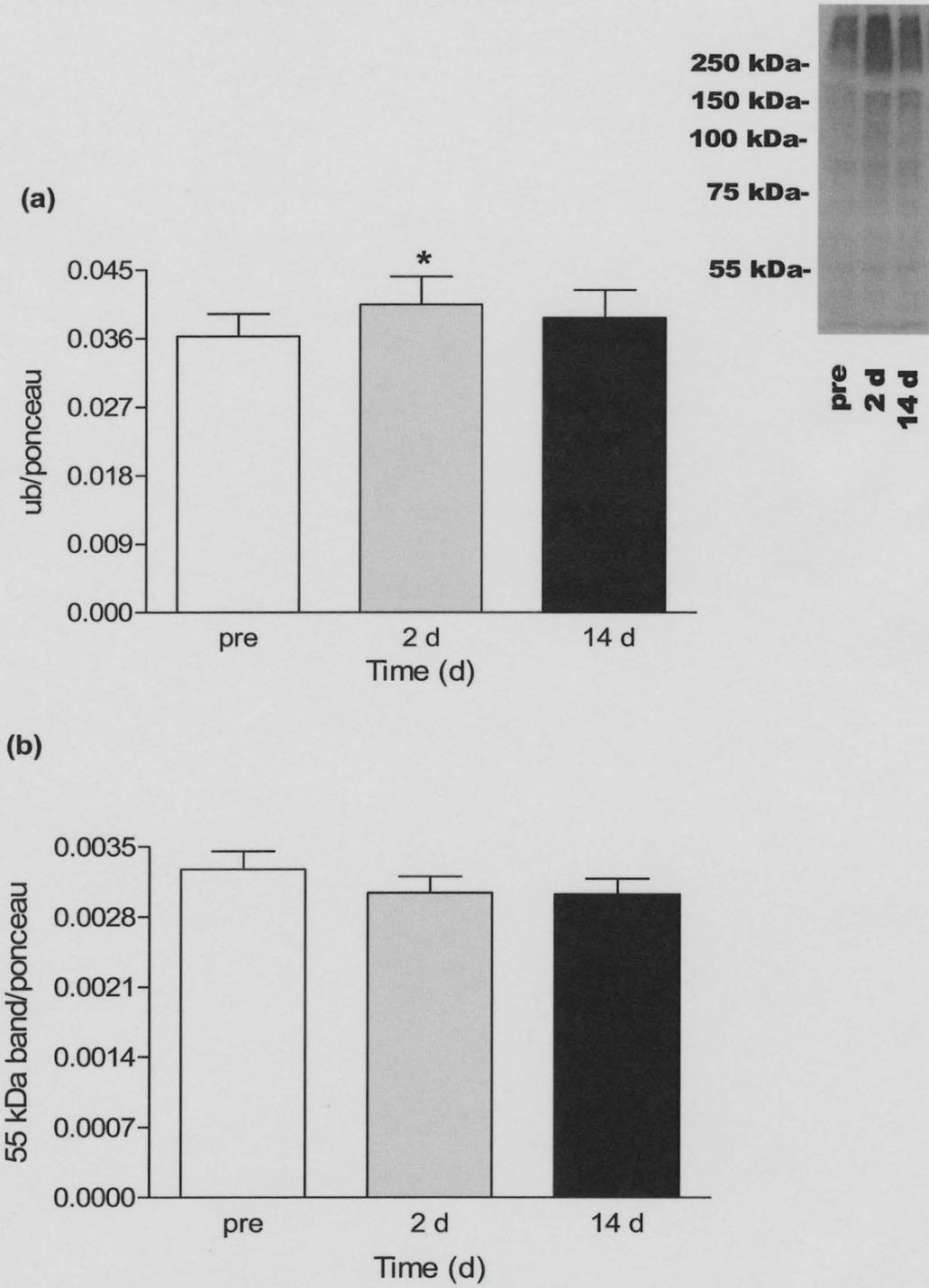


Figure 1

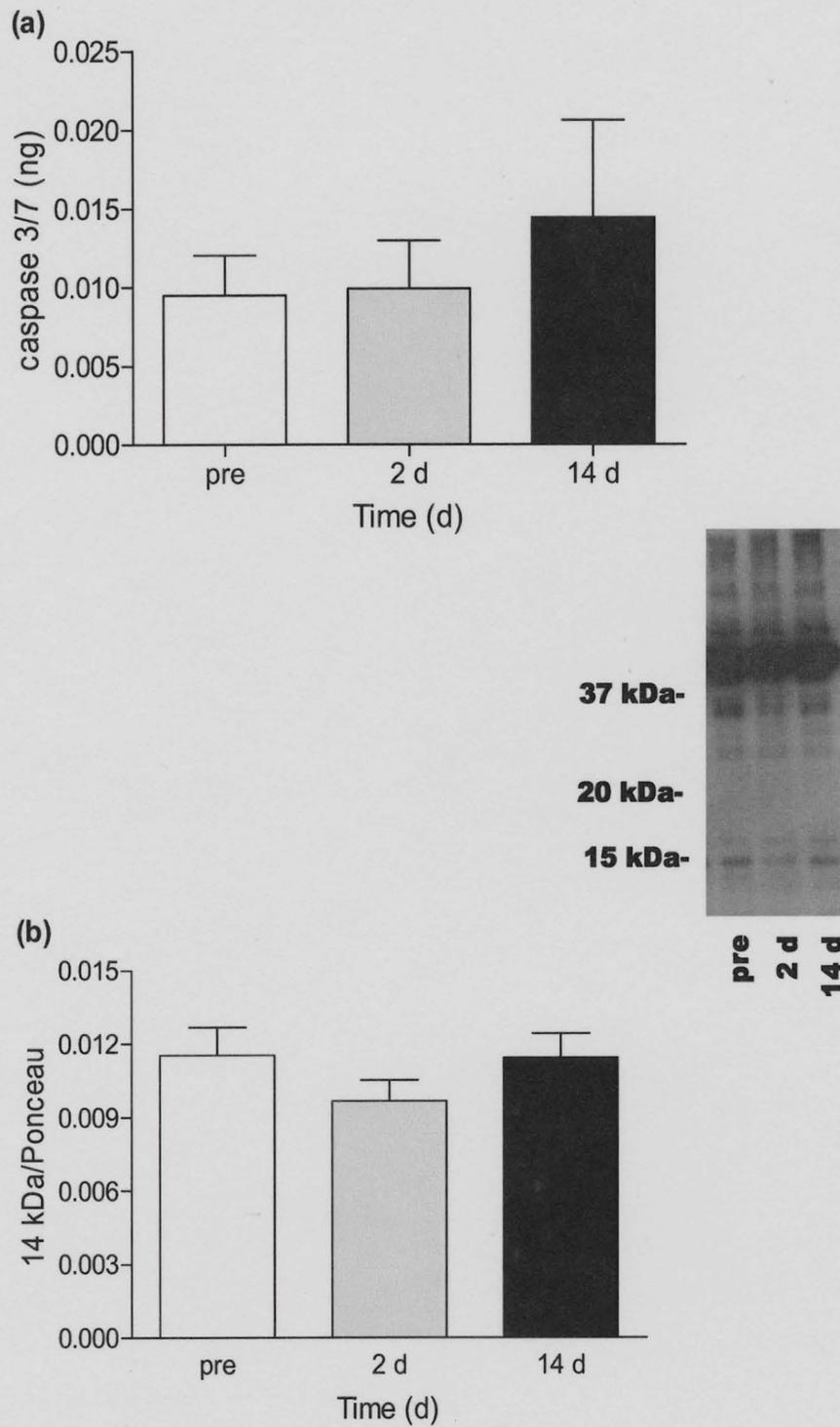


Figure 2

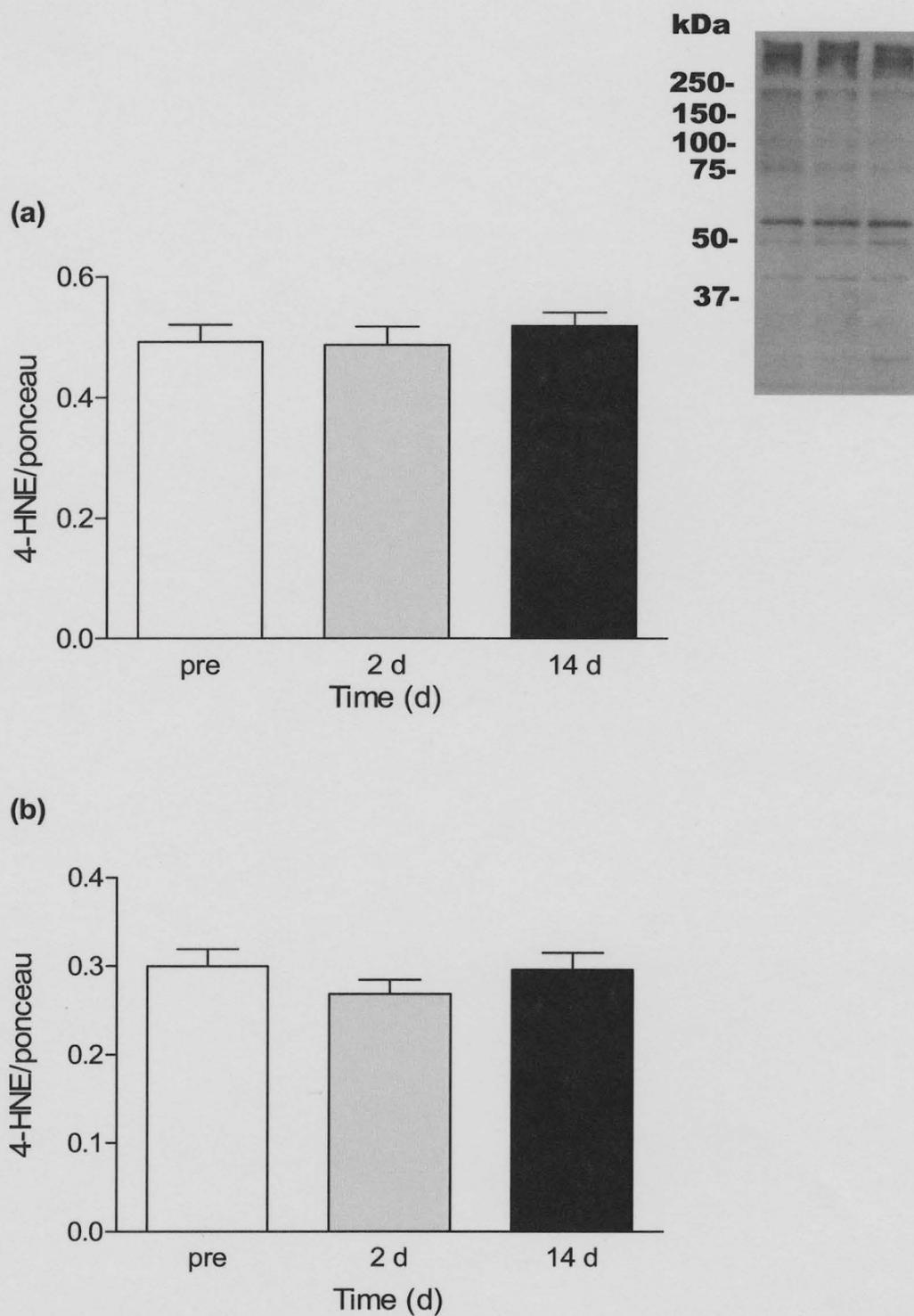


Figure 3

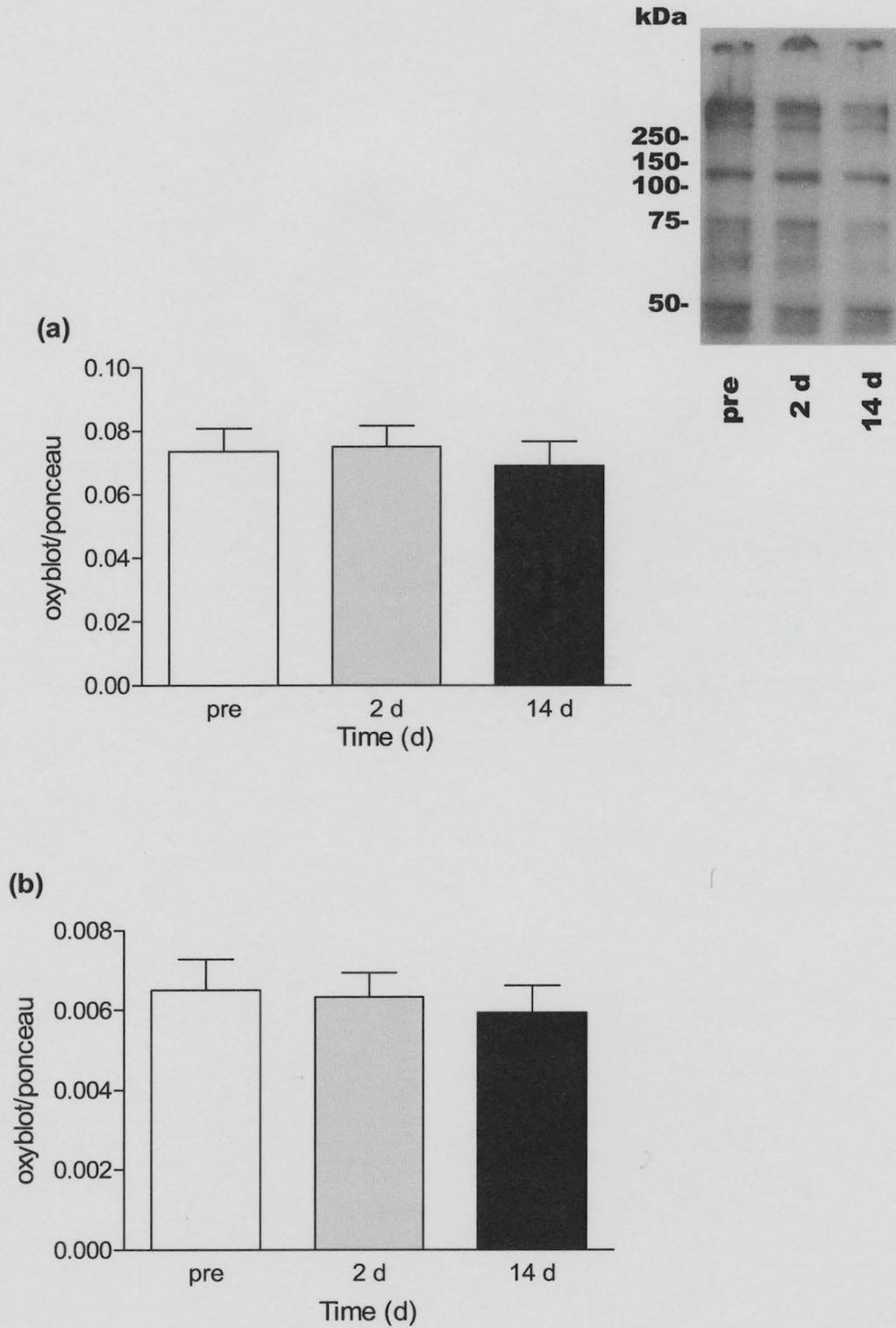


Figure 4

Chapter 5: General Discussion

The studies detailed in this dissertation had the general aim of examining selected aspects of human muscle protein turnover and its regulation across the activity/loading continuum. While the examination of muscle protein turnover responses to altered states of loading was not comprehensive, these investigations made novel contributions that advanced our understanding of human muscle protein turnover and generate additional questions to answer in future work. This chapter summarizes the main findings from the previously presented studies and provides recommendations for future work in the area of muscle signaling and protein turnover responses to variations in loading and amino acid feeding.

In Chapter 1 (Study 1) we reported on the acute (6h) signaling response following resistance exercise with and without feeding using a validated unilateral leg design that minimizes variability due to different subject groups and multiple trials and is devoid of substantial hormonal changes (84). Thus, we improved upon earlier investigations that did not compare all four nutritional-activity states in the same subjects and using similar loading/feeding protocols. Our focus shifted to the synthetic and signaling responses to intravenous feeding after a 14 d period of unilateral immobilization in Chapter 2, extending our knowledge and reporting for the first time that immobilization-induced a depression in synthesis to the fed state. Therein Chapter 3 we reported on static markers of muscle protein breakdown and oxidative damage in the resting state at early (48h) and late (14d) periods, in which we previously reported that atrophy occurs in both men and

women (87), after the onset of immobilization, providing novel findings in an area for which human information is scarce.

Chapter 2: Effect of feeding and resistance exercise on phosphorylation of key translational signaling proteins

In study 1 we reported an additive effect of exercise and feeding with respect to two signaling protein intermediates, S6K and rps6. We also showed for the first time an exercise-induced dephosphorylation of eIF2B ϵ at a GSK3 β -mediated inhibitory phosphorylation site, Ser⁵⁴⁰. This was not further modified by feeding which may be a consequence of the timing of feeding, or perhaps because feeding has little effect on modulation of phosphorylation at this site in humans. In contrast to our expectations, we did not observe changes in residue phosphorylation for the feeding alone condition, aside from an increase in Akt (Ser⁴⁷³). The pattern of feeding in this study was rather unique, as three boluses were given 90 minutes apart, with provision of the first bolus 90 minutes post exercise and a final bolus 90 minutes prior to the biopsy. We reasoned that bolus feeding would be more realistic and prevent muscle protein synthesis from becoming refractory to feeding, as observed with a constant infusion of amino acids (10). However, in light of the 1h delay in rise in feeding-induced FSR recently observed in response to consumption of a single bolus of whey protein, it may be that the periodic bolus consumption pattern was not optimal for observation of a 'feeding' effect, either some degree of refractoriness had nevertheless developed, or a more rapid fall in synthesis occurred by the third bolus. Of course, it is also entirely possible that MPS, which was not measured, and signaling protein phosphorylation were not associated (see discussion

in Chapter 1). Exercise and feeding combined enhanced S6K1 and rps6 phosphorylation which is in line with the well established synergistic interaction between feeding and exercise. In the absence of synthetic measures, however, it is impossible to make any statements about the relationship of the phosphorylation of signaling molecules to MPS with our study design.

As our data in Chapter 2 showed we did not observe a sustained elevation in FAK phosphorylation, evidence for which is beginning to accumulate as an important potential transducer of extracellular stretch. Since the completion of this study our group has demonstrated that FAK phosphorylation was strongly elevated 1h after exercise, with a drop 4h post exercise such that in trained subjects levels are not different from basal (83). The participants in our study were relatively trained which may account for the lack of elevation in FAK phosphorylation by 6 h post exercise.

Future Work

The study reported in chapter 2 was designed to examine changes in signaling at a time when MPS is known to be elevated post exercise (6h). The period over the first hour post exercise or immediately after onset of feeding has not been systematically characterized for humans in terms of changes in phosphorylation of the putative signaling factors or MPS at least in response to a physiological feeding pattern. As the most extensively studied candidates are involved in translation initiation, this would be a crucial time during which major changes are likely to occur. Biopsies every 5-15 minutes may be required to resolve important changes since phosphorylation cascades may be activated and terminated in the order of minutes. Such a time course has been performed

in operantly conditioned rats, revealing that members of the Akt-mTOR-p70 pathway are maximally phosphorylated within 10-15 minutes after exercise (11).

At the time of writing, additional information on the regulation of eIF2B with exercise and feeding in humans is unavailable. Given the significant role accorded to eIF2B activity in regulation of muscle protein synthesis, especially in response to resistance work in animals (35), further work on the regulation of the eIF2 GTPase eIF2B and its subunits is clearly warranted. While we did not observe changes in Akt-mediated GSK3 β phosphorylation in this study, the decrease in phosphorylation of eIF2B ϵ could only have been the result of net phosphatase action, as withdrawal of kinase activity is usually not considered sufficient for a protein to lose the covalent phosphate bond. An understudied area in the regulation of signaling intermediates is the relative contribution of phosphatases in maintaining target proteins in the phosphorylated state. For example, the MAPK proteins are inactivated by MAPK-specific phosphatases (MKP), and dysregulation of their function may, like kinase overactivity, have potentially deleterious physiological consequences (34). In addition, eEF2 is rapidly dephosphorylated in response to insulin (64), likely by protein phosphatase 2A (PP-2A) (65).

To date a significant correlation between the degree of phosphorylation of translational signaling intermediates or initiation/elongation factors and actual synthetic rates has not been demonstrated, although recently it was shown that S6K1 phosphorylation correlated MPS rates 1-2h post exercise in young men (40). Moreover, phosphorylation of S6K1 in response to acute exercise has been correlated with the degree of hypertrophy in response to a training program, supporting the notion that S6K1

is potentially rate determining for protein synthesis (2; 74). Confirmation of the relationship between MPS and S6K1 could be cemented with a study in which MPS is measured at multiple timepoints post exercise in conjunction with S6K1 phosphorylation. This would appear to be particularly important in type II fibres, which are more responsive than the type I fibres in resistance training programs in terms of hypertrophy and also display enhanced S6K1 phosphorylation after intense lengthening exercise (38; 72). In addition, p90rsk has not been extensively studied in the context of exercise or nutrition, yet is considered a ‘central node’ for mediating many effects in translational signaling pathways (Figure 2, Chapter 1); future work should include an assessment of the phosphorylation state of this protein. Finally, additional work is required to further elucidate the potential role of FAK in coordinating extracellular stretch with intracellular signals, especially as it appears to be phosphorylated (i.e., presumably activated) by both low load (endurance) and high load (resistance) contractions (83).

Chapter 3: Effect of unloading on the feeding induced rise in FSR

We confirmed the well known (15; 21; 24; 25) suppression of fasted state protein synthesis that occurs with unloading-induced disuse atrophy. The novel contribution from the work we present in chapter 3 was a demonstration of a suppression of MPS across the diurnal fed-fasted continuum such that fed-state myofibrillar protein synthesis was also suppressed. The only previously available information related to disuse in humans and protein turnover and feeding was reported by Biolo and colleagues showing that 14 d of bed rest inhibited the whole body protein synthesis in response to amino acid infusion (6). While MPS accounts for only 27% of whole body protein synthesis (56) and changes

in MPS would contribute to changes observed in whole body protein synthesis (21), Biolo et al's results were not confirmatory that it was muscle that was affected by the bed rest intervention. As a 'systemic' (as opposed to local) intervention, bed rest may provoke changes in a number of tissues such that changes in fed state whole body protein synthesis may not necessarily have reflected changes at the muscle level.

The suppression in resting MPS we observed could not be overcome by a high rate or relatively long-term (4 h) amino acid infusion. The high AA infusion rate used in this study was previously shown to be the highest rate resulting in a plateau in MPS at rest in a dose response study (9). Therefore, assuming a maximal stimulation of synthesis was achieved in our study, our findings suggest that not only does disuse impair the MPS response to submaximal amounts of amino acids, but that the maximal feeding-induced synthetic rate is blunted with disuse. Consequently, an assessment of anabolic sensitivity, analogous to insulin sensitivity, estimated based on the half-maximal stimulating concentration of amino acids, would likely show a decrease.

We chose to use an intravenous feeding model in our study because of its established use in previous mechanistic studies of feeding and MPS (7; 9; 10; 48) and to control for potential issues such as inter-subject variation in gut absorption and first pass hepatic clearance. While perhaps not directly relevant to situations where free living individuals have a limb unloaded and are consuming their typical daily diet, our findings do have applicability to the clinical environment. Our work would suggest that in patients receiving TPN for example, provision of AA even at high rates cannot overcome the anabolic resistance that develops in inactive muscle such as during bed rest.

The relevance of our 14d immobilization model to clinical states of injury-induced disuse is questionable. Our model is useful for mechanistic explorations, but we are aware that uncomplicated disuse atrophy occurs only with immobilization. Muscle wasting in clinical situations is usually a consequence of injury or disease-related inactivity. For example, muscle wasting that occurs in illnesses such as sepsis, cancer cachexia, and HIV-AIDS, are accompanied by elevated catabolic hormone and cytokine secretion and are oftentimes compounded by hypocaloric intake. In addition, even in normal limb immobilization, this is likely accompanied by inflammatory and direct injury-related changes at the site that could contribute to atrophic processes, at least initially. For instance, head trauma, critically ill, and spinal cord injured (SCI) patients have been reported to exhibit elevations in components of the proteolytic systems (29; 51; 80), with fibre atrophy rates reported to be 3-4% d⁻¹ among the critically ill (29). Furthermore, relatively short-term models of disuse employing healthy young volunteers likely do not reflect the reality of chronic atrophic states in which long term illness, age, and disability coalesce to produce a complex atrophy phenotype that may well involve dysregulated proteolysis (Figure 1).

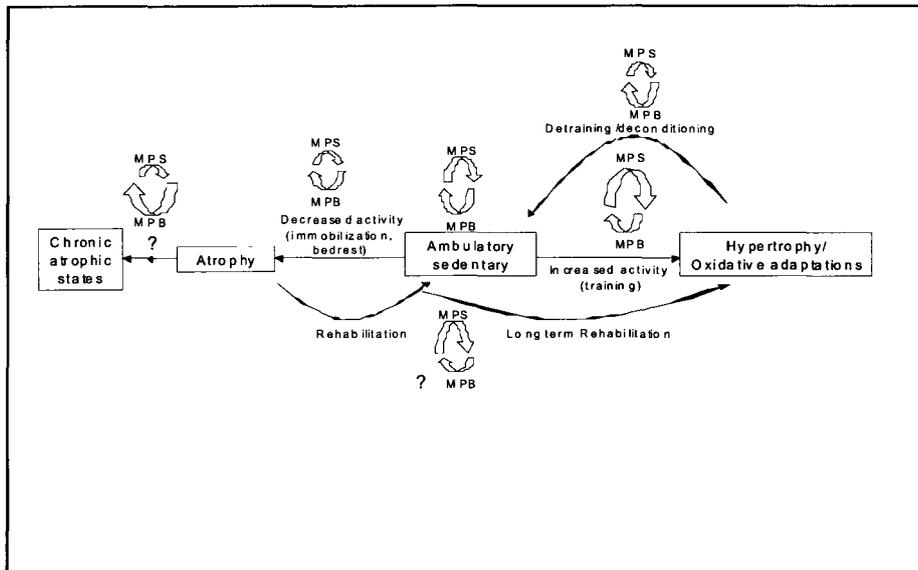


Figure 1. Protein turnover across the muscular activity continuum.

Proteolytic components have been reported to be elevated in ALS (45) and the disabled elderly (16). Nonetheless, in all of these disease states there usually exists a component of hypodynamia and thus decreased MPS would be part of the disease state that contributes to the muscle phenotype. Indeed, the extent to which elevated proteolysis plays a role in progression of these catabolic conditions is unclear- for example cachectic cancer patients do not exhibit elevated MPB (17; 20), even in the face of elevated markers of inflammation, but do show a blunted response to amino acid feeding (17).

The concept of ‘anabolic resistance’ which is essentially defined as a blunted stimulatory response of MPS to feeding and exercise, has been noted with aging (14; 40), and appears to be associated with blunted signaling responses, at least in the elderly. We observed development of anabolic resistance to amino acid feeding with 2 weeks of knee-brace mediated immobilization in young adults, but without striking changes in anabolic signaling factors. Additionally, immobilization/lower limb suspension suppresses fasting

MPS rates in young individuals without decreasing resting concentrations of signaling factors or changes in their phosphorylation (15; 26), suggesting that the lack of regulation does not appear to occur at the level of a global reduction in quantity or responsiveness of translational signaling molecules.

Skeletal muscle is known to rapidly develop reduced insulin action with inactivity (66) while training improves insulin sensitivity (85) in terms of glucose uptake. It is tempting to speculate that anabolic resistance is related to insulin resistance but the blunted signaling response in the elderly to feeding has been shown to occur in the presence of a basal insulin clamp (14), and type II diabetics exhibit MPS responses to increased insulin (4), and protein feeding (50) that are not different from non-diabetic controls. The situation may, however, be different with disuse atrophy. We did not measure phosphorylation of components of the insulin signaling pathway and therefore cannot make any statements about its role and possible cross-talk between the mTOR and insulin signaling pathways in disuse atrophy. No information on changes in insulin signaling is currently available with disuse in humans, but the inhibitory phosphorylation of IRS-1 by JNK is increased with hindlimb suspension in rats (30), promoting rapid degradation of IRS-1, and amino acid-induced activation of S6K1 has been shown to inhibit IRS-1 activity and glucose transport in muscle cells (79). A recent study (28) showed that in humans it appears that the main role in insulin is not a stimulatory one but instead is permissive for protein synthesis and mildly stimulatory only up to a low level beyond which it does not further enhance MPS. In contrast, at higher physiological

concentrations insulin can suppress proteolysis, a process that is highly sensitive to insulin (13; 28).

An interesting prospect is that MPB in disuse states is less sensitive to suppression by insulin and so fed state gains in protein mass are lower, as we have shown, but due not only to a blunted MPS response but also a lesser suppression of MPB. However, we observed no difference in ubiquitination levels between legs in the fed or fasted states (Chapter 4), but as we did not measure MPB, we also cannot state if the feeding/insulin induced suppression of MPB is affected by disuse. A decreased insulin-mediated suppression of thigh tyrosine release was observed after 7 d of immobilization (66), but 14 d bedrest did not affect the amino acid induced suppression of whole body breakdown (6). The main mechanism by which insulin is thought to increase MPS is through an increased AA delivery via increased capillary recruitment and microvascular perfusion (19). We did not measure microvascular volume or bulk blood flow in this study, but it is possible that local insulin-mediated increases in delivery are compromised with disuse, as has been proposed in the elderly (23; 63).

Future Work

As we did not measure MPB the next studies to be undertaken would hopefully assess both MPS and MPB in the fed and fasted states to obtain a complete picture of protein turnover in local (immobilization) and systemic (bed rest) models of disuse. The temporal pattern of the fall in protein synthesis with imposition of inactivity has also yet to be mapped out. In particular, there is a lack of information on protein turnover responses in the early phase (3-5 d) after initiation of unloading. It is expected that such a decrease

would be detected well before a measurable degree of atrophy occurs, but turnover estimates have not been made prior to 10d of lower limb suspension (15). Reductions in MPS with hindlimb suspension have been observed as early as 48 h (75) but protein turnover is ~2.5 fold higher in rodents, lagomorphs and fowl than humans (82). Given that every human undergoes a period of ~8 h of 'bed rest' (i.e., when they sleep) and spends 6-8 h in a postprandial state on a daily basis, it is expected that overt atrophic processes would not be observable until periods of bed rest or fasting would be at least 3-4 times longer than what individuals undergo diurnally. In addition, to date, a relationship between the fall in MPS with the extent of atrophy has not been established, but it would be expected that those who experience greater falls in synthesis would ultimately exhibit greater atrophy.

An important goal in human muscle protein turnover research would be to identify and track the turnover rates of individual proteins. This has been recently achieved for specific mitochondrial and non mitochondrial proteins in rodents, with different proteins exhibiting a broad variability (10 fold) in synthetic rates (32). While we now know all protein sub-fractions (e.g. mito, sarco, myo) in human muscle are stimulated by feeding (9; 54), exercise stimulates the mitochondrial and myofibrillar fractions (54; 83) and disuse suppresses mixed and myofibrillar fractions (15; 24; 26), we do not currently have data on turnover rates of specific muscle proteins, which would be informative in conjunction with information on translational signaling and specifically ubiquitinated proteins under varying states of loading and nutrition. Unfortunately,

breakdown rates of specific proteins, beyond what can be obtained with 3-MH, are not obtainable using current methods.

The results obtained in our study with AA infusion remain to be replicated with oral ingestion of amino acids, intact protein and/or mixed meals. It should be noted, however, that the immobilization model, being a model of local atrophy, is also unlikely to be accompanied by changes that may affect gut absorption and clearance, while it may be an issue for systemic models of disuse such as bed rest and spaceflight, and very likely is affected in disease and injury states. Several studies have attempted to mitigate the effects of disuse on lean mass loss by provision of protein or amino acid supplements (12; 77; 78) and in only one study was it reported that nutrition alone could attenuate muscle protein loss (59). Of potential concern is the observation that elevated protein consumption in long term bed rest studies resulted in increased atrophy of the thigh (5; 78), likely due to upregulation of protein disposal (deamination and oxidation) pathways. The major differences between the study by Trappe *et al.* (78) and Paddon-Jones *et al.* (59) are that Paddon-Jones and colleagues used crystalline essential amino acids, the period of bedrest was shorter (28 v. 60 d) and dual X-ray absorptiometry was utilized by Paddon-Jones instead of magnetic resonance imaging. It is possible with longer term bedrest loss of muscle protein may have been observed by Paddon-Jones *et al.* Therefore it will be important to demonstrate that oral consumption particularly of crystalline amino acids (which have been argued to be particularly stimulatory because of the pronounced and rapid hyperaminoacidemia they induce (59)) during disuse is no more effective than direct intravenous provision, and to measure changes in protein oxidation as well as

levels of protein oxidation enzymes with nutrition counter measures during disuse. In addition, future work in uncovering the mechanisms of disuse-induced anabolic resistance should include examination of the insulin signaling pathway, and measures of both capillary recruitment by assessment of microvascular volume and bulk blood flow.

Chapter 4: Changes in markers of proteolysis and oxidative stress with 14 d immobilization

Our third study was undertaken to examine how protein degradation and oxidative stress might be playing a role with 14d of immobilization. We used the model that we employed previously (26; 87), as so all of our observation are of course derived in a situation of uncomplicated disuse atrophy. We observed an early (48 h) increase in ubiquitin protein conjugates which was subsequently decreased at 14d. Our observation is in contrast to a report in which ubiquitinated proteins were found to be elevated after 20d of bed rest (58). Although muscle protein breakdown has not been found to increase with 21-28 d bed rest (21; 70), the bed rest model, being different in many ways from the ambulatory models of local atrophy, may result in accumulation of ubiquitin conjugates in the muscle, without necessarily increasing bulk proteolysis. Inhibition of proteolysis via proteasomal degradation actually results in accumulation of muscle ubiquitin conjugates (73). Thus, the increase in ubiquitination observed in our study are surrounded by questions about the nature and identity of the ubiquitinated proteins. The increase in ubiquitinated proteins was concentrated in the ~200+ kDa region, which could be interpreted as indicating an increased ubiquitination of large sarcomeric proteins such as myosin or titin. The addition of ubiquitin chains may, however, affect a migration of a

protein through a gel to a greater extent that the molecular weight of the chain would predict (each ubiquitin molecule is 8.5 kDa), or possibly promote aggregates that may not migrate very far into the gel. Furthermore, it is worth noting that mTOR is 289 kDa while its binding partner rictor is 200 kDa, although it is not currently known if either is preferentially ubiquitinated under pro-atrophic conditions. Increased ubiquitin conjugates have been associated with tagging of damaged proteins which would be expected to increase after, for example, intense or lengthening contraction biased exercise. In contrast, with a sudden decrease in muscular activity it is difficult to envision how proteins could become damaged on such a large scale that bulk protein breakdown would be rapidly upregulated, particularly at early times post-immobilization. In fact, rather than increased ubiquitin conjugates representing an increase in proteolysis a reduction in the rate of proteolysis of ubiquitinated proteins is equally as likely.

As part of the study reported in chapter 4, the descriptive results from which have been reported (87), gene array analysis was also conducted. These data are in press (A. Abadi et al., PLoS ONE) and are not part of this thesis; however, I was heavily involved in the study and am the first co-author on the submitted manuscript. Hence, for information and interpretation purposed some of the salient changes in gene transcripts for pathways involved in proteolysis and synthesis are included in this thesis (see Appendix 1) and are reviewed and interpreted since they bring a higher degree of insight into the potential regulation of these processes.

The small (12%) increase in ubiquitin conjugates we observed was accompanied by increases in the gene expression of atrogin-1/MAFbx (60%) and MuRF1 (30%; see

Appendix 1, table 1), two ubiquitin ligases shown to be robustly induced in rodent models of atrophy (8; 27; 44), at 48h as determined by real-time PCR. Interestingly, the probe sets for these genes did not change significantly when analyzed on the Affymetrix array for this study (Appendix 1, Figure 1). A number of ubiquitin ligase (E3) genes appeared as significantly altered from pre levels on the Affymetrix array, but in contrast to MAFbx and MuRF-1, these transcripts were different primarily at day 14 and not at day 2 (Appendix A, Table 1). The expression patterns were mixed, however, with some ring finger and f-box proteins decreasing in expression and others displaying an increase. These data could suggest a highly complex or possibly no truly consistent regulation of E3 enzymes with disuse. However, as the E3 enzymes confer specificity on the ubiquitin-proteasome system it is tempting to speculate that a complex specificity in ubiquitination of target proteins might be occurring rather than global and non-specific increases in protein tagging and bulk proteolysis. Of note, mRNA for FBXO40, a muscle specific f-box protein (f-box proteins are components of E3 ligase complexes that confer substrate specificity (71) whose expression is decreased in limb girdle muscular dystrophy, was also found to decrease early and remain suppressed through to 14 days of immobilization. In contrast, upregulation of the same gene has been observed in a rat denervation model (88). Interestingly the ubiquitin ligase cullin7 was modestly increased by 14 d and it is known that IRS-1 is a specific target of cullin7 (86).

Upstream of the ubiquitin ligases, a ubiquitin activating enzyme (E1C) was upregulated at 14d, but like the E3 members, ubiquitin conjugating enzyme (E2) transcripts exhibited variable changes, with roughly half decreasing and half increasing in

their expression. Most proteasome subunit mRNAs increased 12-25% by 14d, although expression of 2 subunits decreased (PSMD 9 and 10).

Interestingly, the largest class of deubiquitinating proteins in the genome, the ubiquitin specific peptidases (USPs) (57), showed, on average, an increased pattern of expression by 14d suggesting a counter regulatory response to increases in components of the ubiquitin-proteasome system. In addition, the large subunit of calpain 2 and cathepsin O were upregulated 14-16% by day 14. Of note, the muscle-specific calpain p94 decreased at 14d, which was also noted by Jones et al (33). This calcium-activated protease is absent or defective in limb girdle muscular dystrophy IIA, and activated (autolysed) after eccentric exercise (55) possibly implying a role for this protease in maintenance of normal muscle ultrastructure.

Autophagy has recently emerged as a potentially important proteolytic process in atrophy. In support of this thesis the autophagosomal/proapoptotic gene bnip3L, which is upregulated in fasting and denervation (89), as well as a number of genes coding for vacuolar protein sorting (Vps) genes were increased by day 14. The gene encoding an autophagy associated protein, ATG5 (49) showed an early and sustained decrease. Transcripts for a protein required for prevention of autophagy in denervated rodent muscle, RUNX1, (81), decreased by 45% at 14 d. There is currently no data examining the expression of these proteins with disuse atrophy in humans and further investigation is warranted.

While changes in phosphorylation status, and presumably activity, of the translational signaling molecules is thought to be the level at which synthetic control is

regulated acutely, an examination of the transcriptional changes of translational regulators with immobilization can suggest coordinate regulation of certain pathways and uncover new directions for research. Perhaps somewhat surprisingly, mRNA for the translational inhibitor 4E-BP1 significantly decreased 36% by 14d. Transcripts for eIF1, eIF3 and eIF5 subunits were largely downregulated by 14d on the Affymetrix array. The activity of eIF1 has not been examined in the context of hypertrophy or atrophy in humans, but it controls start codon selection (53) and therefore has the potential to play a pivotal role in translational control.

Interestingly, transcript abundance of most of the eEF1 subunits were decreased by 14 d. Changes in eEF2 phosphorylation, which mediates ribosomal translocation, have been investigated in studies of feeding and exercise but eEF1, responsible for aminoacyl-tRNA transfer to the ribosome (43) has not been studied.

Finally, mRNA coding for rps6 was found to be slightly downregulated by 14d while expression of its upstream effector, p90^{rsk3}, also declined, although S6K1 did not change significantly on the gene array. The mTOR binding partner rictor increased expression from 48h to 14d, while rheb mRNA, which acts on mTOR when in the mTORC1 complex with raptor, also increased. These changes may be attempts to compensate for early falls in mTOR activity (mTOR phosphorylation was found to decrease at 48 h (A. Abadi et al., PLoS ONE, In Press)).

These array findings have not been confirmed with real-time PCR, but the relationship between array changes and real-time PCR was very good for a selection of 6 metabolic genes ($R^2=0.996$). The extent to which the change in gene expression might

impact expression of the proteins they encode and the subsequent functional implications for skeletal muscle protein remodeling await confirmation.

A relatively popular concept in disuse atrophy research is that oxidative stress may be a contributing process or even a trigger for the onset of atrophy (61; 62). Thus, in chapter 4, we looked at markers of oxidative stress. Two markers of oxidatively modified proteins were measured (4HNE, protein carbonyls), but neither were found to increase over the immobilization period. In this analysis we did not measure these markers of oxidative stress in the soluble protein fraction, or antioxidant enzyme content or activity as markers of antioxidant capacity, therefore we cannot state for certain that changes did not occur in those proteins. We would speculate that if any proteins are being oxidatively damaged they are likely the more susceptible sarcoplasmic proteins and not myofibrillar.

The treatment of myotubes with hydrogen peroxide increases ubiquitin-protein conjugates and expression of ubiquitin-proteasome pathway enzymes such as MAFbx (41; 46), while animal studies have reported changes in antioxidant enzymes, antioxidant capacity and sarcoplasmic markers of oxidative stress in conjunction with disuse atrophy (37; 42; 62). Levels of mitochondrially generated reactive oxygen species (ROS), produced during muscular activity, would be expected to decrease substantially under conditions of muscle inactivity (61) but there is some evidence to suggest that mitochondria generate more reactive oxygen species during basal, state 4, respiration than during state 3 maximal respiration (60). Hence, a lower but persistently elevated rate of ROS production at rest could trigger or change the basal function of any number of processes linked to cell damage.

Administration of antioxidants that increased muscle antioxidant capacity did not attenuate hindlimb unloading–induced atrophy (36), but administration of vitamin E partially reduced soleus atrophy by 17% in another study (68). The authors concluded that vitamin E exerted its anti-catabolic actions through suppression of the upregulation of caspases, calpains and ubiquitin ligases typically observed in this model, rather than by functioning as a direct antioxidant, as several markers of oxidative stress (reduced:oxidized glutathione, increased uncoupling protein mRNA and in antioxidant enzymes) were unaffected by supplementation (68). The lack of an effect on oxidative stress markers in the study by Servais et al., would seem to be contrary to some notions (3) that large scale oxidative stress is a causative factor in disuse atrophy. Nevertheless, Powers has raised the possibility that oxidative stress may play a role in atrophy via suppression of protein synthesis, but to date all ROS and atrophy research has focused on proteolysis (61). In fact, the mTOR pathway has been shown to be redox sensitive *in vitro* (67)

Future work

The findings from the microarray provide a number of potential targets in the ubiquitin-proteasomal, autophagic, and translational signaling pathways that have not been previously examined in the context of disuse atrophy in humans, and should be confirmed on the protein level. Microarray data, however, only provide a snapshot of RNA content at a particular timepoint. These data provide no information on the rates or transcription or RNA degradation or protein expression/function; however, it is an important first step in understanding potential regulation. The study of microRNA (μ RNA), short stretches of

RNA encoded in the genome that bind with varying affinity to transcripts and regulate their stability and transcription is beginning to be applied to the field of exercise physiology, and future studies of disuse atrophy should include measurement of these molecules to gain a better understanding of transcriptional regulation during disuse(18). Future work on atrophy and oxidative stress and breakdown markers should focus on the identification of the specific proteins that are oxidatively modified and/or ubiquitinated/degraded in order to gain a better understanding of the metabolic and structural remodeling that atrophying muscle undergoes. For example, MyoD is ubiquitinated by MAFbx (76) and degraded by the proteasome (1), FAK is ubiquitinated and degraded (47), while MuRF1 localizes to titin (52) and targets myosin heavy chain (22). Oxidatively modified and ubiquitinated proteins may even perform some kind of signaling function during disuse atrophy, rather than being quantitatively important in bulk proteolysis.

To date, no human study has examined the use of an antioxidant or anti-proteolytic drug as a means of attenuating atrophy during the course of disuse. Potential interventions to investigate the role of ROS production in atrophy would include a cocktail of vitamin antioxidants such as vitamin E and C, and iron chelators to control non-heme iron-induced oxidative stress (31; 61), or pharmacological inhibitors such as allopurinol to prevent reactive oxygen species production via xanthine oxidase (61). The major pathways of proteolysis itself could be targeted by administration of agents such as dantrolene, amlodipine or nifedipine to inhibit increases in intracellular calcium (69), the proteasome inhibitor velcade (39), and chloroquine, to decrease lysosomal

acidification, although potential side effects would likely preclude administration of several of these compounds to healthy individuals.

Summary

This dissertation summarizes the effects of altered loading on protein turnover or markers of protein turnover. A single bout of resistance exercise of an intensity known to stimulate muscle protein synthesis increased phosphorylation of the key signaling molecule S6K1 and its target rps6, as well as the ϵ subunit of the eukaryotic initiation factor 2B, with enhancement of phosphorylation of S6K1 and rps6 in the fed state. Fourteen days of immobilization reduced both fasted and fed state myofibrillar protein synthesis, without major differences in signaling to account for this anabolic resistance. In addition, fourteen days of immobilization were not found to result in sustained elevations in static markers of breakdown and oxidative stress.

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APPENDIX 1

Significant gene changes from the Affymetrix array (HG U133 plus) of Study #3
(Chapter 4)

Table 1. Significant gene changes from the Affymetrix array (HG U133 plus) of Study #3 (Chapter 4)

<u>Class</u>	<u>timepoint</u>	<u>fold change</u>	<u>Gene Symbol</u>	<u>Unigene ID</u>
<u>Protein Translation</u>				
Eukaryotic initiation factors				
eIF1	14D	0.85-0.66	EIF1	Hs.150580
eIF1B	14D	0.86	EIF1B	Hs.315230
eIF3subunit 1 alpha	14D	0.87-0.91	EIF3S1	Hs.404056
eIF3subunit 2 beta	14D	0.91	EIF3S2	Hs.530096
eIF3subunit 5 epsilon	14D	0.81	EIF3S5	Hs.516023
eIF3subunit 6 IP	14D	0.89	EIF3S6IP	Hs.446852
eIF3subunit 9 eta	48H	1.17	EIF3S9	Hs.371001
eIF3subunit 10 theta	14D	1.22-1.25	EIF3S10	Hs.523299
eIF3subunit 12	14D	0.76-0.77	EIF3S12	Hs.314359
eIF4EBP-1	14D	0.64	EIF4EBP1	Hs.411641
eIF5	14D	0.84-0.87	EIF5	Hs.433702
Eukaryotic elongation factors				
eEF1 alpha 1	14D	1.22-1.31	EEF1A1	Hs.586423
eEF1 beta 2	14D	0.86	EEF1B2	Hs.421608
eEF1 delta	14D	0.76-0.77	EEF1D	Hs.333388
eEF1 epsilon 1	48H	0.84	EEF1E1	Hs.631818
eEF1 gamma	14D	0.88-0.89	EEF1G	Hs.144835
Ribosomal proteins/kinases				
rps6	14D	0.91	RPS6	Hs.408073
rps6k, 90kDa polypeptide 2	14D	0.80-0.86	RPS6KA2	Hs.655277
mTORc interactors				
rapamycin-insensitive companion of mTOR	48H	1.40	RICTOR	Hs.407926
rapamycin-insensitive companion of mTOR	14D	1.23	RICTOR	Hs.407926
Ras homolog enriched in brain	14D	1.72	RHEB	Hs.647068
<u>Protein Degradation</u>				
Calpain system				
calpain 2, large subunit	14D	1.14	CAPN2	Hs.350899
calpain 3, p94	14D	0.88-0.89	CAPN3	Hs.143261

Class	timepoint	fold change	Gene Symbol	Unigene ID
Lysosomal enzymes				
cathepsin O	14D	1.16	CTSO	Hs.75262
Autophagic genes				
ATG5 autophagy related 5 homolog	48H	0.91	ATG5	Hs.486063
ATG5 autophagy related 5 homolog	14D	0.87	ATG5	Hs.486063
BCL2/adenovirus E1B interacting protein 3-like	14D	1.21-1.32	BNIP3L	Hs.131226
Runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	14D	0.55	RUNX1	Hs.149261
Vacuolar protein sorting yeast homologs				
Vps13	14D	1.20	VPS13B	Hs.191540
Vps24	14D	1.17	VPS24	Hs.591582
Vps29	14D	1.14	VPS29	Hs.632735
Vps36	14D	1.13	VPS36	Hs.109520
Vps45	14D	1.13	VPS45	Hs.443750
Vps72	14D	1.15	VPS72	Hs.2430
Ubiquitin-proteasome system				
Ub activating enzymes (E1s)				
E1C	14D	1.12	UBE1C	Hs.154320
Ub conjugating enzymes (E2s)				
E2 variant 1	48H	0.82	UBE2V1	Hs.420529
E2 variant 1	14D	0.87	UBE2V1	Hs.420529
E2B	14D	0.84-0.86	UBE2B	Hs.644421
E2D 4	14D	0.85-0.88	UBE2D4	Hs.19196
E2E 3	14D	1.32	UBE2E3	Hs.470804
E2G 1	48H	1.3-1.33	UBE2G1	Hs.462035
E2H	14D	1.13	UBE2H	Hs.643548
E2W	14D	1.39	UBE2W	Hs.597486
Ub ligases (E3s)				
E3 C	14D	0.83	UBE3C	Hs.118351
Ring finger proteins				
ring finger protein 5	48H	0.82	RNF5	Hs.534342
ring finger protein 5	14D	0.76	RNF5	Hs.534342
ring finger protein 7	14D	0.89	RNF7	Hs.134623

Class	timepoint	fold change	Gene Symbol	Unigene ID
Ring finger proteins cont'd				
ring finger protein 19	14D	1.26	RNF19	Hs.292882
ring finger protein 20	14D	1.20	RNF20	Hs.656088
ring finger protein 34	14D	0.79	RNF34	Hs.292804
ring finger protein 103	48H	1.22	RNF103	Hs.469199
ring finger protein 103	14D	1.21	RNF103	Hs.469199
ring finger protein 123	14D	0.88	RNF123	Hs.553723
ring finger protein 145	14D	1.17	RNF145	Hs.553723
membrane-associated ring finger (C3HC4) 8	48H	1.22	MARCH8	Hs.499489
membrane-associated ring finger (C3HC4) 7	14D	1.11	MARCH7	Hs.529272
ring finger and FYVE-like domain containing 1	48H	1.43	RFFL	Hs.13680
ring finger and FYVE-like domain containing 1	14D	1.44	RFFL	Hs.13680
Cullin proteins				
cullin 7	14D	1.16	CUL7	Hs.520136
F-box proteins				
F-box protein 3	14D	0.81	FBXO3	Hs.406787
F-box protein 11	14D	1.12	FBXO11	Hs.352677
F-box protein 30	48H	1.20	FBXO30	Hs.421095
F-box protein 30	14D	1.22	FBXO30	Hs.421095
F-box protein 40	14D	0.75-0.8	FBXO40	Hs.272564
Proteasome				
ATPase subunit 6	14D	1.12	PSMC6	Hs.156171
non-ATPase subunit 1	14D	1.14	PSMD1	Hs.3887
Proteasome cont'd				
non-ATPase subunit 2	14D	1.20	PSMD2	Hs.518464
non-ATPase subunit 4	14D	1.22-1.23	PSMD4	Hs.505059
non-ATPase subunit 5	14D	1.25	PSMD5	Hs.193725
non-ATPase subunit 9	14D	0.86	PSMD9	Hs.131151
subunit beta type 10	14D	0.70	PSMB10	Hs.9661
Ub specific peptidases (USPs)				
USP 1	14D	1.26	USP1	Hs.35086
USP 2	14D	0.88	USP2	Hs.524085
USP 5	14D	1.23	USP5	Hs.631661
USP 6	48H	1.33	USP6	Hs.448851

Class	timepoint	fold change	Gene Symbol	Unigene ID
Ub specific peptidases (USPs)				
USP 6	14D	1.30	USP6	Hs.448851
USP 9, x-linked	14D	1.31	USP9X	Hs.77578
USP 10	48H	0.82	USP10	Hs.136778
USP 14	48H	1.09	USP14	Hs.464416
USP 34	14D	1.13	USP34	Hs.654807
USP 38	48H	1.30-1.36	USP38	Hs.480848
USP 38	14D	1.28	USP38	Hs.480848
USP46	14D	1.21	USP46	Hs.331478
USP54	14D	1.19	USP54	Hs.657355

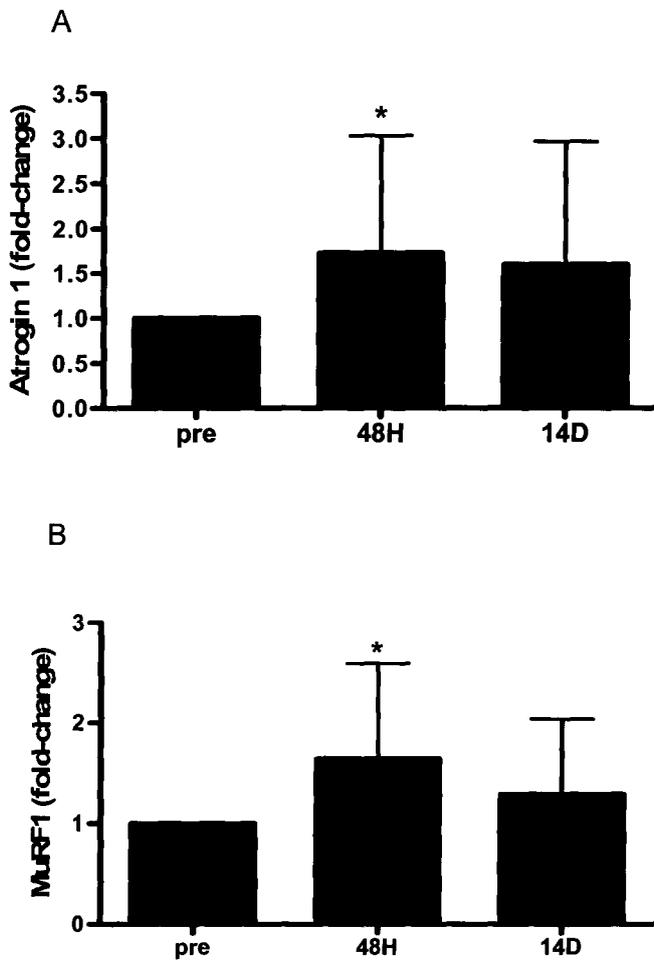


Figure 1. Panel A, atrogin-1 mRNA levels prior to, 48h and 14d after immobilization. Panel B, MuRF1 mRNA levels prior to, 48h and 14d after immobilization.