PROTEIN TURNOVER AND REGULATION IN RAT SKELETAL MUSCLE DURING IN VITRO HYPO-OSMOTIC STRESS

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A thesis submitted for completion of the requirements
for the degree of Master of Science in Applied Health Sciences
(Kinesiology)

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ABSTRACT

PROTEIN TURNOVER AND REGULATION IN RAT SKELETAL MUSCLE DURING IN VITRO HYPO-OSMOTIC STRESS

Hypo-osmolality influences tissue metabolism, but research on protein turnover in skeletal muscle is limited. The purpose of this investigation was to examine the effects of hypo-osmotic stress on protein turnover in rat skeletal muscle. We hypothesized increased protein synthesis and reduced degradation following hypo-osmotic exposure. EDL muscles (n=8/group) were incubated in iso-osmotic (290 Osm/kg) or hypo-osmotic (190 Osm/kg) modified medium 199 (95% O_2, 5% CO_2, pH 7.4, 30±2 °C) for 60 min, followed by 75 min incubations with L-U[^{14}C]phenylalanine or cycloheximide to determine protein synthesis and degradation. Immunoblotting was performed to assess signalling pathways involved. Phenylalanine uptake and incorporation were increased by 199% and 169% respectively in HYPO from ISO ($p < 0.05$). This was supported by elevated phosphorylation of mTOR Ser^{2448} (+12.5%) and increased Thr^{389} phosphorylation on p70s6 kinase (+23.6%) ($p < 0.05$). Hypo-osmotic stress increased protein synthesis and potentially amino acid uptake. Future studies should examine the upstream mechanisms involved.

Key Words: *Cell swelling, hydration, protein turnover, skeletal muscle, metabolism*

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List of Abbreviations

APS – Ammonium Persulfate

AQP – Aquaporin Water Channel

ATP – Adenosine Triphosphate

BSA – Bovine Serum Albumin

Ca^{2+} - Calcium

Cl^- - Chloride

CO_2 – Carbon Dioxide

EDL – Extensor Digitorum Longus

EGTA – Ethylene Glycol Tetra-acetic Acid

GLUT – Glucose Transporter

H^+ - Hydrogen Ion

HYPER – Hyper-osmotic

I – Iodide

IGF-1 – Insulin-like Growth Factor

ISO – Iso-osmotic

K^+ - Potassium

KCC – Potassium Chloride Cotransporter

KCl – Potassium Chloride

K_2HPO_4 – Potassium phosphate

mTOR – Mammalian Target of Rapamycin

NaOH – Sodium Hydroxide

NKCC – Sodium Potassium Chloride Cotransporter

O_2 - Oxygen

PBST – Phosphate Buffered Saline Tween

PCr - Phosphocreatine

PI - Phosphatidylinositol

PI3K – Phosphatidylinositol-3-kinase

PVDF – Polyvinylidene Flouride

RBC – Red Blood Cell

RVD – Regulatory Volume Decrease

RVI – Regulatory Volume Increase

SDS – Sodium Dodecyl Sulfate

SR – Sarcoplasmic Reticulum

TBST – Tris Buffered Saline Tween

TEMED - Tetramethylethylenediamine

Introduction:

Physiological Relevance of Cellular Hydration:

Cellular water plays a vital role in biological life by providing the aqueous environment necessary for cellular reactions to occur. In some instances, it is also used directly as a substrate or product within enzymatic reactions (Hoffmann, Lambert et al. 2009). Skeletal muscle is an important tissue to be considered during water compartment shifts. Diseases and activity levels can drastically alter the basal and stimulated metabolic activity and consequently the flux of water molecules across tissue membranes. Various pathological conditions, diseases and physiological states have effects on the fluid volume within the different water compartments of the human body (Verkman 2008).

During recovery from burns or sepsis the release of chemical messengers of tissue damage is extremely high, stimulating the recruitment of white blood cells and other proteins required to deal with tissue repair. This process known as inflammation, increases plasma osmolality causing skeletal muscle and/or the injured site to lose intracellular water stores (Rajapakse and Wijewickrama 2009). Inflammation is associated to an overall loss of lean muscle mass in the initial immediate recovery following such insults.

Cancer, type 2 diabetes, and obesity also demonstrate elevated plasma osmolality due to differing mechanisms, but the end result is a hyper-osmotic external environment for peripheral tissues including skeletal muscle (Ritz and Berrut 2005). In cancer, not only does the disease itself causes dehydration, but so do many of the common treatments employed to fight tumour growth, which actually accelerate the already problematic rate

of dehydration in these individuals (Maltoni, Nanni et al. 2001; Verkman 2008). This is beneficial in slowing tumour growth, however, it comes at the expense of promoting skeletal muscle atrophy. There are also conditions involving hormonal imbalances within the body that can dramatically alter whole body hydration. One example is a condition known as hypernatremia, where excessive Na^+ storage causes the blood to become hyper-osmotic (Lien, Shapiro et al. 1990). The opposite is true during hyponatremia, an inability to absorb and maintain sodium in the body (Grantham and Linshaw 1984).

A hypo-osmotic induced cell-swelling will also occur under hypoxia/ischemia, hypothermia due to the inhibition of the Na^+K^+ ATP-ase pumps, intracellular acidosis (possibly from exercise) and diabetic ketoacidosis (Hoffmann, Lambert et al. 2009). Any changes in water compartments could eventually alter cellular metabolism by affecting substrate and chemical messenger gradients across the membranes within the affected tissue. This means that any processes relying on intracellular substrates that also serve as osmolytes (glucose, amino acids, free-fatty acids) could be altered. The range of dehydration/hypohydration that can accompany these pathological states can be anywhere from 10-18% (of total body water stores), leading to changes in blood osmolarity of up to 100 - 150mmol/kg in extreme cases (Fortney, Wenger et al. 1984). During chronic disease or recovery from stressors, the body will regulate the synthesis and degradation of these osmolytes (substrates, ions, by-products of metabolism, etc.), causing impending alterations in the differing water compartments and pending tissue metabolism (Burg 1995).

Significant physiological results of hyper-osmotic stress include increased muscle atrophy, and metabolic dysregulation of total lean body mass when compared with

healthy-matched controls (Alfieri and Petronini 2007; Dennis, Zhu et al. 2009). In diabetes and obesity, it is postulated that the hyper-osmotic environment may partially block correct intracellular volume changes from hormonal binding, and this may be a contributing factor to the inability of skeletal muscle to correctly respond to the insulin intracellular signalling cascade (al-Habori, Peak et al. 1992; Bjornholm and Zierath 2005). In contrast, various studies on athletes, athletic performance, and protein accretion and inflammation have all demonstrated the requirement for proper hydration (or the inflammatory response leading to cell swelling) to allow the beneficial physiological adaptations to occur that accompany training (muscular hypertrophy; both myofibrillar and non-myofibrillar) (Lang, Ritter et al. 1993; Dennis, Zhu et al. 2009).

Studies investigating full body hydration and its effects on metabolism have demonstrated strong support for the Haussinger "cellular swelling" theory (Ritz, Salle et al. 2003; Schafer, Gehrmann et al. 2007). This theory proposes that cell size plays a regulatory role in cellular processes, with shrinkage activating catabolic cellular activities and swelling activating anabolic processes (Haussinger, Lang et al. 1994). During a study on malnutrition, a positive correlation was found between rates of protein degradation and the level of dehydration in the individual even after resuming a correct diet and hydration regimen (Baracnieto, Spurr et al. 1978). The recovery to baseline/pre-intervention protein synthesis values did not occur until hydration levels recovered to pre-malnutrition values suggesting the importance of cell water in maintaining lean mass in free-living humans. Another study involving trauma and sepsis patients showed a 15-20% reduction in lean mass water content, with a concomitant decrease of 15% in overall total body protein content (Finn, Plank et al. 1996). In contrast, a study investigating hypo-

osmotic induced cell swelling in humans demonstrated increased protein sparing and an improved maintenance of lean mass with increased lipid oxidation (Berneis, Ninnis et al. 1999). These authors concluded that cellular hydration state played a significant role in mediating protein metabolism, but were criticized for having only measured overall hydration and inferring those changes to reduced cellular hydration status (Ritz, Salle et al. 2003).

Other studies employing pharmacological agents (NSAIDS) to block the inflammatory process post-exercise have demonstrated reductions in the long-term gains and maintenance of lean muscle mass in response to training (Trappe, White et al. 2002; Wellen and Hotamisligil 2005). In contrary, aging individuals often display chronic low grade inflammation, and this could possibly de-sensitize their bodies' ability to adapt to hypertrophy based training programs due to the altered inflammatory response (Barbieri, Ferrucci et al. 2003). This de-sensitization from chronic low-grade inflammation has also been demonstrated in individuals suffering from metabolic disorders; type 2 diabetes, cardiovascular disease and obesity (Wellen and Hotamisligil 2005). These results suggest that the inflammation process is extremely important and necessary for the full adaptive response of muscle hypertrophy to physical training programs.

Although all of these studies used indirect measurements of muscle water content (with the exception of the Finn study), they all lend support to the concept that cellular hydration status plays some type of role in protein metabolism in both healthy and diseased states. Improving our understanding of how hydration affects protein metabolism is an important area of research as many clinical disorders, diseases and their treatments lead to tissue and whole body dehydration. On the other hand, hydration status

is an important factor in allowing athletic performance and full physiological adaptations to training. Therefore, with a better understanding of how hydration can affect skeletal muscle mass maintenance, researchers and clinicians will gain further insight into the development of more effective treatment programs, methods and techniques (Maltoni, Nanni et al. 2001; Glass 2005). This thesis will examine the effect of hypo-osmotic exposure on protein turnover and the associated regulatory mechanisms in mammalian skeletal muscle.

Literature Review:

Importance of Cell Volume Regulation & Maintenance:

Approximately 70% of a cell is composed of water, and almost all intracellular reactions require the aqueous environment it provides for these reactions to occur (Hoffmann, Lambert et al. 2009). In light of this, both cellular and whole organism water balance are precisely regulated processes. This assures the maintenance of a suitable functional environment for enzymatic reactions and cellular processes to occur (Macknight and Leaf 1977).Water molecules move both passively and actively (through indirect mechanisms) across the cellular membrane. These molecules will always follow the concentration gradient set by intracellular/extracellular compounds and osmotically active molecules defining the compartments osmolality or concentration (Hoffmann, Lambert et al. 2009). The basal metabolic activity of a cell will produce by-products from the breakdown of various substrates and as a result, the uptake or release of these substances will lead to corresponding water shifts between the intra and extracellular environment of a cell (Wilkerson, Gutin et al. 1977; Convertino, Keil et al. 1981; Miles,

Sawka et al. 1983; Grantham and Linshaw 1984; Convertino 1987; Maw, Mackenzie et al. 1995). The effect of cellular volume perturbations on cellular metabolism and energy status have been examined primarily in tissues that deal with, or make use of osmotic gradients to control their metabolic activities. Examples of such tissues include individual blood and liver cells, and nephrons within the kidneys (Baquet, Hue et al. 1990; Clegg, Jackson et al. 1990; Baquet, Lavoinne et al. 1991).

Conversely, there is limited research regarding the effect of osmotic stress on skeletal muscle metabolism and more specifically protein metabolism even though many drug and therapy treatments for diseases leave tissues subject to hyper-osmotic stress, commonly displayed as overall dehydration (Low, Rennie et al. 1996; Low, Rennie et al. 1996; Brunmair, Neschen et al. 2000). In humans, skeletal muscle contains 75% of total body water stores, and demonstrates extremely large fluid shifts during various pathophysiological and physiological states such as metabolic disorders, simple postural manipulations, space flight and physical exertion (Sjogaard and Saltin 1982; Miles, Sawka et al. 1983; Convertino 1987; Hamilton, Ward et al. 1993; Maw, Mackenzie et al. 1995; Leach, Alfrey et al. 1996). Chronic fluid shifts, as seen in pathological conditions, have often been associated to reduced ability to regulate and maintain functional lean muscle mass (Finn, Plank et al. 1996). It is therefore imperative to understand how altered cell volume can affect the activity and regulation of skeletal muscle mass not only because it is a primary tissue contributing to overall basal metabolic rate, but because it is also often a primary effected tissue in metabolic disorders (type II diabetes, obesity).

Cell Volume Detection Mechanisms:

Due to the role of cell water in providing a suitable functional environment for proteins and macromolecules, the cell has mechanisms in place to recognize perturbations in cell volume and initiate corrective responses to restore normal cell hydration (Haussinger, Lang et al. 1994). During situations involving aniso-osmotic stress, the cell is subjected to an external environment that is either of higher (hyper-osmotic) or lower (hypo-osmotic) osmolality than the intracellular environment. The term osmolality refers to the overall concentration of ions, solutes, organic osmolytes, and substrates within an aqueous environment and their combined concentrations will determine the osmolality of that compartment (Lord 1999). The main theories regarding how a cell responds to a volume change are categorized in two major domains: mechanically-induced and osmotically-induced detection and signalling (Lang, Ritter et al. 2000). The former would involve cytoskeletal and membrane elements responsive to stretch from cellular swelling. The latter would involve changes in key intracellular osmolyte concentrations that the cell may be controlling or monitoring in order to regulate intracellular water balance (Parker 1993).

Certain cell lines have demonstrated cell volume regulatory responses at as little as 4% increase in overall cell volume, suggesting that osmotic signalling is likely responsible for such early responses. This is because biological membranes have a large degree of compliance, and such minimal changes in volume would be relatively insignificant due to such compliance (Hoffman, Eden et al. 1958; Colclasure and Parker 1991; Watson 1991; Sadoshima and Izumo 1997; Tzima 2006). It has been demonstrated that changes in volume, whether small or large, are accompanied by cell membrane

folding/unfolding further demonstrating this structures large degree of compliance, likely making precise detection of small volume changes difficult through changes in membrane stretch (Hoffmann, Lambert et al. 2009). Accordingly, the remainder of this section will focus on osmolyte detection mechanisms in response to changes in cell volume.

Perturbations in cell volume affect the physical relationship of macromolecules with each other, the membrane, and the various substrates and ions required for their function (Zimmerman and Minton 1993; Minton 2001). During hyper-osmotic stress the cell will shrink causing macromolecular crowding which can eventually lead to the initiation of apoptotic signalling pathways (Lang, Ritter et al. 2000; Bevilacqua, Wang et al. 2010). This could possibly be due to the inability of the internal structures of the cell to correctly perform basic cellular processes required for survival. It is hypothesized that the cell recognizes this crowding by the interaction of protein residues on the peripheral aspects of macromolecules. It is postulated that this recognition occurs either directly through changes in protein folding or through the release of chemical messengers denoting macromolecular damage (Tokuriki, Kinjo et al. 2004; Banks and Fradin 2005). In contrary, studies investigating the effects of reduced pressure between membrane phospholipids from swelling or stretch demonstrated changes in the fatty acid chains position and direction (Perino, Ghigo et al. 2011). Changes in the fatty acid tails positioning can alter protein function because many enzymes are activated through their interaction with the various phospholipid species composing the membrane (Wymann and Marone 2005). These results suggest an optimal range of physical location between proteins, macromolecules and the various membranes and compartments within the cell.

During aniso-osmotic stress, changes also occur in the cells phosphagen levels leading to changes in the activation of regulatory kinases and phosphatases responsible for guiding cell metabolism and gene expression to correct for the volume change (Antolic, Harrison et al. 2007). The changes in energy status further support the cell swelling theory; during cell swelling ATP and PCr levels are slightly higher than during resting conditions, whereas they are significantly depleted during cell shrinkage (Gual, Le Marchand-Brustel et al. 2003; Antolic, Harrison et al. 2007). This suggests cell shrinkage detection initiates stress response and survival pathways leading to above normal (iso-osmotic) utilization of high energy phosphates within the cell. This contrasts the situation in cellular swelling where ample availability of these energy substrates allows for anabolic processes such as growth and proliferation to be initiated or continued (Carling 2004; Gual, Gonzalez et al. 2007).

One major cell energy sensor kinase, AMPK, has also been implicated in the cellular response to osmotic stress, demonstrating increased phosphorylation/activation during hyper-osmotic stress as a result of the decreased cellular energy status. (Gual, Le Marchand-Brustel et al. 2003; Smith, Patil et al. 2005). Increases in the AMP/ATP ratio activate AMPK by AMP allosterically inducing phosphorylation of a threonine residue by the upstream kinase, the tumour suppressor LKB1 (Carling 2004; Hardie 2004; Kahn, Alquier et al. 2005). Activation of AMPK by CAMKK (Calcium/calmodulin-dependent kinase kinase) is also triggered by a rise in intracellular calcium ions, without any detectable changes in the AMP/ATP ratio suggesting multiple and complex regulatory inputs on AMPK (Hawley, Pan et al. 2005; Fogarty, Hawley et al. 2010). Increased intracellular calcium concentrations have been demonstrated in response to both hyper

(myofibers and myocytes) and hypo-osmotic stress (human epithelial cell cultures) (Boudreault and Grygorczyk 2004; Martins, Shkryl et al. 2008). When activated through reversible phosphorylation, AMPK favours increased muscle fatty acid oxidation and glucose uptake, while decreasing mobilization of adipose tissue stores and inhibiting protein synthesis and other anabolic processes (Barnes, Ingram et al. 2002; Tokunaga, Yoshino et al. 2004). In general, AMPK activation triggers catabolic pathways to produce ATP, while turning off anabolic pathways that consume ATP in an attempt to maintain cellular energy stores (Carling 2004). Because of these characteristics, AMPK can be viewed as promoting catabolic effects on cell metabolism including inhibition of anabolic processes such as protein synthesis through its inhibition of the mammalian target of rapamycin (mTOR) (Hardie 2003).

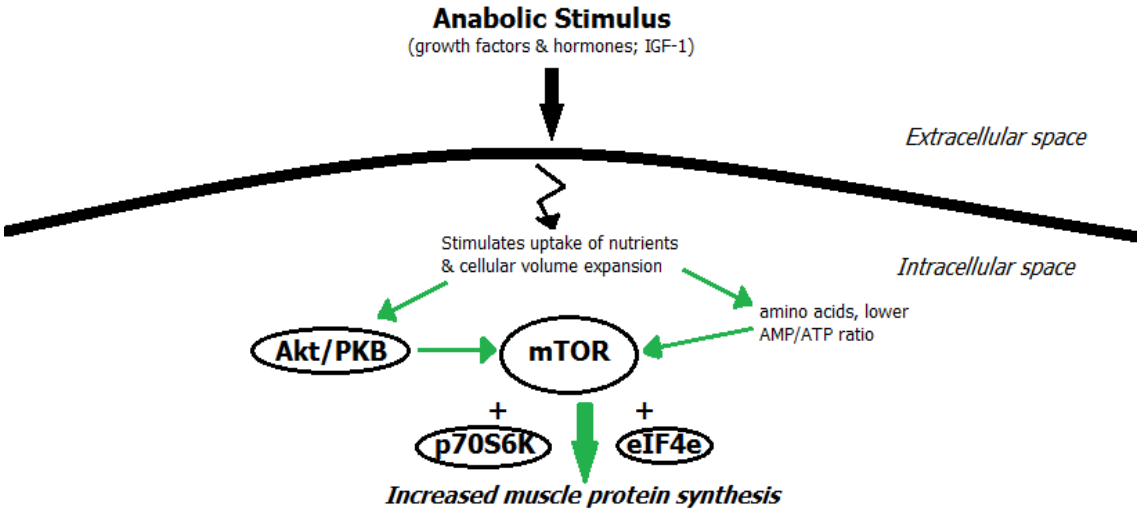
mTOR is another regulatory complex involved in cell proliferation and growth. It accomplishes this process by integrating information on substrate availability, in this case amino acids, to energy and redox status of the cell (Tokunaga, Yoshino et al. 2004). mTOR is involved in regulating the anabolic process of protein synthesis, and has shown responsiveness to cell stressors such as increased oxidative damage and increased intracellular calcium levels that have also been demonstrated to occur as a result of aniso-osmotic stress (Martindale and Holbrook 2002; Boudreault and Grygorczyk 2004; Apostol, Ursu et al. 2009). The response of mTOR to hypo-osmotic stress has yet to be investigated within skeletal muscle models.

Protein kinase B (Akt), a serine/threonine protein kinase which plays a role in cell proliferation and growth, has also been shown to be dephosphorylated and inactivated during hyper-osmotic stress. The opposite occurs during hypo-osmotic stress (Meier,

Thelen et al. 1998). Increased Akt phosphorlyation should in theory increase glucose uptake, but our lab has demonstrated an increase in glucose uptake during hyper-osmotic stress suggesting other players such as p38 kinase, AMPK or the MAP kinase family are likely involved in this phenomenon (Gual, Le Marchand-Brustel et al. 2003; Gual, Gonzalez et al. 2007)(Farlinger, unpublished data). The literature on cell volume suggests that the residual cell swelling induced by hypo-osmotic stress may serve as a proliferative growth signal. With the opposite being true for cell shrinkage leading to activation of apoptotic signalling mechanisms, this possibly explains the increased glucose uptake and utilization (Schreiber 2005). AMPK and Akt both have downstream effects on p70s6 kinase (through their phosphorylation activities on mTOR), a key enzyme involved in protein translation and elongation at the ribosomal complex (Davis 1993; Wang, Li et al. 2001).

Eukaryotic translation initiation factor 2 alpha (eIF2a) is a major transcriptional factor involved in allowing protein elongation when it is phosphorylated (in its active form). eIF2a assists in the delivery of the initiation tRNAs to cellular mRNAs to allow the use of an AUG initiation codon, which allows protein translation initiation to proceed (Hamanaka, Bennett et al. 2005). This protein has demonstrated increased activation during hyper-osmotic stress and is required for the initiation of apoptotic cell processes (Bevilacqua, Wang et al. 2010). Although eIF2a facilitates protein initiation of individual tRNAs, it is not the rate-limiting step in protein synthesis. Eukaryotic initiation factor 4E (eIF4E) is a three sub-unit transcription factor that is necessary for the binding of an mRNA to the ribosome to allow for protein translation to begin (Hara, Yonezawa et al. 1997). The activity of this protein is regulated by its interaction with another

transcriptional factor, eukaryotic initiation factor 4G (eIF4G) (Shahbazian, Parsyan et al. 2010). Following activation, eIF4E directly binds to the mTORc1 complex and when complexed allows for protein initiation and subsequent translation to proceed (Hara, Yonezawa et al. 1997). The activated mTORC1 protein complex initiates a series of reactions to phosphorylate these associated proteins once they have adjoined the complex. The mTORc1 complex will then regulate which mRNAs will be selected to proceed to the ribosomal complex and finally translated into functional proteins (Holz, Ballif et al. 2005; Foster and Fingar 2010). The diagram below gives a theoretical model for the response of these regulatory proteins and downstream effectors at rest in response to growth factors and nutrient availability.



Adapted from original image at (http://www.efdeportes.com/efd131/leucine-stimulates-mtor-and-muscle-protein-synthesis.htm)

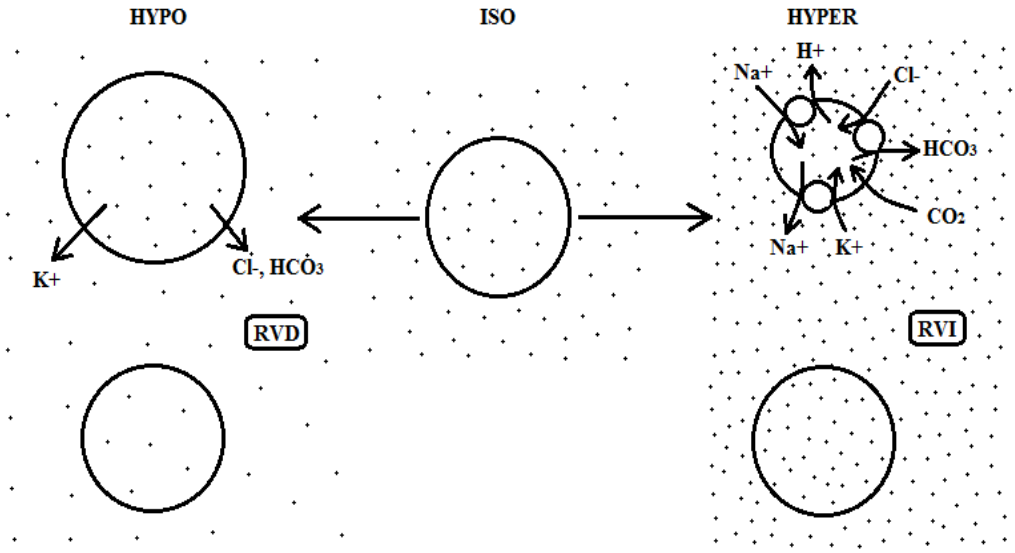
Figure 1. Signalling pathways involved in the regulation of skeletal muscle protein synthesis through the Akt/mTOR/p70s6 kinase pathway in response to anabolic extracellular influences (eIF4e = eukaryotic initiation factor 4e, mTOR = mammalian target of rapamycin).

The activation of these protein kinases has been shown to play a role in mediating the cellular response to aniso-osmotic stress. Uncovering the molecular regulation of mTOR and p70s6 kinase during aniso-osmotic stress will provide a better understanding of protein synthesis during physiological water balance perturbations. This information is pertinent in the development of successful treatment and management programs for conditions associated to muscle atrophy.

Response Following Detection of Changes in Cell Volume:

Because the cell is separated from the external environment by a phospholipid bilayer membrane, it must undertake mechanisms requiring ATP to move ions and non-essential substrates into or out of the cell to initiate secondary movements of water across the membrane (Haussinger, Lang et al. 1994). Water molecules follow the changes in ionic and substrate concentrations in an attempt to create iso-osmolality (equal internal and external osmolality) between the intracellular and extracellular solutions (Moyes & Schulte 2008). Although it is well known that the cell initiates such specific responses in order to cause these changes in cell water contents, the molecular mechanisms connecting the detection to response is still poorly understood (McManus, Churchwell et al. 1995; Schreiber 2005).

During hypo-osmotic stress the cell swells, and the impending activities that restore cell volume back to normal are referred to as the regulatory volume decrease (RVD). The primary mechanisms involve release of K^+, Cl^- and $HCO3^-$, and it is believed that phosphorylation of AMPK and Akt play important roles in mediating the RVD response in liver tissue (Boyer, Graf et al. 1992; Tilly, Vandenberghe et al. 1993). During more extreme swelling, the mechanical stretch of the membrane is proposed to activate non-selective cation channels allowing Ca^{2+} to enter the cell and activate channels responsible for the release of K^+ and Cl^- (Bear and Petersen 1987; Sackin 1989). The diagram below illustrates the processes of RVI and RVD for a general cell type.



Adapted from original (Haussinger, Lang et al. 1994)

Figure 2. Regulatory volume increase and decrease in mammalian cells in response to water stress.

The short-term use of such ion transport systems is beneficial for acute volume regulation, but over time the changes in ionic gradients across the membrane can effectively reverse exchangers like the Na^{+}/Ca^{2+} exchanger. This can lead to increased cytosolic calcium levels leading eventually to the initiation of apoptotic signalling pathways (Trump and Berezesky 1995; Schreiber 2005).

The organic osmolytes offer a better regulatory pathway during chronic changes as they do not affect the intra/extracellular charge, meaning they maintain membrane potential viability and avoid negative secondary effects on pH that can alter the structure and function of proteins (Haussinger, Lang et al. 1994; Sheikh-Hamad, Garcia-Perez et al. 1994; McManus, Churchwell et al. 1995). In skeletal muscle, the primary non-active osmolytes that exist and can be synthesized/degraded from macromolecules within

muscle are amino acids. Long-term adjustments to the free amino acid pool could be used to deal with chronic osmotic stress, and this could in turn influence protein turnover as the substrate availability (amino acid pool size) for synthesis would be altered (Low, Rennie et al. 1997). The following section will discuss the pathways that are affected during cell swelling and shrinkage while focusing mainly on the effects on cellular metabolism during swelling.

The Effect of Cell Volume on Cell Substrate Metabolism:

In a study performed on isolated hepatocytes, it was demonstrated that hypo-osmotic swelling was associated with increased amino acid uptake (Baquet, Lavoinne et al. 1991). Interestingly, the investigators discovered in a following study that the change in cell volume was completely responsible for this effect on amino acid uptake and subsequent protein synthesis. When the volume changes were blocked by the introduction of NaCl or raffinose (plant-based sugar) into the incubation medium, the apparent effect on protein synthesis was abolished (Stoll, Gerok et al. 1992). In line with this evidence, it was also demonstrated that when volume changes associated with the action of insulin were blocked, the up-regulation of protein synthesis and increase in cell volume normally observed from insulin administration was also abolished (Hallbrucker, vom Dahl et al. 1991). Conversely, during hyper-osmotic cell shrinkage, mimicking the effects of glucagon on protein degradation, it was shown that this volume change in the cell decreased protein synthesis and increased proteolysis (Hallbrucker, vom Dahl et al. 1991). Care should be taken in extrapolating and interpreting such data as all of these studies used concentrations of amino acids and hormones at least twice that of

physiologically relevant levels, but they still provide considerable evidence suggesting that changes in cell volume may be a mechanism exerting control on cellular protein turnover.

Cellular swelling effects on glucose metabolism have been previously observed in our lab. Hypo-osmotic incubation demonstrated slightly but not significantly elevated values of high energy phosphates (ATP & PCr) compared to the iso-osmotic condition (Antolic, Harrison et al. 2007). Alternatively, hyper-osmotic stress resulted in increased glucose uptake (Farlinger et al, 2007) and lower high energy-phosphate levels than both iso and hypo conditions (Antolic, Harrison et al. 2007). Other studies have also demonstrated that liver cell swelling promotes glycogen synthesis, and decreases glycogenolysis both measured directly and through the activity of the enzymes responsible for regulating these pathways (G-6-phosphatase, glycogen synthase) (Haussinger, Lang et al. 1990; al-Habori, Peak et al. 1992; Peak, al-Habori et al. 1992). Work performed on the signalling pathways involved in glycogen metabolism has demonstrated that glycogen synthase kinase activity is related to a reduction of inhibition on the ubiquitin-proteasome degradation pathway (Glass 2005). This evidence demonstrates the apparent relationship between glycogen and protein metabolism signalling pathways during aniso-osmotic exposure.

Cellular swelling in hepatocytes causes increased rates of lipogenesis (Baquet, Lavoinne et al. 1991) and increased Akt activity (Schliess, Schreiber et al. 1995). This evidence suggests favouring fat utilization for metabolic processes, and initiation of prolific activities through Akt, again suggesting an anabolic environment during cell swelling. Experimental results regarding cell hydration and volume suggest that various

anabolic activities are initiated during cell-swelling, and the opposite during cell-shrinkage (Haussinger, Lang et al. 1994). Although much evidence supports this principle and theory, no experiments have defined the effect of cell swelling on skeletal muscle tissue protein turnover and regulation. A study involving hypo and hyper-osmotic infusion in humans displayed increased protein synthesis and protein sparing at the whole body level during hypo-osmotic infusion (Berneis, Ninnis et al. 1999). Whether this was a result of intracellular signalling through the mTOR/p70s6 kinase pathway or a result of changes in the amino acid pool remains to be determined.

Regulation of Protein Synthesis:

Protein synthesis is required for the growth and progression of a living tissue through from juvenile to adult form, for regeneration following various types of stress or injury (Bolster, Jefferson et al. 2004). At the whole body level, protein synthesis is regulated by a number of different factors, from nutrient availability, to mechanical stress, and hormonal changes (Biolo, Tipton et al. 1997; Ritz, Salle et al. 2003; Burd, Tang et al. 2009). The net result of either muscle hypertrophy or atrophy will be defined by the relative difference in activity between the rates of protein synthesis and protein degradation that are both continually active to some extent (Biolo, Tipton et al. 1997).

Substrate availability (amino acids) is the primary point of regulation in the whole body, as feeding will lead to the sufficient and continual availability of the amino acid building blocks in the bloodstream of the animal to produce proteins (Millward, Nnanyelu.Do et al. 1974). Other nutrient related regulatory processes involve hormones like insulin, and more specifically insulin growth factor-1 (IGF-1). IGF-1 has been shown

to strongly activate the Akt/mTOR pathway, which is involved in regulating protein synthesis and muscle growth (Bodine, Stitt et al. 2001; Musaro, McCullagh et al. 2001). These factors in combination can lead to a larger increase in protein synthesis following both endurance and resistance exercise. Various studies have demonstrated that post-exercise meal consumption stimulates an increase in the exercise-induced potentiation of protein synthesis and concomitant suppression of protein degradation (Burd, Tang et al. 2009).

It has now been well established that IGF-1 and insulin play important signalling roles in mediating the response of skeletal muscle to mechanical stress and also both contribute to basal regulation of protein synthesis (Burd, Tang et al. 2009; Dillon, Sheffield-Moore et al. 2009). Not only does IGF-1 promote the activity of various protein synthesis mechanisms, but it has also has been shown to attenuate protein degradation via inhibition of the ubiquitin-proteasome pathway (Bodine, Stitt et al. 2001; Stitt, Drujan et al. 2004). IGF-1 accomplishes this by activating Akt which directly inhibits FOXO3a resulting in a reduced expression of atrogin-1/MuRF1 transcript expression (Rommel, Bodine et al. 2001). This highlights the regulatory interplay between protein synthesis and degradation, and how the coordination between these opposing systems will define whether muscle mass is gained or lost.

Akt has also been directly implicated in cell growth as mice containing a constitutively active form of the protein demonstrated two-fold increases in muscle fibre cross-sectional area compared to controls (Lai, Gonzalez et al. 2004). In addition, during physiological hyper-osmotic stress such as burn, sepsis, or cachexia in humans, there is a decreased activation of the PI3K/Akt pathway and this is thought to play a role in the

increased rates of muscle atrophy seen during these pathological conditions (Sugita, Kaneki et al. 2005). In vitro studies with rat neonatal cardiomyocytes suggest the PI3K/Akt signalling cascade as a survival pathway allowing for osmotic preconditioning following initial exposures (Pastukh, Ricci et al. 2005). Therefore, it would seem an attractive hypothesis that the PI3K/Akt pathway is involved in regulating growth and responding to cellular energy status, but also demonstrates possible integrated regulation from changes in the cellular hydration status.

Following Akt activation, the downstream effectors mTOR and p70s6K are activated leading to increased rates of protein synthesis. IGF-1, PI3K, Akt, mTOR and p70s6K have been directly implicated in regulating cell size as studies involving site-directed mutagenesis decreasing the expression of these genes all led to decreases in overall cell size in cell culture models (Leevers, Weinkove et al. 1996; Bohni, Riesgo-Escovar et al. 1999; Montagne, Stewart et al. 1999; Zhang, Stallock et al. 2000).

Although IGF-1 can activate mTOR through the PI3K/Akt pathway, amino acids have also been shown to directly activate mTOR, suggesting a central function of the protein in integrating a variety of growth signals from nutrient status to humoral growth factors (Burnett, Barrow et al. 1998; Tokunaga, Yoshino et al. 2004). Phosphorylation of mTOR on the Ser^{2448} residue causes mTOR to actively bind to 5' capped mRNAs. Based on this relationship, mTOR is suggested to act as a selective regulator, defining specificity and priority for the mRNAs within the cytosolic mRNA pool (Foster and Fingar 2010). mRNAs that have been capped will attract mTOR's downstream targets, the translational machinery and therefore would have translational priority. mTOR also phosphorylates and activates p70s6 kinase also involved in protein synthesis (Thr389),

and will phosphorylate and de-activate PHAS-1, a transcriptional factor involved in protein degradation (Glass 2003; Proud 2004).

Following activation of mTOR, downstream activation of p70s6K leads to the phosphorylation of ribosomal S6 kinase allowing protein translation and elongation to proceed by combining with mRNAs labelled by mTOR within the cytosol (Holz, Ballif et al. 2005). As previously mentioned above, the eukaryotic initiation factors (eIF4/2) are also up-regulated as a result of mTOR phosphorylation, and are involved in regulating protein initiation and elongation processes. The diagram on the following page depicts the major proteins, kinases, and transcription factors involved in protein synthesis and degradation.

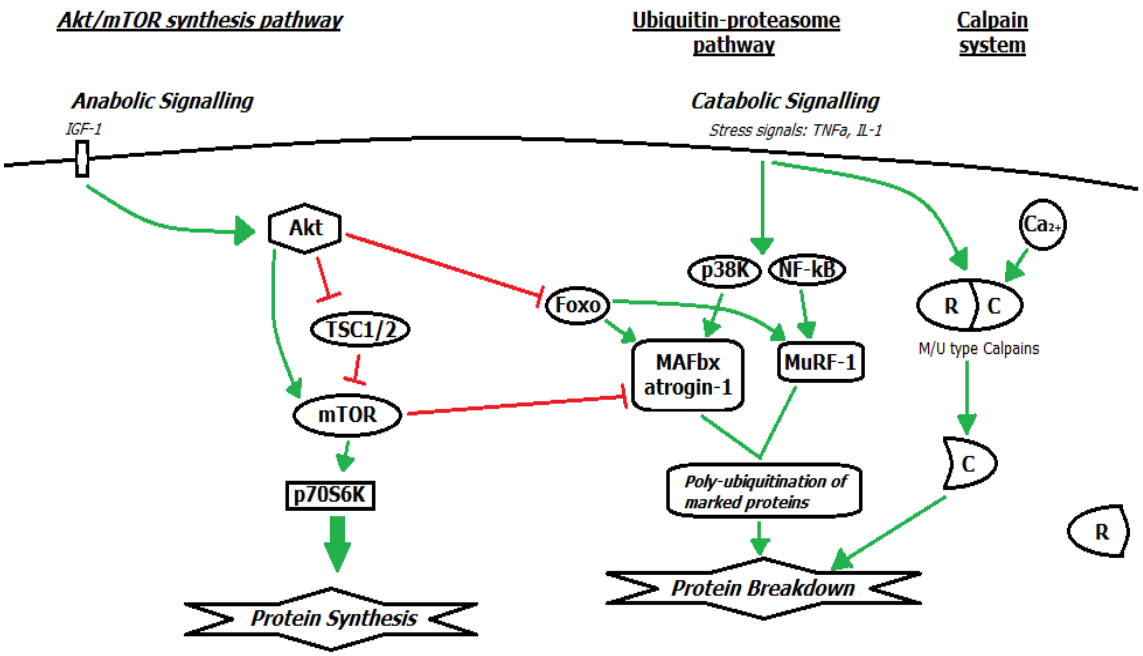


Figure 3. Major hypertrophy (protein kinase B/Akt, TSC ½ = tuberous sclerosis ½, mTOR = mammalian target of rapamycin, p70s6 kinase), and atrophy (ubiquitin-protein conjugates, forkhead Box 3 [FOXO], E-3 ligases of the ubiquitin-proteasome pathway [MuRF1 & atrogin-1] & μ-calpain autolyzed [C subunit] and non-autolyzed [R/C complex]) signalling pathways regulating protein turnover in skeletal muscle. *Adapted from original in (Glass 2005).*

Regulation of Protein Degradation:

Protein degradation involves three separate pathways that each contributes to the overall rate of degradation at varying levels during different physiological situations. These are the ubiquitin-proteosome ATP-dependant pathway, the lysosomal proteolytic pathway, and the calpain pathway and all contribute to the total level of degradation in biological tissues (Attaix, Aurousseau et al. 1998; Bechet, Tassa et al. 2005; Dupont-Versteegden 2006).

The ubiquitin-proteasome pathway is the largest contributor to protein breakdown in skeletal and cardiac muscle tissue. The pathway itself is a 26 subunit complex known as the 26S proteasome (Pickart and Eddins 2004). Protein degradation via this pathway occurs by labelling a protein for degradation by covalent linkage of ubiquitin molecules. The active components of the pathway are three enzyme families; E1, E2 and E3 ligases, these three enzyme families complete the initiation and activation processes for labelling proteins with ubiquitin for degradation (Pickart 2004). The activity of the E1 subunit is ATP-dependant and this enzyme is responsible for forming the first thiol ester bond between the E1 enzyme itself and ubiquitin, prepping the ubiquitin molecule for full binding to the breakdown machinery (Han, Zhong et al. 2008).

Following this process, multiple E2 enzymes in the complex can create another thiol ester bond between E1 and a specific E2 cysteine residue. The final step is a third bond created between E2 and a cysteine residue on E3, completing the linkage between the labelled protein and the ubiquitin molecule through the ligases (Hershko and Ciechanover 1992). This process must be repeated until at least four ubiquitin molecules are bound to the protein or macromolecule, a process known as poly-ubiquitination (Pickart and Eddins 2004). The protein polypeptide is then degraded into smaller peptide molecules, and the subsequent ubiquitin molecules are cleaved off for re-utilization in the breakdown of other cellular proteins and macromolecules.

Two specific E3 ligases that have been shown to be largely active in mammalian skeletal muscle atrophy are MuRF1 and atrogin-1 (Skurk, Izumiya et al. 2005). Both of these ligases have demonstrated to be up-regulated during skeletal muscle models of disuse and denervation atrophy (Stevenson, Giresi et al. 2003). Investigation of protein

breakdown in skeletal muscle will commonly examine the expression of MuRF1 and atrogin-1 mRNA levels as markers for the activity of the ubiquitin-proteasome pathway (Cao, Kim et al. 2005; Chinkes 2005).

The lysosomal pathway is responsible for minimal protein degradation in almost all tissues, but its exact role and contribution to overall degradation in skeletal muscle remains unclear. This pathway involves lysosomes, which are membrane bound vesicles that exist in the cytosol of cells and contain a plethora of hydrolases that cause breakdown of the endocytosed molecules (Tsujinaka, Ebisui et al. 1995). Hydrolases contribute largely to creating the acidic environment that allows for macromolecular denaturation inside the lysosomal vesicles (Kirschke and Wiederanders 1987). Proteases are the active component involved in protein degradation and the main proteases involved in skeletal muscle are the cathepsins; B, D, H and L which target proteins that have accumulated damage, have been tagged for degradation or have been modified in a way that negatively effects their function (Costelli, Carbo et al. 2003). Cathepsins must be synthesized by the rough endoplasmic reticulum as pre pro-enzymes rendering them inactive until they reach the lysosome (Hasilik 1992). Once synthesized, cathepsins undergo translocation by binding to the mannose residue of the Golgi apparatus following its phosphorylation (Rosorius, Issinger et al. 1996). The acidic environment created by the lysosome weakens the bond between the pro-cathepsin and Golgi apparatus and dissociation occurs allowing the cathepsin to be taken up by the lysosome through phagocytosis, pinocytosis or endocytosis. Upon uptake into the lysosome, the cathepsins become activated by the decreased pH weakening the bond between the pro-peptide and the active cathepsin isoform (Turk, Janjic et al. 2001).

Following activation, cathepsins freely degrade protein substrates that are delivered to the lysosome. Two types of proteases exist within the lysosome; endopeptidases and exopeptidases. As the name denotes, endopeptidases hydrolyze inner peptide bonds specifically aspartic and cysteine residues, while exopeptidases hydrolyze terminal ends of proteins (Turk, Janjic et al. 2001). Differing cathepsins have demonstrated increased activation in models of muscle atrophy, and they also demonstrate preferential degradation of specific proteins and protein families, most commonly structural and cytoskeletal elements (Schwartz and Bird 1977; Matsukura, Okitani et al. 1981; Towatari and Katunuma 1983). Current opinion is divided on whether cathepsins activity contributes significantly to overall levels of muscle protein degradation, and further research is required to clearly define the role that this system may play in overall rates of protein breakdown in skeletal muscle. This system is required for maintaining muscle mass in times of energy deprivation by regulating amino acid availability from intracellular pools, and may possibly be regulated by the mTOR pathway (Schliess, Richter et al. 2006).

The third pathway involved in protein breakdown in skeletal muscle is the calpain pathways. Calpains are cysteine proteases that undergo activation by the increased presence or local concentration of Ca^{2+} (Guroff 1964). Skeletal muscle contains three different calpain isoforms; ubiquitous μ-calpain, m-calpain, and calpain-3 (Bartoli and Richard 2005). Calpains have been shown to be involved in many cellular processes, from cell migration to cellular apoptosis. Two subunits complex to form the calpain enzyme, a larger catalytic subunit, and a smaller subunit responsible for regulatory processes (Smith, Lecker et al. 2008). As intracellular calcium concentrations rise in the

cytosol, calcium binds to a site on the regulatory unit of the calpain enzyme causing autolysis (self-digestion of a portion of each subunit of the calpain weakening the association between the regulatory and catalytic subunits) (Cong, Goll et al. 1989). This process is a primer to activation, significantly lowering the required concentration of calcium needed to further activate calpain degradation activity. The activity of this pathway lies on a continuum, and factors other than calcium such as intracellular location and proximity to phospholipids and channels (primarily Ca^{2+}) can play a role in the regulation of these enzymes (Moldoveanu, Jia et al. 2004).

Following partial activation by calcium, calpains translocate to the plasma membrane where phosphotidylserine (PS) and phosphotidylinositol (PI) dissociate the two subunits that compose the calpain complex (Yoshizawa, Sorimachi et al. 1995). This process results in the release of the calpain subunits from the plasma membrane allowing activation of the calpain catalytic domain to hydrolyze protein substrates marked for degradation (ubiquitin-labelled, glycosylated proteins, irreversibly oxidized proteins). Calpains have been shown to be primarily involved in the degradation of myofibrillar proteins in vitro, and μ and m-calpains both require differing activational concentrations of calcium providing another level of regulation to this pathway (Toyo-Oka, Shimizu et al. 1978; Huang and Forsberg 1998; Goll, Thompson et al. 2003). Calpain activity usually leads to modification or adjustments of a protein target, and rarely will calpain degradation result in the full digestion of a protein to its constituent amino acids. A third calpain specific to skeletal muscle, calpain-3, does not require Ca^{2+} for activation and is regulated mainly through the redox and energy status of the cell (Bartoli and Richard 2005).

Various lines of research have demonstrated an inhibition of protein degradation pathways during hypo-osmotic stress and other environments that elicit cellular swelling such as; anabolic hormones and creatine supplementation (Haussinger, Roth et al. 1993; Tipton and Ferrando 2008; Hoffmann, Lambert et al. 2009). But, the majority of current and past research has focused on cardiac and liver tissue, and has also made extensive use of cell culture models (Haussinger, Roth et al. 1993; Burg 1995). The true inhibition and regulation of this phenomenon during hypo-osmotic stress in intact skeletal muscle and the contribution of key signalling pathways involved has yet to be fully elucidated. Gaining further understanding of the regulation of protein degradation pathways will provide the necessary information to produce pharmacological agents or treatments to fight or offset the atrophy associated to aging, cancer, and various other pathological conditions.

Methods for Measuring Protein Turnover:

There are a variety of methods available to investigate protein synthesis and degradation in vivo with varying levels of accuracy. In vitro methods are usually more controlled offering less room for measurement error in estimations and calculations due to the greater level of control afforded to the investigator (Sugden and Fuller 1991). The following paragraphs will use FSR to define protein synthetic rate or protein incorporation over time (fractional synthetic rate), and FBR (fractional breakdown rate), or the amount of amino acids incorporated/released into the media & from a tissue relative to that tissues mass over a defined time interval (Toffolo, Foster et al. 1993; Navegantes, Resano et al. 2004).

FSR refers to the rate of total protein synthesis in the cell, but combined with the correct separation methodologies it is also possible to determine the rate of synthesis for individual proteins, cellular fractions and even specific organelles and their contents (Sugden and Fuller 1991). However, there are benefits and downfalls to the current methods available for investigating the phenomenon of overall muscle protein turnover in vivo. In vivo refers to the animal or organism in its integrated physiological state with multiple organ systems working together in synergy. Because of this in vitro models are the most representative of real-life situations and environments (Basu, Samanta et al. 2008).

To quantify protein metabolism in a human or intact laboratory animal, labelled amino acids are administered through either an oral dose (flooding-dose technique) or constant intravenous infusion into the organism. The amino acids are traceable because they contain stable or radioactive isotopic atom(s), commonly carbon, phosphorus, or hydrogen. This means the stable isotopic atoms will not be reduced or altered while in the body making them useful as stable markers for calculating protein turnover (Rennie 1999). In both methods, the baseline concentrations and enrichments of all amino acids must be quantified by pre-infusion blood draws and subsequent protein content analysis (Lobley, Milne et al. 1980). Estimations of total incorporation or degradation can then be calculated from the delta change from pre to post infusion concentrations of the stable isotope.

These approaches require that an isotopic enrichment steady state be achieved within the endogenous pools and this can take considerable time, even as long as 4-6 hours after the initial dose (Martin, Rabinowitz et al. 1977). A priming bolus is often

given to decrease the time required for equilibration, but with long protocol durations another issue is the possible re-utilization and futile cycling of labelled amino acids leading to underestimations of the actual FSR and FBR values (Melville, Mcnurlan et al. 1989).

The constant infusion method will give lower values in humans and rats when compared to the flooding-dose method, and this is likely a result of attaining equilibrium in the endogenous pools faster in the flooding dose protocol compared to the infusion protocol (Garlick, McNurlan et al. 1980; Garlick, Wernerman et al. 1989). The rate obtained by either method will also vary largely depending on the radio-active/stable isotopic amino acid used for measuring FSR and FBR (Obled, Barre et al. 1989). Higher rates (41%) were found when using phenylalanine and tyrosine when compared to lysine (26%) and threonine (31%) for FSR measures. These results stem from the now available knowledge that oxidation and/or transformation of leucine and threonine will occur while in the body and therefore these amino acids do not offer the same stability as a non-oxidized amino acid such as phenylalanine (Obled, Barre et al. 1989). The authors also suggested that this may be evidence of preferential uptake and higher rates of turn-over for certain amino acids, or that the tissues involved in contributing to the measured turn-over may be higher in one protein than another (Williams, Sugden et al. 1981; Sugden and Fuller 1991). More likely is the fact that phenylalanine and tyrosine are not significantly metabolized in many tissues in the body other than the liver and kidneys, thus explaining their higher reliability as internal markers (Kussmann, Affolter et al. 2007).

Tritium (^{3}H) was a popular label for studying protein synthesis early on following its use for measuring total energy expenditure in free-living human subjects. But, during the early 90's this labelling method was demonstrated to be less reliable in protein turnover measures due to the inconsistent loss of the label during other reactions involving water within the intracellular environment (Williams, Sugden et al. 1981; Toffolo, Foster et al. 1993). This is because the tritium label is associated to a side chain on the amino acids compared to being integrated into the carbon backbone of the amino acid as other atoms such as carbon allow to reduce the loss of label in side reactions. The tritium label is still commonly used in human studies because it contains radioactive hydrogen atoms with low beta wave activity, and therefore presents less risk for the health of study participants compared to other radioactive isotopes (Penner, Klunk et al. 2009).

More recently, the use of ^{13}C stable isotopes has increased in measures of whole body protein synthesis and myofibrillar protein synthesis (West, Kujbida et al. 2009). This usually will involve analyzing human muscle biopsies following initial label bolus administration and can also be used for subcellular fractionation and subsequent analysis with chromatographic techniques to determine incorporated and non-incorporated amino acid components (Phillips, Parise et al. 2002; Tang, Moore et al. 2009). Both of these above mentioned methods alleviate the issues observed with tritium or other non-radioactive tracers and eliminate any risk for the health of the human subject participants from exposure to the label.

Researchers employing in vitro models will commonly make use of ^{14}C (Carbon 14) as a label because it is a component of the carbon backbone of the amino acid and can be read

easily through scintillation counting. This largely reduces the time required for subsequent protein turnover measures due to the brevity of the method compared to others such as chromatographic techniques requiring amino acid concentrations to be measured individually.

Although the full body environment provides a good tool for investigating a representative physiological model, there are also a number of negative side effects; the inability to control extracellular environments, to directly quantify/modify the make-up of the extracellular pool, and to test and quantify error through loss of label (Sugden and Fuller 1991). Due to these limitations, experiments that involve investigating the molecular mechanisms of protein turnover or how these processes are affected during different extracellular stressors will commonly make use of in vitro models, allowing for greater experimental control.

The models available for in vitro study of muscle protein turnover are cultured cells, and incubated intact muscle or muscle strips obtained from laboratory animals (Ekins, Ring et al. 2000). Cell culture represents the most modifiable, and controllable model to investigate protein synthesis in vitro, allowing direct access and greater surface area of the tissue when compared to a muscle or muscle strip (Ong, Blagoev et al. 2002). This enhanced surface area reduces the time required for isotopic enrichment and equilibrium and greater long-term tissue viability compared to whole muscle or muscle strip models (Dmitrieva, Michea et al. 2001). But, cell culture is not as closely representative of a true physiological living tissue, and even more importantly cell cultures demonstrate less dramatic responses to external cellular stresses, including osmotic shock, when compared to full intact muscle or muscle strips (Schreiber 2005).

This is likely attributable to the lack of fully intact microtubule and actin filament structures within the cultured cells leading to less efficient linking of cell surface-detection to intracellular response pathways (Lang, Busch et al. 1998; Hoffmann, Lambert et al. 2009). With either model chosen, the measurement technique involves incubations with amino acids containing radioactive or stable isotopes, and subsequent analysis for the incorporation or release of the labelled amino acids. Chromatographic methods are also available that can be used to quantify naturally occurring amino acid concentrations within the tissue. As previously mentioned, this method requires significantly more time as each individual amino acid must be analyzed separately. But, this also provides a full picture of the turnover for all amino acids rather than one or two labelled amino acids and inferring changes based on these limited observations.

For reliable protein turnover measures in vitro, there must be rapid and complete isotopic equilibration between the exogenous precursor pool and the endogenous aminoacyl-tRNA pool (Sugden and Fuller 1991). To accomplish this with intact muscle or muscle strips, investigators often use perfusate media containing amino acid concentrations at least 2 times that of physiological levels (McKee, Cheung et al. 1978). This can be problematic, as the infusion of the label will also require an aqueous media infusion leading to changes in the hydration state of the tissues as well as tissue insult at the point of injection. For studies investigating changes in cell hydration status, they will commonly employ media incubations containing the label to avoid these issues.

Once isotopic equilibration has occurred the experimental duration can begin, measuring the rate of incorporation or release of the radioactive/stable isotope amino acids into the muscle homogenate or incubation medium over time (Bolster, Jefferson et

al. 2004). The total amount of amino acid incorporated or released can then be calculated and used to estimate possible changes in protein synthesis and degradation. At lower concentrations of the radiolabel in the media, the concentration of radiolabel in the muscle can rapidly reach a plateau value. This can lead to underestimating the true rate of incorporation due to limitations in transport (peripheral vs. central fibers) or preferential charging of degraded amino acids for reutilization (McKee, Cheung et al. 1978; Stirewalt and Low 1983; Stirewalt, Low et al. 1985). This problem can be minimized through the use of two different radioactively labelled proteins to allow for correction of protein reutilization and give better estimates of FSR and FBR (Ong, Blagoev et al. 2002). But this is much more difficult to attain and not overly applicable to full muscle incubations due to the shorter duration of viability with this model as compared to cell cultures (Sugden and Fuller 1991).

Finally, aside from examining protein turnover at the cellular level, there are also methods to measure activity and quantity of individual enzymes and proteins involved in the signalling pathways of protein turnover. This could involve proteins like Akt and further downstream active components such as the enzymes involved directly in protein translation, such as p70s6k. As with most major regulatory protein kinases and phosphatases, their activity is regulated by various phosphorylation sites. The amount of phosphorylated protein out of total protein can be quantified using protein separation and immunoblotting techniques (Cohen 1985). This methodology involves isolating the protein fraction of the cell homogenate, and using gel electrophoresis and antibody labelling to sort and visually quantify the proteins based on their molecular weights and identities (Burnette 1981).

Assay-based methods have been developed to compare the reaction rate for p70s6K by measuring the appearance of its product (through fluorescence or spectroscopy). Although this is a very useful tool for investigating individual protein activity, it does not give a full picture or representation of overall protein turnover. Based on the current literature, researchers should make use of the incubation method at physiological concentrations of either uniformly labelled phenylalanine or tyrosine to obtain the most accurate and representative results on protein turnover with in vitro models.

Statement of the Problem:

The current literature supports an effect of cell swelling to increase protein synthesis and reduce protein degradation as observed in cell culture, individual hepatocytes, and liver tissue (Stoll, Gerok et al. 1992; Alfieri and Petronini 2007; Bevilacqua, Wang et al. 2010). The majority of investigations to date have used cell culture, and when intact whole muscle has been used, the extracellular agent used to manipulate osmolality and its downstream effects were the primary variables examined (insulin and it's signalling cascade), rather than the end point measure of protein turnover.

We proposed to investigate the effect of increased cell volume, through hypo-osmotic extracellular stress, on rat skeletal muscle protein turnover and the contribution of select regulatory pathways involved in protein synthesis and degradation. This model allowed for the maintenance of the microtubule and actin cytoskeletal structures considered essential in the response to osmotic stress (Hoffmann, Lambert et al. 2009). The results of this work are important to further define the relationship of cell hydration and protein turnover in a skeletal muscle model, and may contribute to a better understanding of how water shifts in pathological situations affect skeletal muscle protein turnover.

Purpose:

The purpose of this experiment was to determine if hypo-osmotic stress, resulting in cellular swelling, would increase protein synthesis and/or decrease protein degradation and whether these changes would be supported by increased activation of the mTOR/p70S6k pathway and suppression of the ubiquitin/μ-calpain pathways, respectively.

Hypothesis:

It was hypothesized that HYPO exposure would elicit increased protein synthesis as measured by L-U[^{14}C]phenylalanine incorporation and would be associated with increased mTOR Ser^{2448} phosphorylation. Downstream of mTOR activation, p70s6 kinase was expected to demonstrate increased Thr^{389} phosphorylation/activation in the HYPO condition also, possibly contributing to the increased protein synthesis.

Furthermore, it was expected that HYPO exposure would elicit a reduction in protein degradation as measured by tyrosine appearance in the media and this would be associated with a decrease of ubiquitin-protein conjugates and a reduction in the autolysosomal ratio for μ-calpain; suggesting lower degradation activity of these pathways. The diagram below gives a theoretical representation of what we hypothesized would occur with the protein synthesis signalling pathways following HYPO exposure.

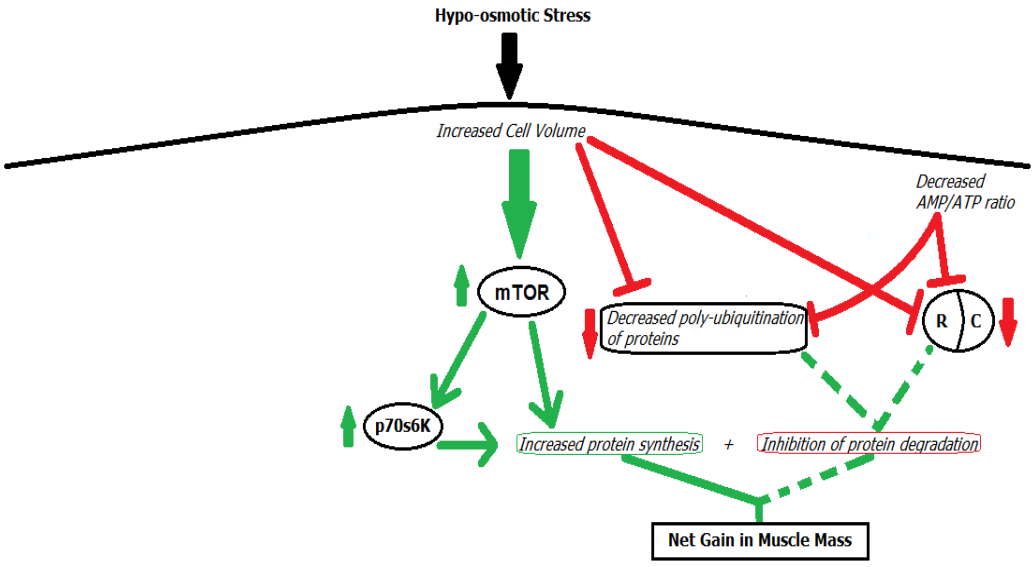


Figure 4. Proposed activation of protein synthesis through the mTOR/p70s6k (mTOR = mammalian target of rapamycin, p70s6 kinase) and inhibition of degradation through the ubiquitin-proteasome and μ-calpain degradation pathways (ubiquitin-protein conjugates = polyubiquitinated proteins, R/C = full calpain complex; R = regulatory subunit, C = catalytic subunit) by hypo-osmotic exposure.

Legend: ***Up arrows = increased activity, Down arrows = decreased activity, broken line = inhibition.***

Methods

Animals and Housing:

The current investigation required a total of 22 Long Evans rats (Charles River Laboratories, St. Constant, QC) with an average body mass of 126.6 ± 8.9 grams (4-6 weeks of age). All rats were housed with littermates (4-6 animals/cage) on a 12:12 light-dark cycle with ad libitum access to water and food until 1.5 hours prior to surgery when they were brought to our laboratory (5012 rat diet, PMI Nutrition Inc. Brentwood, MO). All experimental protocols were approved by the Brock University Animal Care and Use Committee and conformed to all Canadian Council for Animal Care guidelines.

There were 2 experimental conditions (ISO & HYPO) both requiring 8 animals (a total of 16 muscles). 8 muscles were put through incubations involving the osmotic condition without the ^{14}C-L[U]phenylalanine (with 0.5mmol/L cycloheximide) and were used for protein degradation, and western blot analysis, while the other 8 were used for protein synthesis measures with the ^{14}C-L[U]phenylalanine label present in the media. The additional 6 animals provided 8 muscles (4 muscles/group) for pilot testing of the protein synthesis and degradation methods and the additional 2 animals (4 muscles) for any errors or complications that occurred during experimentation requiring repeat analysis. 2 ISO and 2 HYPO were run at the same time in the bath set-up, having one day dedicated to synthesis measures, and a separate day for degradation.

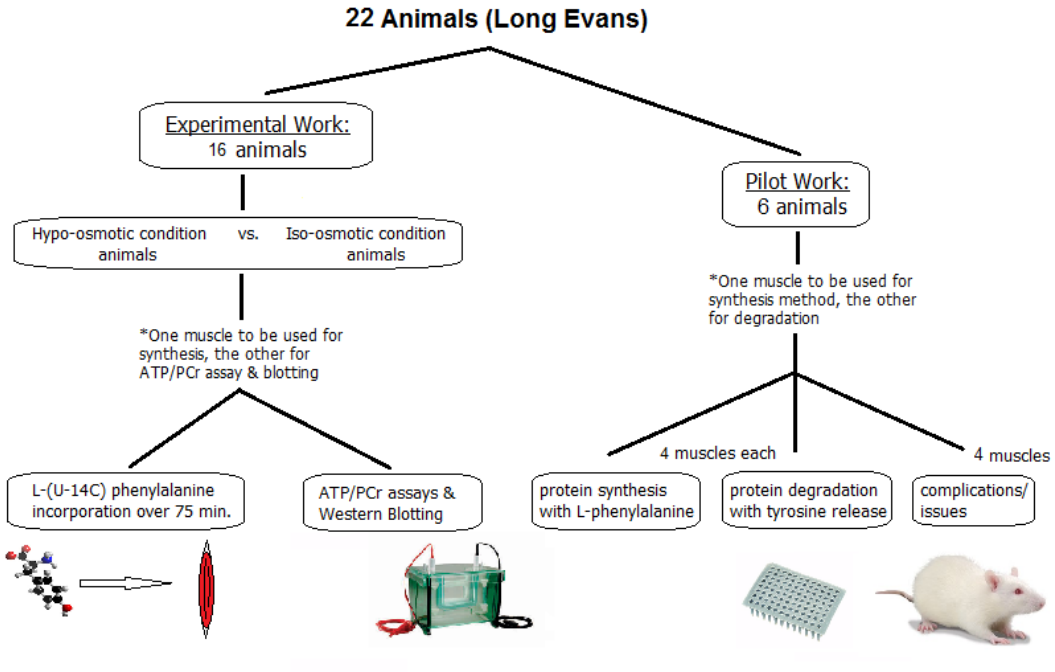


Figure 5. Animal use breakdown for experimental data collection and pilot testing

Incubation System & Medium:

Muscles were incubated using a previously described system (Antolic, Harrison et al. 2007). This system allows for whole muscles to be incubated in modified media while maintaining tissue viability (Antolic, Harrison et al. 2007). Samples were incubated in modified medium 199, absent of phenol-red dye and tyrosine (Gibco Custom Mediums; Quebec, ON, see appendix for detailed description of medium). This allowed for determination of tyrosine release into the medium at the end of the incubation. This medium contained extracellular concentrations of ions, but also the necessary substrates (glucose, amino acids, fats) and other compounds required to maintain tissue viability.

The media was continuously bubbled with a 95% O_2, 5%CO_2 gas mixture to

provide an environment to sustain tissue metabolism and viability. Deionized H_2O and mannitol were added to the incubation medium to achieve the targeted osmolalities [HYPO (190 ± 10 mmol/kg) and ISO (290 ± 10 mmol/kg)]. Media osmolality was verified using a standard osmometer (5520 VAPRO Vapor pressure pressure osmometer; Wescor, Inc). The incubation conditions used were similar to previous work from our laboratory investigating the effect osmotic stress on skeletal muscle metabolism (Antolic, Harrison et al. 2006).

Mannitol was used to manipulate osmolality because it represents a non-active osmolyte, only exerting effects on the osmolality of a solution, avoiding alterations to metabolism or the charge of the membrane. It has also been demonstrated that mannitol will not pass through cell membranes and effect the intracellular compartment, making it useful for eliciting isolated changes in cell volume (Stuart, Torres et al. 1970). Skeletal muscle is also unable to metabolize the compound, making it a consistent agent for osmotic manipulations (Wick, Morita et al. 1954).

Previously, the optimal incubation temperature to maintain muscle viability in Soleus and EDL muscles from rats weighing up to 140 grams has been shown to be 25 – 30 °C (Segal and Faulkner 1985; Antolic, Harrison et al. 2007; Cermak, LeBlanc et al. 2009). A number of studies have used similar temperature ranges successfully for other metabolic studies involving full rat muscle tissue incubations (Bonen, Luiken et al. 1998; Dyck, Steinberg et al. 2001). To assure a representative resting muscle tension and length during the incubation, 1 g of tension on a 50 mg or smaller muscle was used and has been previously shown to successfully imitate in vivo length/tension relationships (Clausen, Dahl-Hansen et al. 1979).

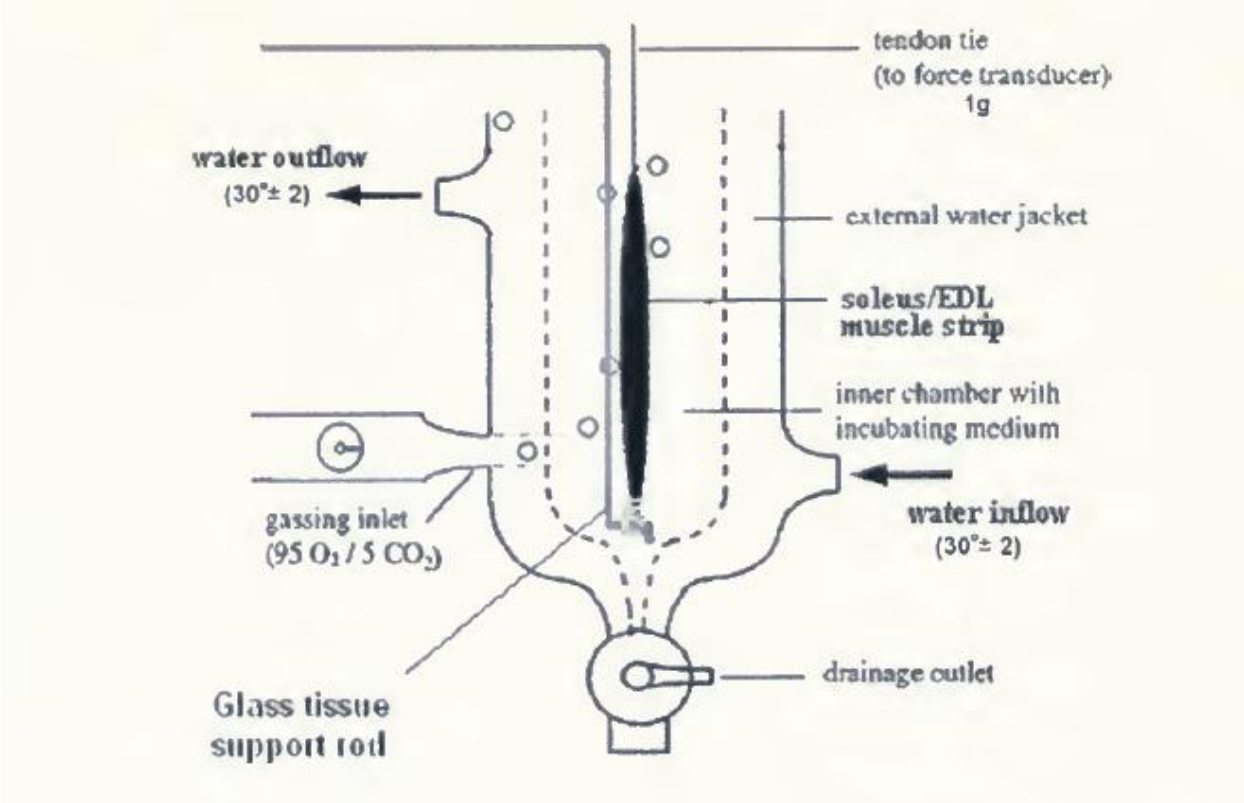


Figure 6. Muscle incubation bath containing isolated full skeletal muscle.

From (Antolic, Harrison et al. 2007)

Skeletal Muscle Model & Incubation Conditions:

The extensor digitorum longus (EDL) muscle from Long-Evans rats was used for all experiments. Our lab has previously demonstrated that the EDL responds more significantly to osmotic stress compared to more oxidative muscles such as the soleus (Antolic, Harrison et al. 2007). We chose 190mmol/kg for our HYPO condition because it has previously been demonstrated to significantly alter glucose metabolism and water volume content in rat extensor digitorum longus, compared to ISO medium at 290 mmol/kg (Mulligan et al. 2007; Antolic, Harrison et al. 2006).

Experimental Protocol:

Rats were administered anaesthetic (Sodium pentobarbital, 55mg/kg^{-1}, IP) prior to isolation and removal of EDL muscles from both hind-limbs. Removal of the muscle involved removing the skin from the hind-limb, isolating the distal tendon of the EDL muscle at the ankle, and removing the surrounding tissues to allow for isolation of the proximal tendon. Once both tendons were isolated and the muscle was free from the surrounding connective tissue, silk sutures (4-0 silk) were tied around each tendon prior to final excision and removal of the muscle. This allowed for the muscle to be isolated from the animal very briefly prior to placement into the incubation bath (<10sec).

Following muscle extraction, the rats were euthanized with an overdose of sodium pentobarbital. Muscles were divided into 4 groups; ISO/Synthesis, ISO/Degradation, HYPO/Synthesis and HYPO/Degradation. All conditions started with the ISO or HYPO condition for a 60 min incubation, immediately followed by a 75 min incubation with 0.5mM L-U[^{14}C]phenylalanine or 0.5mmol/L cycloheximide added to the media to quantify protein synthesis and protein degradation, respectively. At the end of the incubations, muscles were removed from the bath and immediately snap frozen in liquid nitrogen for subsequent protein synthesis, protein degradation, metabolite, and/or western blot analysis.

Post-Incubation Analysis

Protein Turnover measurement

Protein synthesis and breakdown were estimated through the measurement of L-U[^{14}C]phenylalanine incorporation and tyrosine appearance into the media respectively (Tischler, Desautels et al. 1982; Dardevet, Sornet et al. 1994; Dardevet, Sornet et al. 2000; Marzani, Balage et al. 2008). Muscles were pre-incubated for 60 min in the appropriate experimental condition medium. Muscles were then incubated in identical media with the addition of 0.5mM L-U[^{14}C]phenylalanine (9.25MBq/L) for 75 minutes to assess protein uptake (non-incorporated) and protein synthesis (incorporated) in the tissue. Immediately following the incubation, muscles were homogenized in 0.61 M trichloroacetic acid (TCA) and centrifuged at 10,000g for 10 min at 4 °C to isolate the protein-bound and non-bound fractions (Marzani, Balage et al. 2008). TCA-insoluble material was washed 3 times in 0.61 mol/L TCA and the resulting pellet was solubilised in 1 mol/L NaOH at 45 - 50 °C for 15 minutes to isolate protein-bound and non bound fractions. Following isolation of the protein-bound and non-bound fractions, the radioactivity of these fractions was assessed through liquid scintillation counting. Total protein synthesis was then calculated by dividing the protein-bound radioactivity by the specific activity of free labelled phenylalanine in the incubation media and expressed as nanomoles of phenylalanine incorporated/mg protein/75 min (Dardevet, Sornet et al. 2000). The media reading was used to find the specific activity of a known concentration of our label. The values obtained from reading the samples could therefore be compared to the initial medium and the specific activity used to calculate the enrichment of the label within the sample.

$$\frac{\text{Sample reading}}{\text{Medium reading}} \times \underset{\text{(media conc.)}}{0.5\text{umol/L}} \times \underset{\text{(nmol/mg/75 min)}}{1000} \times \underset{\text{(dilution from washes)}}{2 \text{ for non-bound only}}$$

Protein degradation was estimated by quantifying tyrosine release into the incubation media. Tyrosine is not significantly metabolized in skeletal muscle, making it a stable marker of overall breakdown as the incubation medium was devoid of this amino acid at the onset of the incubation (Dardevet, Sornet et al. 1995). Separate 75 min breakdown incubations were performed to allow for the addition of cycloheximide at 0.5mmol/L, which blocked protein translation and eliminated the possible re-utilization of any degraded/released tyrosine. Therefore, the difference between tyrosine concentration pre (fresh medium containing no tyrosine) and post-incubation (muscle released during 75 min) in the medium was used to calculate the tyrosine released over the 75 min duration.

The tyrosine in the medium was measured using nitroso-2-naphtol as previously described (Dardevet, Sornet et al. 1994). Briefly, prior to the completion of the incubation, 0.1% nitroso-naphtol solution and a 0.05% sodium nitrate/nitric acid (1:5 d H_2O dilution) solution were prepared (20ml and 25ml, respectively). Following completion of the incubation, a 1 ml sample of pre-incubation medium was used as a blank and all subsequent sample media were reacted with 1ml of each of the previously mentioned solutions. The samples were then placed into a water bath at 65° C for 30 min to allow for the chemical reaction between the tyrosine in standards or in the medium and the nitroso-2-naphtol. Samples were then cooled for 10 min and 10ml of ethylene dichloride was added. The samples were then centrifuged at 2000rpm at 4° C for 10 min, and the fluorescence of the remaining supernatant was determined by activation at the 460nm wavelength and was measured at the 570nm wavelength in a standard

fluorometer. Tyrosine concentrations were determined through comparison of sample values to a standard curve made through serial dilutions of an initial tyrosine concentration. The pre and post-incubation samples and standard curves were all run in triplicate. See the appendix section for a detailed description of the synthesis and degradation methods.

Metabolite Analysis:

A small portion of the frozen muscle was chipped off (10-20mg) from the central region of each muscle belly and then lyophilized (16-20 hours), powdered and cleared of any blood and/or connective tissue. The powdered samples were then acid extracted (in 1 M PCA) and neutralized with 2.3 mol/L $KHCO_3$. The samples were then used for measurements of muscle metabolites. ATP and PCr concentrations were determined to confirm muscle viability post-incubation. Fluorometric techniques developed by Harris et al. (Harris, Hultman et al. 1974) and modified by Green et al. (Green, Thomson et al. 1987) were used to analyze the concentrations of the metabolites (See method protocols in appendix, pgs. 94-98). To assure accuracy and reliability of the measures, each sample was analyzed in triplicate during the same experimental session (Roy, Green et al. 2000).

Relative Muscle Water Content

After chipping off the piece of muscle used for metabolites, the sample portion was weighed pre and post lyophilisation to attain wet and dry weight values. The relative muscle water content was then calculated by the following equation:

$$\text{Wet Weight} - \text{Dry Weight} = \frac{\text{Water Weight}}{\text{Wet Weight}} = \% \text{ Muscle Water Content}$$

Western Blot Analysis:

Frozen muscle samples were hand homogenized in an ice cold EDTA or EGTA base buffer (See appendix method for volume/weight ratio used) with the addition of 1 protease and phosphatase inhibitor tablet to 10ml of homogenization buffer (Complete Mini, EDTA-free protease inhibitor; Roche Diagnostics & PhosSTOP phosphatase inhibitor cocktail Tablets; Roche Diagnostics). Total protein content of each sample was determined using the Bradford assay (Hartree 1972). To allow for equal loading onto the gels, each sample was diluted with a buffer containing sodium dodecylsulphate (SDS) and β-mercaptoethanol to a concentration of 1µg/µl or 2µg/µl (mTOR antibodies only). The samples were denatured using boiling water (5 minutes for u-calpain only) and then re-cooled for 5 minutes on ice.

Samples were then separated by SDS gel electrophoreisis (SDS-PAGE) on gels varying between 7.5 – 10% polyacrilamide (dependent on individual protein of interest's molecular weight; see method protocol section) for 90 minutes at 100 – 160V. Following protein separation they were then transferred to either nitrocellulose (µ-calpain only) or polyvinylidene fluoride (PVDF) membranes, depending on the optimized protocol for the protein of interest, for 90 – 180 minutes at 100 – 120V (See pg. 106-109)

Following transfer, the membranes were blocked with either 1 – 5% BSA (bovine serum albumin) or 1 – 5% milk-TBST with addition of a detergent (Tween-20 at 0.025%), for 60 minutes at room temperature. Membranes were then washed (5 x 5 min) each and then incubated with the primary antibody of interest diluted (1:1000 to 1:6000) with either 1 – 5% BSA or milk-TBST solution and agitated overnight at 4 °C. The membranes were then rewashed again (5 x 5 min) and then incubated with an appropriate secondary antibody (horseradish peroxidise-conjugated antibody [HRP]) for 60 minutes at room temperature to allow for visual detection. The final step involved imaging the protein bands by administering chemiluminescent (Immobilon Western Chemiluminescent HRP Substrate: Millipore, Missisauga, ON) substrates and quantifying the bands of labelled protein using band densitometry calculations from "Image J" software.

Proteins were normalized to the total actin content from the same lane as measured by western blotting (ubiquitin-protein conjugates) or to the total content of the protein of interest for the phosphorylated signalling proteins (mTOR & p70s6k). For the μ-calpain autolysis ratio, the 78 & 76 kDa (autolyzed active components) bands total density value was divided into the 80 kDa (non-autolyzed non-active component) bands density value to calculate the autolysis ratio. The 76/78 kDa versions recognized by this antibody are cleaved or autolyzed μ-calpain catalytic domains and can actively degrade proteins. In contrast, the 80 kDa band represents the full complex of regulatory and catalytic subunits that would represent inactive μ-calpain. Therefore, the autolyzed proportion was divided by the non-autolyzed portion, giving a representation of the pathways overall degradation activity.

Statistical Analysis

All experimental results were analyzed using unpaired two-tailed t-tests to compare the two experimental groups on a variety of individual variables. All groups were examined for significant differences in bodyweight, ATP and PCr concentrations, protein synthesis and degradation and signalling protein content (mTORSer^{2448} & p70s6k Thr^{389} phosphorylation, content of ubiquitin-protein conjugates, and the μ-calpain autolysis ratio). The level of statistical significance was set at $p < 0.05$ for all tests. All statistical analyses were conducted using GraphPad Prism 5 (La Jolla, CA) and all values are expressed as mean ± standard deviation (SD).

Results:

General Observations:

No significant differences were noted in rat bodyweights between groups ($p<0.05$) (Table 1). For all of the incubations, media osmolalities were maintained in HYPO (188 ±5.0 mmol/kg) and ISO (287 ± 5.2 mmol/kg), while incubation temperature was maintained at 30.0 ± 2° throughout.

Table 1. Rat total bodyweight and individual muscle weights for animals included in the experiment

	ISO	HYPO
Rat Weight (g)	125.5±5.0	121.2±5.7
EDL Wet Weight (mg)	58.7±4.7	62.4±6.8

n = 8 - 10 animals/group. Data is represented as Mean ± SD. No significant difference between groups. EDL = extensor digitorum longus.

Metabolites:

Adenosine triphosphate (ATP) and phosphocreatine (PCr) concentrations (mmol/kg dry weight or d.w.) were measured to assure that the incubation protocol maintained metabolic viability. This also allowed for examining differences in the tissues overall energy status at the completion of the incubations. The HYPO condition mildly increased ATP and PCr concentrations compared to ISO (5.7 and 2.4% respectively) ($p < 0.05$). The HYPO condition also induced a significant increase (8.5%) in the cell water percentage of total muscle weight ($p<0.05$) (Table 2).

Table 2. Adenosine triphosphate and phosphocreatine concentrations and relative water content of muscles.

Experimental Measure	***ISO***	***HYPO***
ATP (mmol/kg d.w.)	32.1±1.1	34.0±1.5*
PCr (mmol/kg d.w.)	77.7±1.1	79.6±1.3*
Relative Water Content (%)	77.4±1.9	84.3±0.71*

** Denotes significant difference from ISO; HYPO. Data represented as mean ± SD n = 6 muscle, ISO n = 5 muscles ($p<0.05$).*

Protein Turnover:

Protein synthesis was determined by assessing the incorporation of L-[^{14}C]-U-phenylalanine into the protein-bound cellular fraction. Protein synthesis was robustly increased by 169% following HYPO exposure compared to ISO ($p<0.05$).

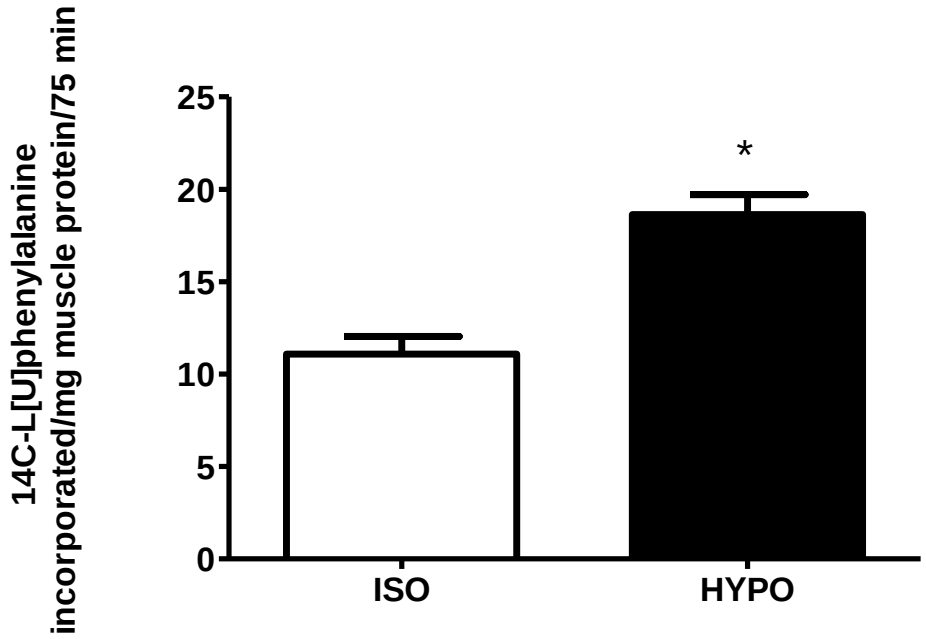


Figure 7. L-U[^{14}C]phenylalanine incorporation per mg muscle over the 75 min incubation. Data represented as mean ± SD, n = 8 muscles/group. * Denotes significant difference from ISO (p<0.05).

In support of the incorporated phenylalanine values, non-incorporated phenylalanine in the muscle was significantly increased 199% in HYPO compared to ISO ($p<0.05$). This suggests that the incubation condition not only favoured increased synthesis mechanisms, but also increased uptake of amino acids.

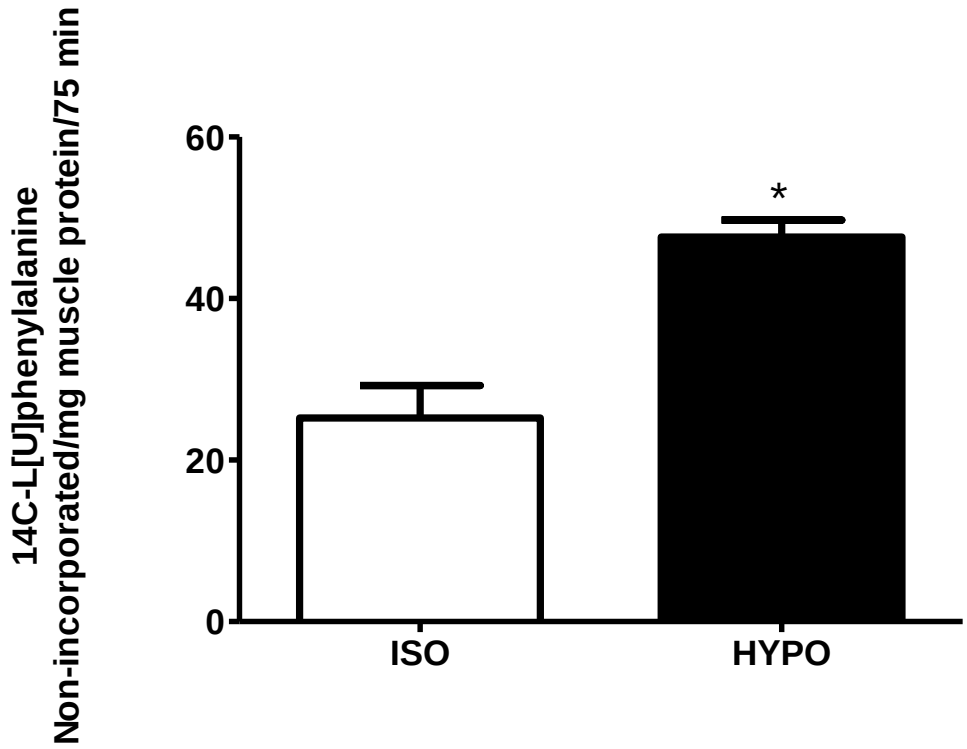


Figure 8. Non-incorporated L-U[^{14}C]phenylalanine uptake during the 75 min incubation protocol. Data represented as mean ± SD, n = 8 muscles/group. * Denotes significant difference from ISO condition ($p<0.05$).

Tyrosine release into the medium was used as a marker of overall protein degradation in the presence of cycloheximide in the incubation medium. No significant differences were noted in the media tyrosine concentrations at the completion of the incubations between ISO and HYPO ($p<0.05$).

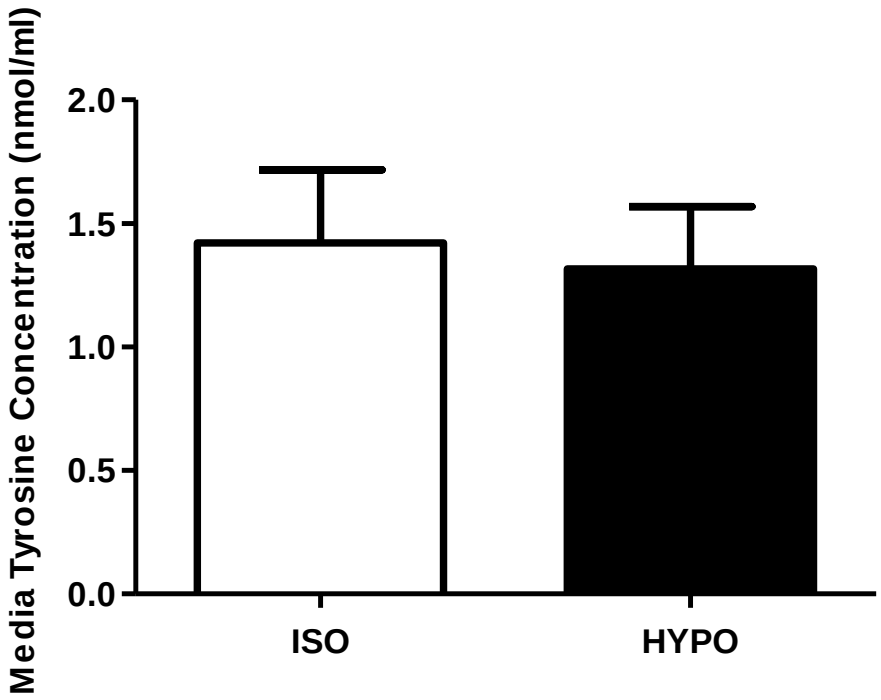


Figure 9. Tyrosine concentration in the incubation medium at the conclusion of the incubation. Data represented as mean ± SD, n = 8 HYPO, 7 ISO muscles/group. No significant difference between groups.

Western Blot Analysis:

Total mTOR was not significantly different between the HYPO and ISO conditions ($p<0.05$). mTOR Ser^{2448} phosphorylation values were slightly elevated following HYPO exposure (12.5%) compared to ISO ($p<0.05$).

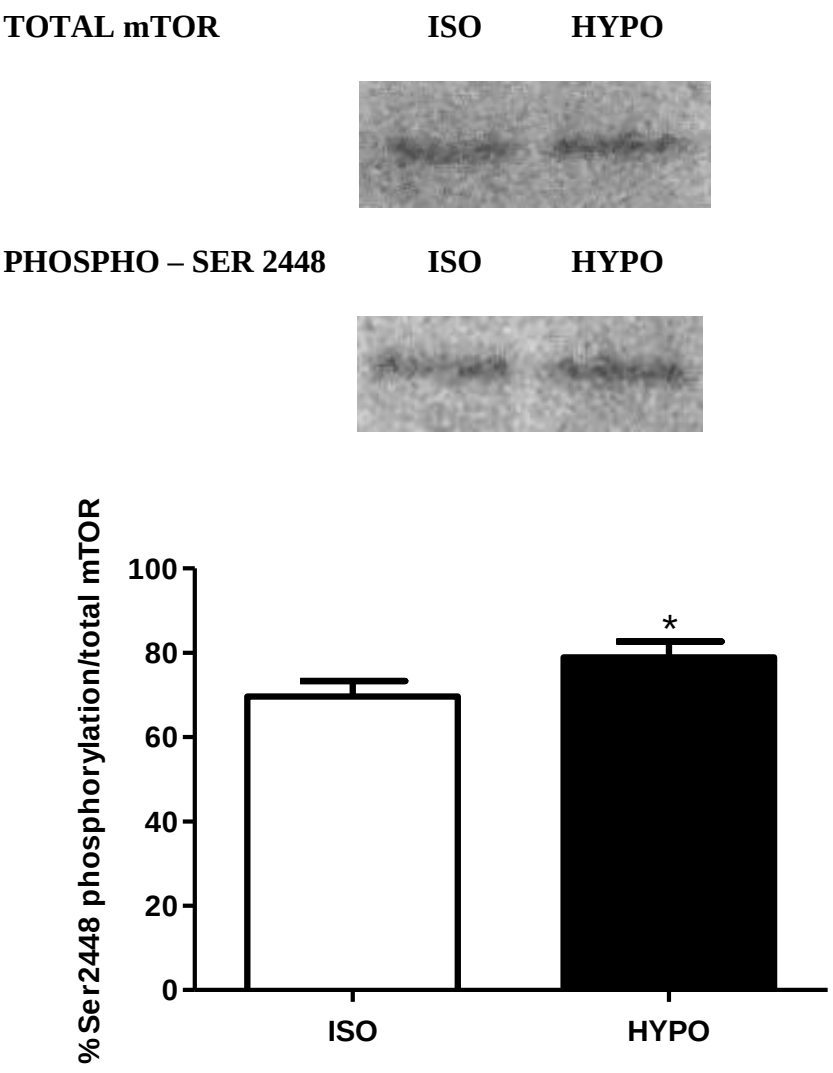


Figure 10. Percent phosphorylation of mTOR ser^{2448} out of total mTOR content. *Denotes significant difference from ISO. Data represented as mean ± SD, n = 7 muscles/group (p>0.05). Above the graph are sample images from the blots used to calculate percentage phosphorylation of total protein for mTOR ser^{2448}

Total p70s6K protein content was not significantly different between the HYPO and ISO conditions ($p<0.05$). The content of phosphorylated p70S6 kinase Thr^{389} was increased following HYPO exposure (23.9%) compared to ISO ($p<0.05$).

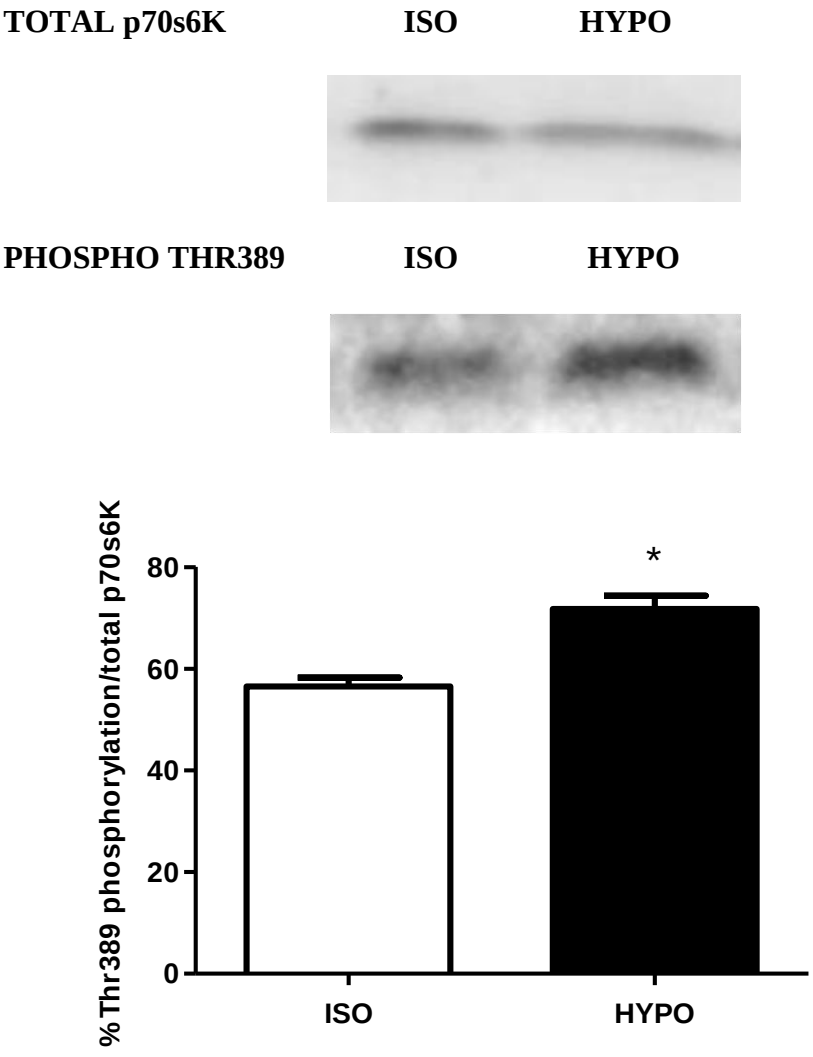
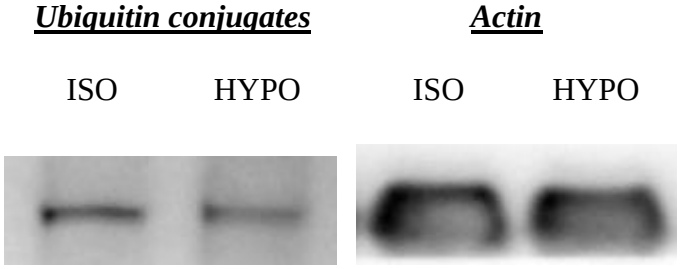


Figure 11. Percent phosphorylation of p70s6 kinase thr^{389} out of total p70s6 kinase content. *Denotes significant difference from ISO. Data represented as mean ± SD, n = 8 muscles/group ($p>0.05$). Above the graph is an image from the blots used to calculate percent phosphorylation of thr^{389} out of total p70s6k content.

The content of ubiquitin-protein conjugates at the 200kDa weight range was significantly reduced by HYPO exposure compared to ISO ($p < 0.05$). Above the graph is a sample image of both ubiquitin-protein conjugates, and actin blots used in creating the graph.



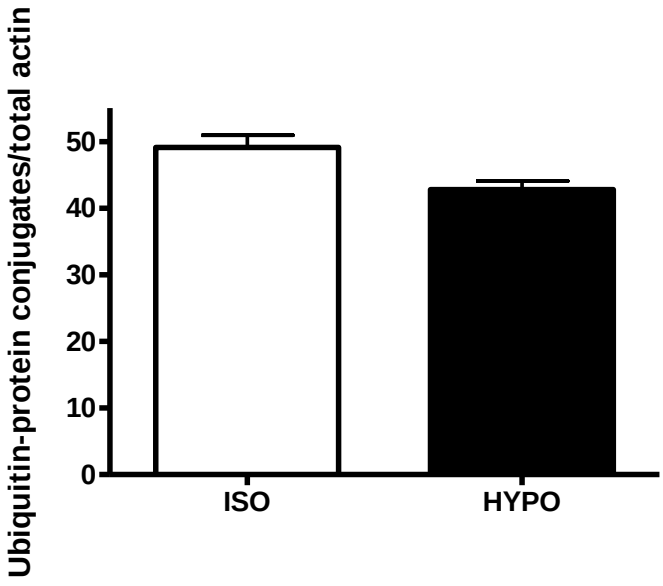


Figure 12. Ubiquitin-protein conjugates normalized to total actin content. No significant difference between groups. Data represented as mean ± SD, n = 5 HYPO, 8HYPO muscles/group (p 0.05).

The μ-calpain autolysis ratio (78 & 76 kDa band/80 kDa band) was significantly higher (189%) in the HYPO condition compared to the ISO condition ($p<0.05$). This increased autolyzed activated u-Calpain ratio in HYPO may suggest an increase in the activity of this pathway.

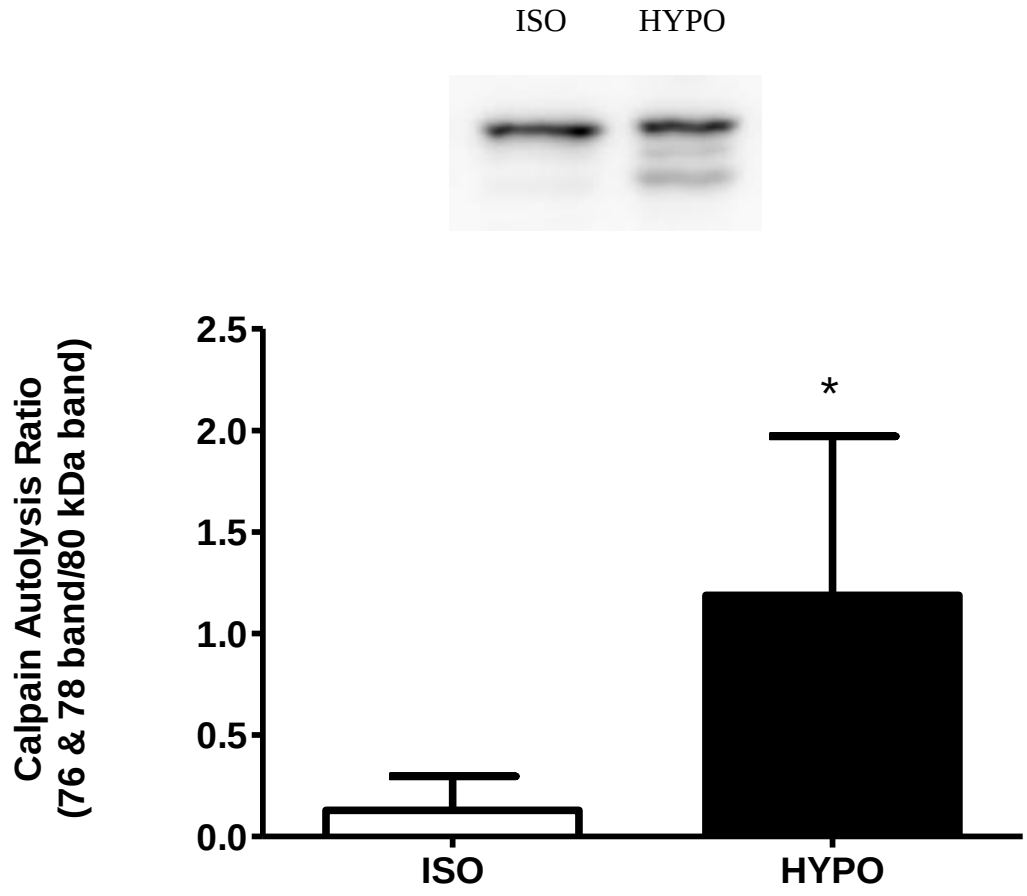


Figure 13. μ-calpain autolysis ratio (78 & 76 kDa band/80 kDa band) of HYPO and ISO conditions. Above the graph are sample blots used to calculate the autolysosomal ratio. Data represented as mean ± SD, n = 6 muscles/group. * Denotes significant difference from ISO (p<0.05).

Discussion:

The HYPO exposure resulted in significantly increased intracellular non-bound and bound phenylalanine, with an associated small elevation in mTOR ser^{2448} phosphorylation and slightly greater elevation in p70s6 kinase thr^{389} phosphorylation. No significant differences were observed between HYPO and ISO for protein degradation. Interestingly, there was a reduction in ubiquitin-protein conjugates in HYPO and an unexpected increase in the average μ-calpain autolysis ratio. In general, HYPO stimulated large increases in protein synthesis, possibly through an expansion of the intracellular amino acid pool, while eliciting no observable differences in protein degradation as measured by tyrosine release into the media.

Muscle Water Content and Phosphagen Levels Response to Hypo-osmotic Exposure:

The results of our current study are similar to past investigations and lend further support to the idea that hypo-osmotic exposure leads to significant changes in cell volume and phosphagen levels; HYPO osmotic stress increased the intracellular water weight content by 8.5%, and ATP & PCr by 4-6% from the ISO condition ($p<0.05$) (Haussinger, Lang et al. 1994; Hoffmann, Lambert et al. 2009). These results closely resemble previous observations from our lab in which a 6-7% increase in muscle water content was observed during 1 h of exposure to HYPO and a trend toward elevated ATP & PCr (although not significant) compared to ISO. In the current investigation we confirm the previously observed change in water content is maintained for up to 135 min (Antolic, Harrison et al. 2007) and ATP and PCr concentrations are slightly elevated. Previous work demonstrated that these changes can be attributed to intracellular water content

changes and not simply increased water content in the interstitial fluids (Antolic, Harrison et al. 2007). This change in intracellular water content was associated with mild alterations in muscle metabolites and robust increases in protein turnover, suggesting a possible regulatory role for cell volume in the regulation of protein turnover.

Protein Synthesis:

Exposure to the HYPO condition resulted in a 199% and 169% increase in non-bound intracellular L-U[^{14}C]phenylalanine and incorporated L-U[^{14}C]phenylalanine, respectively. The proteins being synthesized during this acute exposure to hypo-osmotic stress would likely be proteins associated to cytoskeletal structural elements. The mRNAs of cytoskeletal and microtubule proteins have been shown to be up regulated in various tissues in response to osmotic stress (Pedersen, Hoffmann et al. 2001; Hoffmann, Lambert et al. 2009). HYPO exposure may also be increasing the expression or activity of metabolic enzymes that have previously been shown to be activated upon hypo-osmotic exposure, such as GSK (al-Habori, Peak et al. 1992).

These results demonstrate that hypo-osmotic exposure is associated with robust increases in both intracellular phenylalanine and incorporated phenylalanine in rat skeletal muscle, suggesting a possible regulatory role for cell volume in protein synthesis in skeletal muscle tissue. This is the first study to display this phenomenon in skeletal muscle tissue, confirming previous results from investigations involving protein synthesis in response to HYPO exposure in humans, hepatocytes and liver perfusion, and cell culture models (Stoll, Gerok et al. 1992; Haussinger, Roth et al. 1993; Berneis, Ninnis et al. 1999). These results in collaboration with future projects could potentially lead to the

use of hypo-osmotic infusion therapy in humans to deal with conditions of skeletal muscle atrophy.

The large increase in non-incorporated phenylalanine content was unexpected, although in an indirect way supported our original hypothesis. This result suggests that the HYPO environment is possibly stimulating mechanisms to increase intracellular amino acid content to assist with the increased protein synthesis that was also observed following HYPO exposure. This could likely be a result of signalling leading to increased uptake mechanisms. The possibility that amino acid transporter activity and presence on the membrane may be up regulated in response to HYPO exposure seems a plausible explanation. A previous study demonstrated increased V max kinetics for numerous system A Na^+-dependant amino acid transporters in response to insulin and aniso-osmotic shock in L6 muscle cells (Kashiwagi, Yamazaki et al. 2009). These Na+-dependant transporters show specificity to neutral, non-charged amino acids, like the L-U[^{14}C]phenylalanine used in the current investigation. It seems an attractive explanation for our results and future studies should examine the content, expression and enzyme kinetics of system A amino acid transporters in skeletal muscle in response to HYPO exposure.

Other studies have shown implications for increased SNAT2 activity, a non-specific system A amino acid transporter, in response to increased mTOR activity during cell stress (Pinilla, Aledo et al. 2011). Our current study demonstrated significant but small increase in mTOR Ser^{2448} phosphorylation, however there is the possibility that the activation of this kinase may have occurred much earlier in the incubation or that phosphorylation of mTOR at other key regulatory sites could have greater effect on the

kinase activity of the protein complex. If mTOR signalling had occurred at an earlier point in the incubation, it is possible that it could have been associated with the increase in amino acid uptake mechanisms. Future studies should examine the timeline of mTOR phosphorylation at SER^{2448} and the other known regulatory sites on mTOR in response to HYPO exposure to further determine its possible regulatory role under these conditions.

As hypothesized, HYPO exposure resulted in a significant increase in protein synthesis and demonstrated that the anabolic response observed in other tissues and models is conserved in skeletal muscle and its response to HYPO exposure (Stoll, Gerok et al. 1992; Berneis, Ninnis et al. 1999). We expected this increase to be mirrored by corresponding increases in mTOR Ser^{2448} and p70s6k Thr^{389} phosphorylation as these are commonly used markers of protein synthesis, but our results indicated only mild elevations to these sites following HYPO exposure that may not fully explain the observed changes in protein synthesis.

This falls in line with gene ablation studies in cell culture models demonstrating that mTOR and p70s6k activity contribute to, but are not necessary for, cell growth and protein synthesis (Kawasome, Papst et al. 1998; Wang and Proud 2006). Other factors not measured such as the upstream signals (Akt, ERK ½, MAP K, AMPK), eukaryotic (protein) initiation factors (including eIF and 4EBP), other components of the mTORc1 (raptor) and p70s6k ribosomal complex (FRAP transcription factors), and degradation transcription factors (FOXO, MuRF1, atrogin-1) may lend further explanation to the observed changes. mTOR has been observed to require interaction with other proteins such as raptor and the transcription factor 4E-BP1 for optimal complex activity (Nobukuni, Joaquin et al. 2005). Future investigations should aim to define the exact

signalling events involved in the protein turnover measures observed in the current investigation. It also seems pertinent to determine the signalling involved in the increase in intracellular phenylalanine as it could help explain the observed increase in phenylalanine incorporation.

Previous studies have suggested the transcription factor 4E-BP1 to be a better marker of protein synthesis, as correlations between these factors were maintained regardless of p70s6k ablation or rapamycin administration (Kawasome, Papst et al. 1998). This suggests multiple signalling systems are likely contributing to the overall rate of protein synthesis under such conditions. It is also possible that the inclusion of cycloheximide in the media may have altered the signalling observed for mTOR ser^{2448} and p70s6k thr^{389}. Future investigations are needed to examine cycloheximide free time-line responses of the measured and non-measured factors involved in regulating protein synthesis in response to HYPO exposure in skeletal muscle.

Protein Degradation:

Contrary to our original hypothesis, HYPO exposure led to no significant differences in protein degradation as measured by tyrosine release into the incubation medium. Interestingly, the results for ubiquitin-conjugates showed the expected reduction, albeit small, while the μ-calpain autolysis ratio was unexpectedly increased in response to the HYPO exposure, which would have favoured increased protein degradation. Therefore, based on the current data it appears that HYPO exposure may not have a regulatory role in regards to protein degradation within skeletal muscle tissue. Although the synthesis incubations did not contain cycloheximide, the increased

intracellular phenylalanine during incubation suggests that the intracellular amino acid concentration may be regulated within skeletal muscle. Because the method used for measuring protein degradation relied on the release of tyrosine from the intracellular pool, it is possible that our measure may have slightly underestimated the true quantity of degradation occurring in this condition. This is due to the possibilities of different turn-over rates of other amino acids, and also preferential re-utilization of amino acids cleaved from intracellular proteins for new protein synthesis (Chinkes 2005). This method for measuring protein degradation in rodent skeletal muscle has been successfully used in various investigations (Dardevet, Sornet et al. 1994; Marzani, Balage et al. 2008; Balage, Dupont et al. 2010) and future investigations could employ alternate methods of analysis examining the variation in all amino acids during protein degradation in HYPO exposure to confirm the observed results in the current investigation.

The small reduction in ubiquitin-conjugate labelling indicates a potential reduction in protein degradation, but previous studies have demonstrated this marker does not necessarily correlate to changes in protein degradation and that its relationship to protein degradation is often species and condition specific (Hershko and Ciechanover 1992). Studies investigating protein degradation in cardiac myocytes have demonstrated its use and reliability as a reliable marker of the rate of protein degradation (Perino, Ghigo et al. 2011). In contrast a study on humans demonstrated that although protein degradation increased immediately following mechanical tissue insult in humans, ubiquitin-protein conjugates were not significantly elevated until 48 hrs post-injury (measured at 0, 6, 12, 24, and 48 hrs post insult) (Majetschak, Seiffert et al. 2007).

These results suggest that although ubiquitin labelling plays a role in marking proteins for degradation, it may not necessarily fully explain the proteasomal degradation activity downstream of this ubiquitin labelling, or correlate to short-term measures of protein degradation. In the current investigation muscles were only incubated for 135 min and it is possible that with extended exposure to hypo-osmotic stress the reduction in ubiquitin labelling may provide better correlations to possible reductions in protein degradation. It is also highly possible that the small change in labelling was not sufficient to stimulate an observable decrease in protein degradation as seen in our condition. Further work could employ cell culture or animal models to provide a possible avenue to examine the longer-term associations between this marker and protein degradation in muscle.

In contrast to the observations of the ubiquitin-protein conjugates, HYPO exposure led to an activation of the μ-calpain system as measured by the autolysis ratio. Previous literature has demonstrated that calpains are normally activated under stressful conditions (Goll, Thompson et al. 2003; Bartoli and Richard 2005). Our current results possibly suggest that the HYPO condition was a stressful condition for skeletal muscle, despite the observed increase in protein synthesis. There are two possible explanations for this apparent activation; (1) increases in cell water content are reducing intra-membrane protein and phospholipid pressure, possibly allowing for altered fatty-acid chain orientation and (2) the HYPO environment may be leading to increased cytosolic calcium concentrations activating the μ-calpain autolysis process. Previous studies have demonstrated that calpain-II associates and is activated by phosphoinositol (PI) fatty acid chains in lipid rafts in the membrane (Hood, Logan et al. 2003). It remains to be

investigated whether hypo-osmotic exposure may lead to such changes in PI conformation in the membrane.

The other possible explanation for the μ-calpain activation observed in the current study is increased intracellular calcium concentrations in response to HYPO exposure. Previous studies examining cardiac myocytes and astrocytes demonstrated that HYPO exposure stimulated increases in calcium transients, and that these increases in calcium concentration led to activation of various intracellular pathways associated to cytoskeletal remodelling and alterations in ion pump activity (Whalley, Hool et al. 1993; Schliess, Sinning et al. 1996; Bewick, Fernandes et al. 1999). Although the above mentioned investigations did not examine μ-calpain activity, increased calcium transients in response to HYPO exposure could be a mechanism explaining the observed increase in the autolysis ratio seen in this condition. Future examination of calcium transients in response to HYPO exposure is required to further establish whether this may be the activation signal for the μ-calpain response seen in the current investigation.

There are some possible explanations for the disparity between the increase in the μ-calpain autolysosomal ratio and no observable difference in protein degradation. Because the method of measurement for degradation required amino acids to be released from the muscle, the possibility does exist that μ-calpains were actively degrading proteins possibly contributing to the intracellular pool for re-utilization. The other more likely explanation is that although the autolysosomal ratio is indicative of the quantity of the μ-calpain pool available to contribute to degradation, it does not measure the actual degradation rate (activity) of this pathway. Quantification of the actual rate of

degradation by this pathway would be beneficial to better understand the exact contribution of this system to overall protein degradation in skeletal muscle.

The following diagram illustrates the changes in signalling pathways and protein synthesis observed in the current investigation.

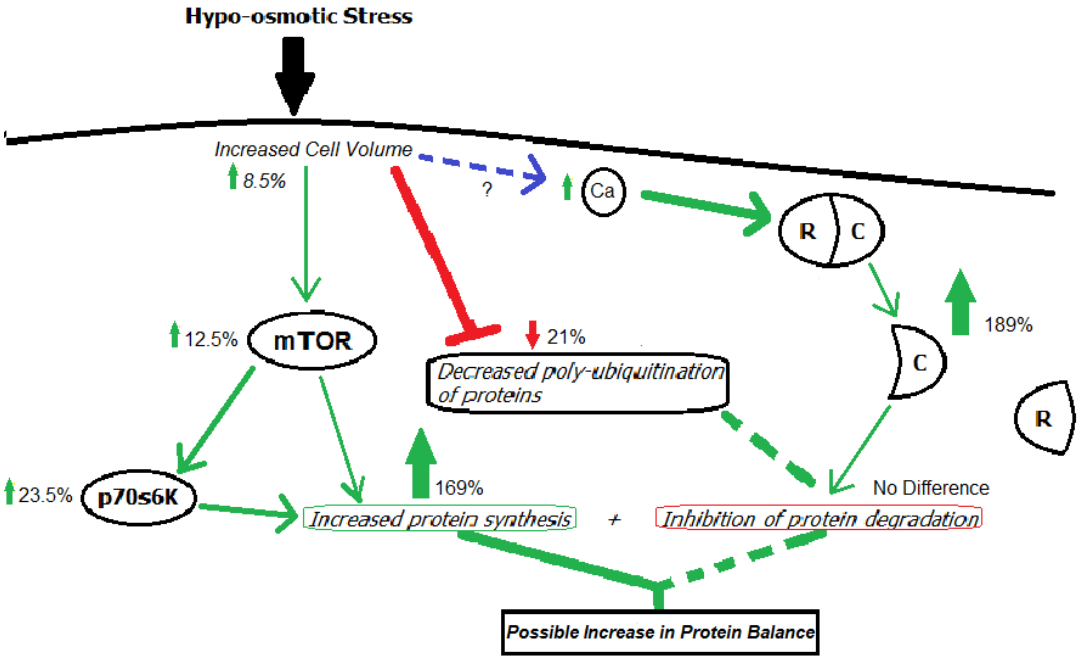


Figure 14. HYPO exposure increased protein synthesis (through mammalian target of rapamycin and p70s6 kinase) and no significant effect on protein degradation, while decreasing ubiquitin-protein conjugates but increasing the μ-calpain autolysis ratio. Up Arrows = upregulation, straight bars = inhibition, dotted line = inhibited response, dotted arrow = potential activation mechanism.

Conclusions and Future Directions:

The primary finding of the current investigation was that HYPO exposure elicited a large increase (169%) in protein synthesis and an unexpectedly large increase (199%) in non-bound phenylalanine uptake compared to the ISO condition. The observed increase in synthesis during HYPO was accompanied by small increases in the levels of mTOR Ser^{2448} and p70s6K Thr^{389} phosphorylation. Further studies are required to determine the time line and involvement of the measured signalling proteins, as well as others involved in protein synthesis but not examined in the current study. Some proteins of interest for future work include possible upstream activators such as Akt, ERK1/2, MAP K, AMPK and the transcription factor, 4E-BP1. Future work should also examine the signalling cascade involved in the increase in amino acid content that could be involved in the observed increase in protein synthesis in the current investigation.

Interestingly, protein degradation, as measured by tyrosine appearance into the media, was not significantly different between the HYPO and ISO conditions, despite an increase in μ-calpain activation. A slight reduction in ubiquitin-protein conjugates were observed with HYPO exposure and this was likely not large enough to elicit significant changes in protein degradation in our acute incubation period. We speculate that the possible activation of the μ-calpain pathway occurred through interactions with the lipid membrane and/or intracellular calcium transients, but additional work is required to verify these hypotheses. We have demonstrated for the first time with incubated skeletal muscle that hypo-osmotic stress has a significant effect on protein turnover, confirming evidence previously observed in human, rodent liver perfusion, and hepatocyte culture work (Haussinger, Hallbrucker et al. 1990; Stoll, Gerok et al. 1992; Berneis, Ninnis et al.

1999). This increase was not fully explained through changes in the signalling pathways examined. Future studies should investigate the time line responses of the examined pathways, as well as pathways not examined in this investigation that could further explain the observed changes in protein turnover. It seems pertinent to understand the possible role of cell volume in regulating protein turnover, as it could possibly lead to the clinical application of hypo-osmotic infusion to deal with skeletal muscle atrophy associated with various pathological conditions.

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Appendix

Method Protocols:

Protein Synthesis In-vitro:

Day 1:

Incubation Preparation:

*Can be made during first or second incubation

Reagents:

Make up 0.61mol/L TCA acid = 4.983g/50ml of dH_2O

Make up 1mol/L NaOH = 1.999g/50ml of dH_2O

1. Prepare your incubating medium (Sigma, KHB etc) and obtain wanted osmolality and pH values and store 2 ml of media for scintillation counting standard (represents 0.5mmol/kg phenylalanine).
2. Want 0.5mmol/L of label into incubation baths (1.5uL/15ml bath).

Day 2:

1. Post incubation the muscle is blotted dry and frozen in liquid nitrogen, then weighed.
2. Next the muscle is homogenized in 0.61mol/L trichoroacetic acid (TCA).
   - Add 100ul of TCA to chilled glass homogenizer.
   - Add 10ul/mg of tissue muscle weight with muscle and homogenize.
3. Then centrifuge at 4 degrees Celsius @ 10,000 g for 10 minutes.
4. Use the TCA acid to wash the supernatant 2X (250ul) from the pellet to eliminate non-bound radioactivity (store this supernatant to analyze free

phenylalanine/amino acid concentration in muscle [*multiply by 2 to account for dilution]).

5. Re-suspend (take your time here to break up all large pieces) the pellet in 1 mol/L NaOH (500uL) by heating at 45-50 degrees Celsius for 15 minutes and use this to measure protein-bound radioactivity by scintillation counting.
6. Load 400ul of sample and medium then top up with 7.6ml of scintisafe fluid so the vials are filled to the top. Vortex gently to mix the sample and scinti-safe fluid.
7. Calculate protein synthesis by dividing the values obtained in muscle by the free phenylalanine reading in the medium and then multiplying by 0.5umol/ml to obtain nmol phenylalanine incorporated/mg muscle tissue/75 minutes.

Labelling: Condition, D, Incorporated (I) vs. Non-incorporated (N) for scintillation vials

Protein Degradation (Media Tyrosine Assay) In-vitro:

1. Make up 0.1% nitroso-napthol solution (20 mg 1-nitroso-2-naphthol into 20mL methanol). Mix thoroughly.
2. Make up 2.5% sodium nitrite solution (25mg sodium nitrite into 1mL of water). Vortex.
3. Make up 1:5 nitric acid solution (5mL nitric acid into 25mL water). Mix thoroughly.
4. Combine 24.5mL nitric acid solution with 0.5mL of sodium nitrite solution. Mix thoroughly.
5. Add 1 mL of nitroso-naphthol solution and 1mL of nitric acid-sodium nitrite solution to all samples. Mix thoroughly.
6. Place samples in water bath of 65°C for 30 minutes. Allow samples to cool post-bath.
7. After cooling, add 10mL of ethylene dichloride to each sample. Shake samples.
8. Centrifuge samples 2000rpm at 4°C for 10 min.
9. Transfer aqueous supernatant layer of samples to a well-plate for fluorometric reading (200ul).
10. Open KC4. Conditions:
    - End-point
    - Fluorescence
    - Read samples from both top and bottom
    - Activation: 485 Emission: 590
    - Sensitivity: 35 from bottom. 44 from top

11. *Create a standard curve*. Run fluorescence readings against the curve.

Western Blotting Protocol:

Sample Preparation

Homogenization

1. Make homogenization buffer (can store at 4 C)
    a. 250mM sucrose = 21.4g
    b. 100mM KCL = 1.865g
    c. 5mM EDTA = 0.4695g

Add 225ml dH_2O, fix pH to 6.8, then top up to 250ml with dH_2O (stored in fridge)

2. Using homogenization buffer – add 1 tablet of both protease inhibitor and phosphatise inhibitor to 10ml of the solution.
3. Break muscle up into small segments if possible and follow chart below for the buffer to weight ratio.

Muscle Weights	***Homogenization buffer added***
50mg or less	700ul
60mg	850ul
70mg	1000ul
80mg	1150ul*
90mg	1300ul*
100mg	1450ul*
110mg	1600ul*
120mg	1750ul*
130mg	1900ul*

*For this muscle size use larger glass homogenizer's to avoid loss of buffer/break muscle up if possible before use, it will homogenize quicker/more evenly

4. Homogenize well on ice, 5 minutes etc until only tendons are left

Now either store the homogenate (freeze in liquid N2 and put in freezer) or perform the protein content assay

Protein Content Determination (Bradford Assay)

1. Make up 1mg/ml BSA standard or get from left -20 C fridge/freezer
2. Make up standard curve:

Final Protein Concentration (mg/ml)	***Volume of BSA (uL)***	***Volume of dH_2O (uL)***
0.5	25	25
0.4	20	30
0.3	15	35
0.2	10	40
0.1	5	45
0.05	2.5	47.5
0	0	50

*Turn on flourometer here as it requires a few minutes to warm-up prior to use

Dilute the muscle sample 10X – 10ul of sample and 90ul of dH_2O

Vortex the BSA standards and samples and load 10ul of each in triplicate into the 96 well plates.

3. Use the multi-channel pipette to add 200ul of diluted reagent (Bio-Rad protein assay Dye reagent in 1:4 dilution with dH_2O

4. Open the KC4 program on the computer and:
    a. Click Wizard, then reading parameters
    b. Click end-point assay, and make sure absorbance is selected
    c. Wavelength = 595, Intensity = 3 and Duration = 5
    d. Click OK, and select Read
    e. Copy results and paste into excel (use paste options in excel to text to column by the semicolon)

Sample Loading Preparation/Dilution

Decide on your total volume (dependant on lanes loaded X load/lane etc)

1. Muscle sample = 1/[protein] X total volume
2. Laemelli/sample buffer = total volume/sample buffer concentration (usually 3X)
3. Water = total volume – sample added – laemelli buffer added

SDS PAGE Procedure:

Electrophoresis Preparation:

1. Prior to using glass plates, wipe dry with methanol and a kimwipe, then wet sides to assist in creating a seal.
2. Level and press down when locking into the gel loading docks (green holders)
3. Prepare Gels:

Percent Gel	**dH_2O (ml)**	**Protogel (ml) (30% acrylamide)**	**Gel Buffer (ml) (in fridge, R & S)**
4 (Stacking)	***6.1***	***1.3***	***2.5***
6	5.3	2.0	2.5
8	4.62	2.67	2.5

7.5 (Running)	***4.85***	***2.5***	***2.5***
10	3.96	3.33	2.5
12	3.29	4.0	2.5
15	2.29	5.0	2.5

4. Before pouring the running gel, add 100ul APS (60mg/600ul) and 10ul TEMED. Swirl and pour until reaching depth of the comb. Use methanol to remove bubbles.
5. Before pouring the stacking gel, add 100ul APS and 20ul TEMED. Swirl and pour until top is reached, then immediately place in combs (after hardening rinse wells with water)
6. Place the gel cassettes into the electrical assembly with short place facing inward.
7. Boil prepared samples for 5 min, then ice for 5 minutes while preparing the running buffer (50ml 10X running buffer + 450ml dH_2O)
8. Fill the inner chamber of the apparatus all the way to the top of the glass plates, then add 200ml to surrounding space *save some in case of slow leakage in set up.
9. Load the standard (5-10ul) and the appropriate amount of sample to each well (this will have been optimized).
10. Run electrophoresis at 120V for 90 minutes or until the blue dye begins to approach the bottom of the gel. Prepare transfer buffer

Transfer:

1. Remove the gels carefully and cut off stacking, allow to equilibrate in plastic dish with transfer buffer
2. Create the transfer sandwich:
    a. Clear side of plastic holder
    b. Sponge/fibre pad
    c. 2 pieces of filter paper
    d. Membrane (PVDF[open pores in methanol] or nitrocellulose)
    e. Gel
    f. 2 pieces of filter paper
    g. Sponge/fibre pad
    h. Black side of plastic holder

*Be sure to roll out any air bubbles, close the cassette firmly, and place the black side of the cassette to the black side of the holding apparatus.

3. Fill the tank with the transfer buffer and the container around with plenty of ice/water to avoid cooking the gels.
4. Run the transfer at 100V for 60 minutes.

Antibodies

1. Discard the gel and filter papers and transfer membrane to small dishes
2. Block the membrane with 20ml TBST-5% skim milk solution (4.5g into 90ml TBST) for 1 hour
3. Add in primary antibody into milk-TBST solution at the appropriate dilution and incubate overnight

4. Wash 3 x 5 minutes in TBST
5. Add secondary antibody (dependent on primary species) in TBST-milk solution for 1 hour at room temperature with the appropriate dilution

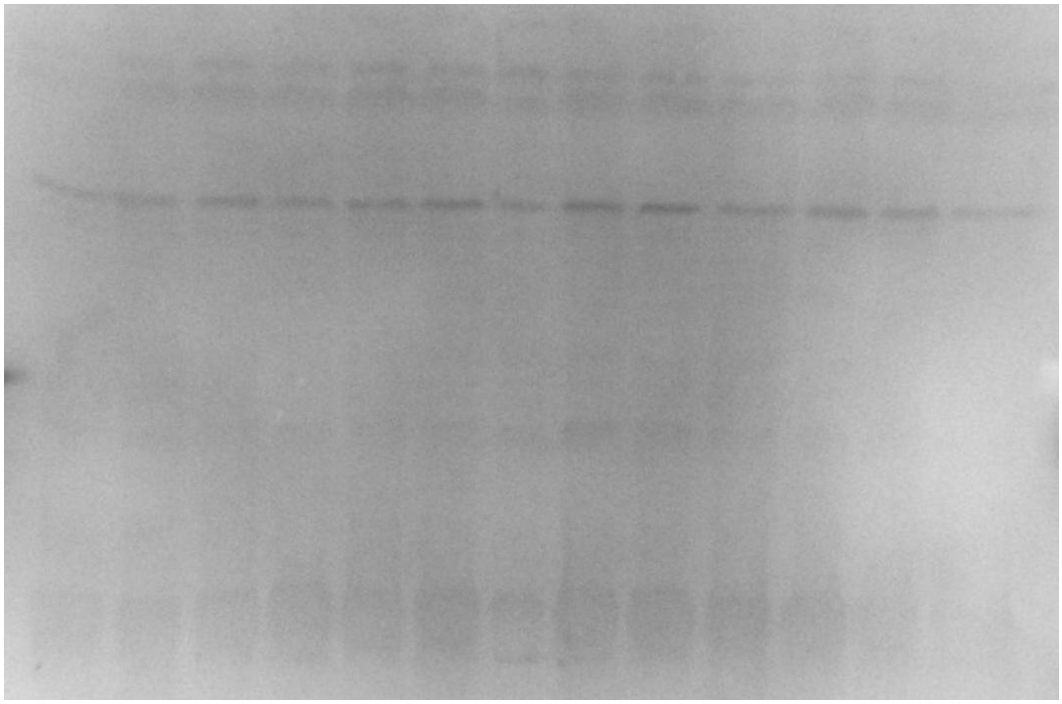
Secondary	Dilution	Amount (ul in 10ml)
Anti-mouse	1:5000	2
Anti-goat	1:20000	0.5
Anti-rabbit	1:10000 *Using(1:6000)	1*Using 3ul into 18ml

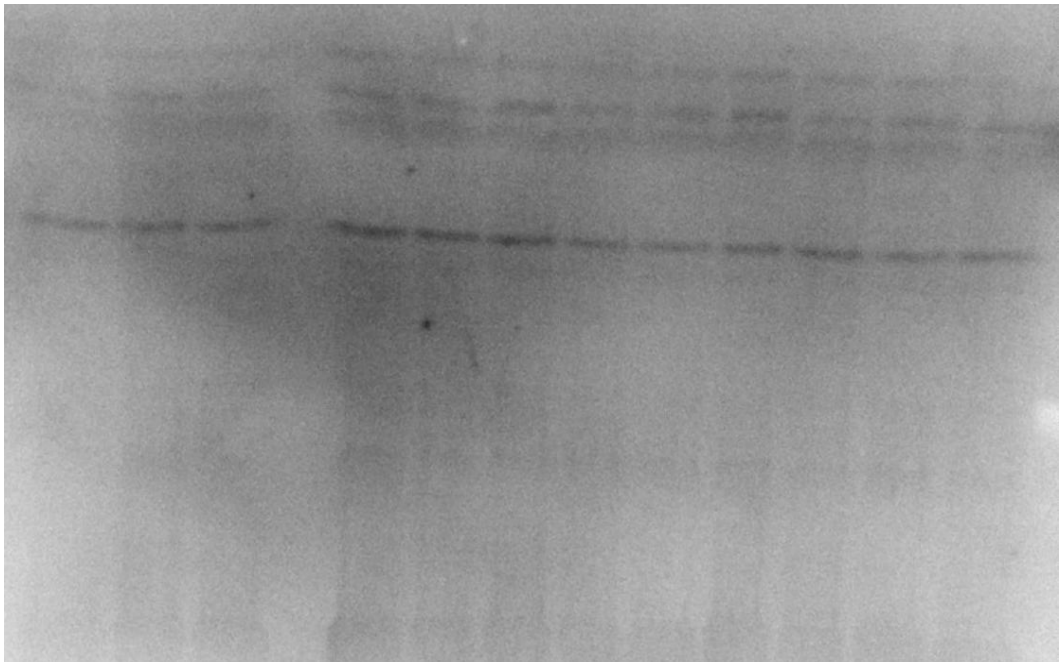
Enhanced Chemiluminesence:

1. Wash 3 x 5 mins in TBST
2. Combine 1ml of each chemiluminesence HPR substrate (peroxide solution + luminal reagent) and pipette over membrane for 5 mins
3. Place membrane protein side down onto thin glass plate and cover back with wax paper
4. Roll out any bubbles, and take a photo with the door open in image J and use the needle tip as a reference to where your protein should fall on the membrane. Take the second photo door closed with the appropriate exposure time and analyze using Image J software.

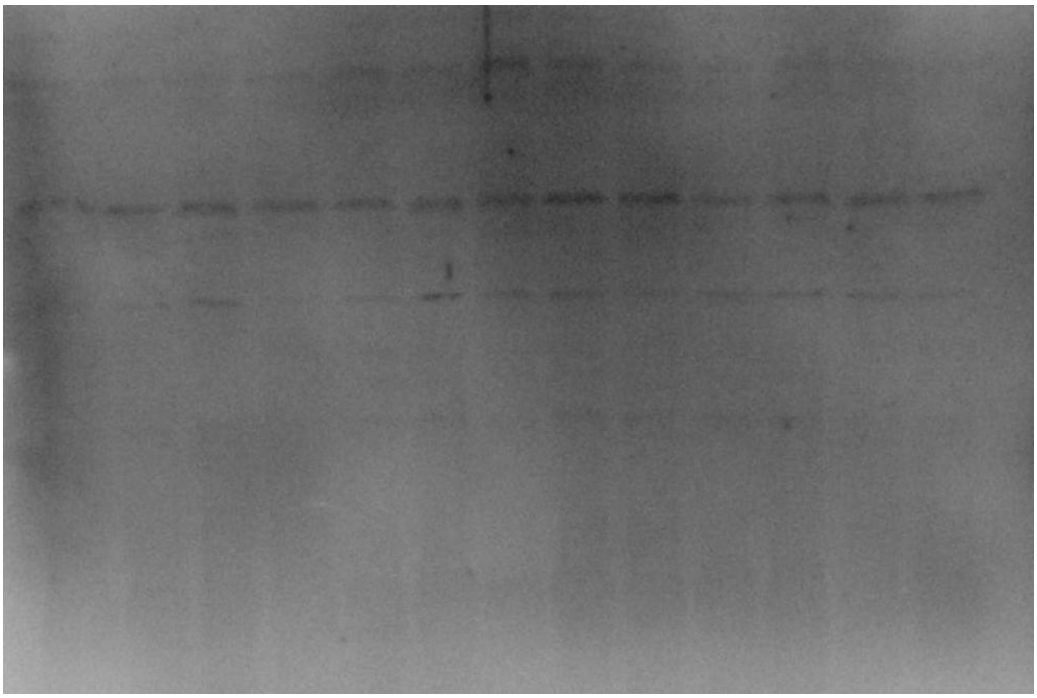
Western Blotting Images

1) Total mTOR (Samples are loaded HYPO, ISO, HYPER)

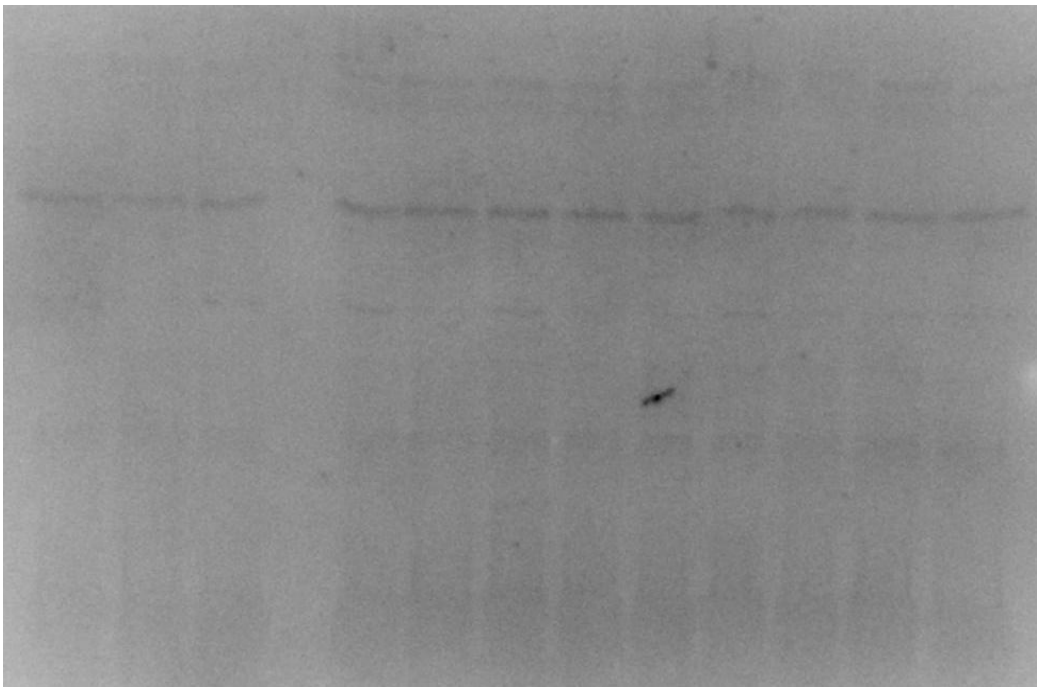




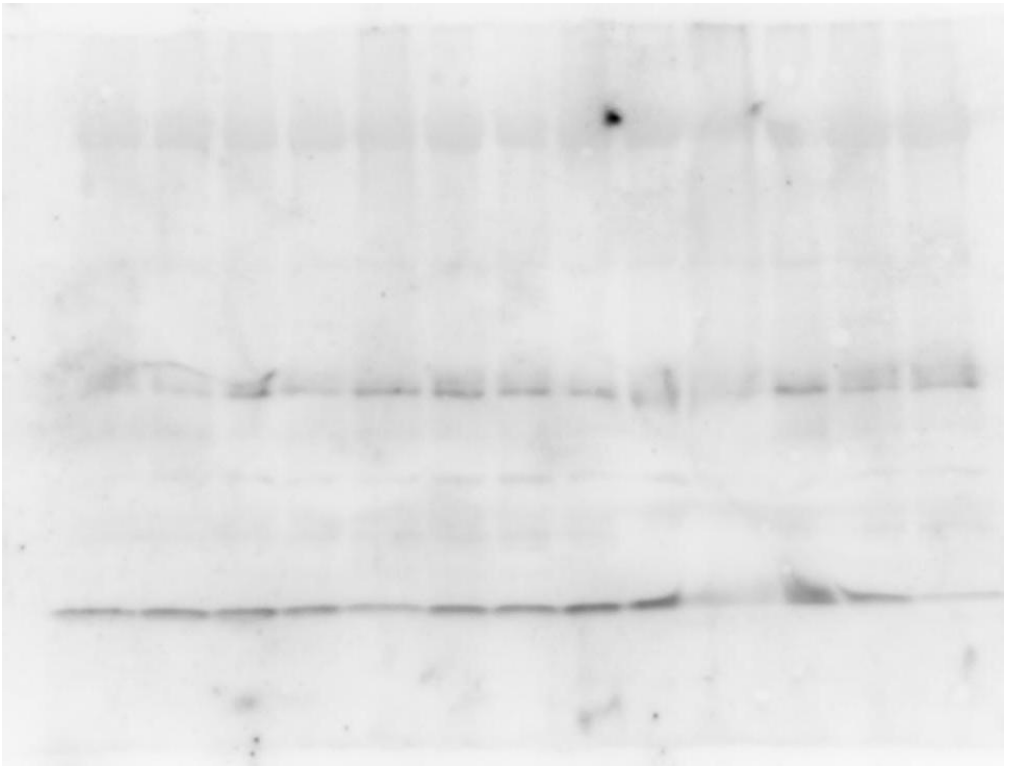
2) Phospho-mTOR (Samples are loaded HYPO, ISO, HYPER)

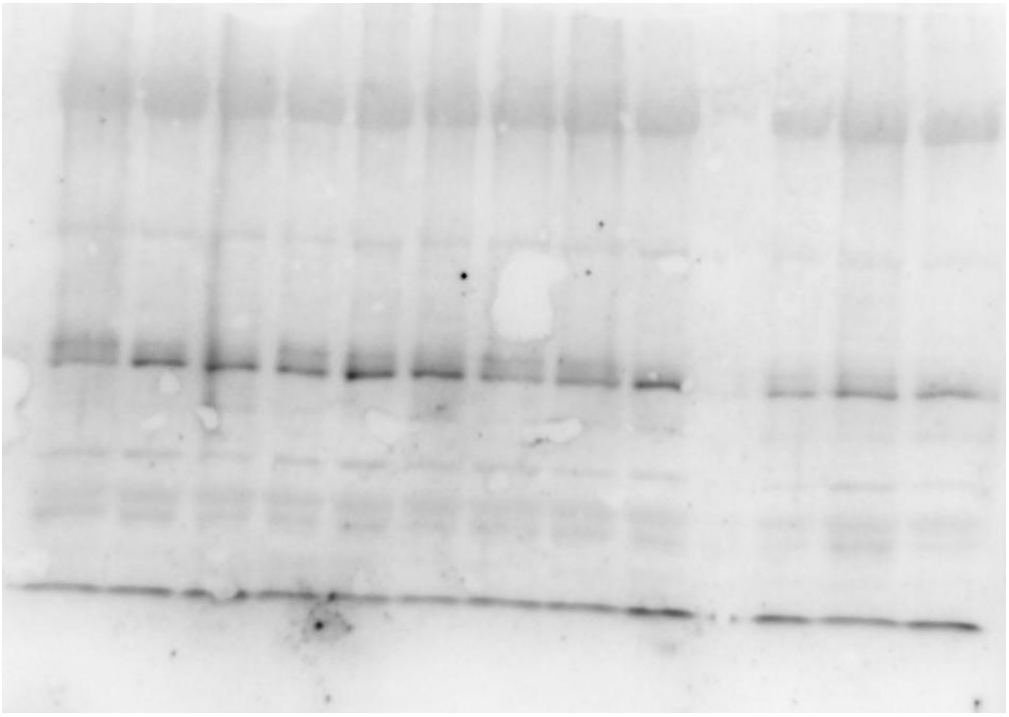


Samples are loaded (HYPER, ISO, HYPO)

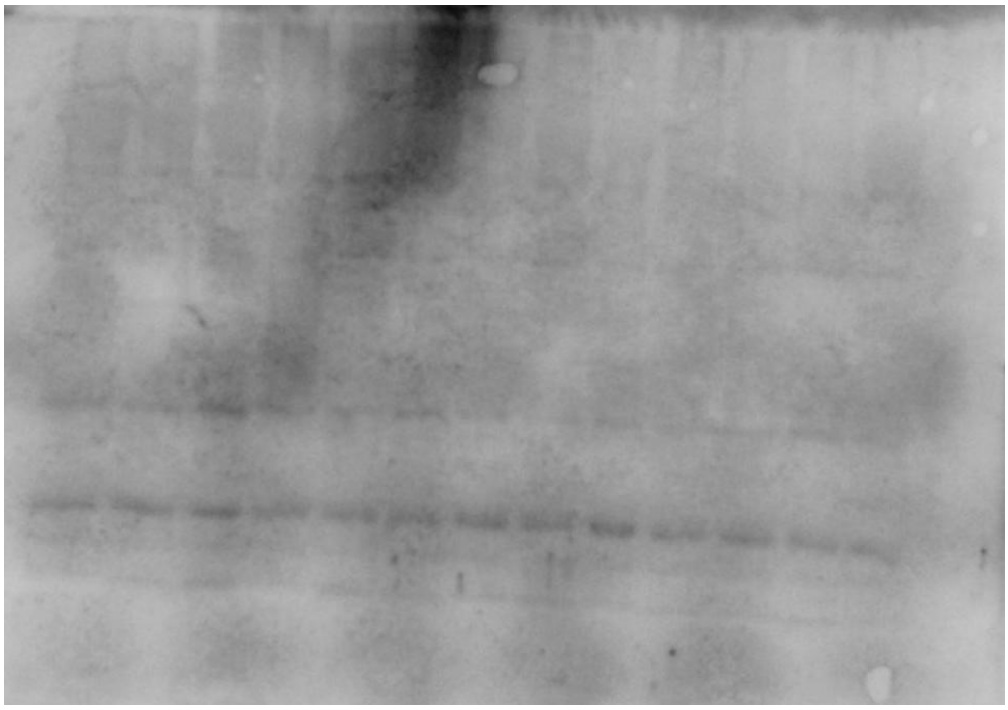


3) Total p70s6 kinase (Samples are loaded HYPO, ISO, HYPER)

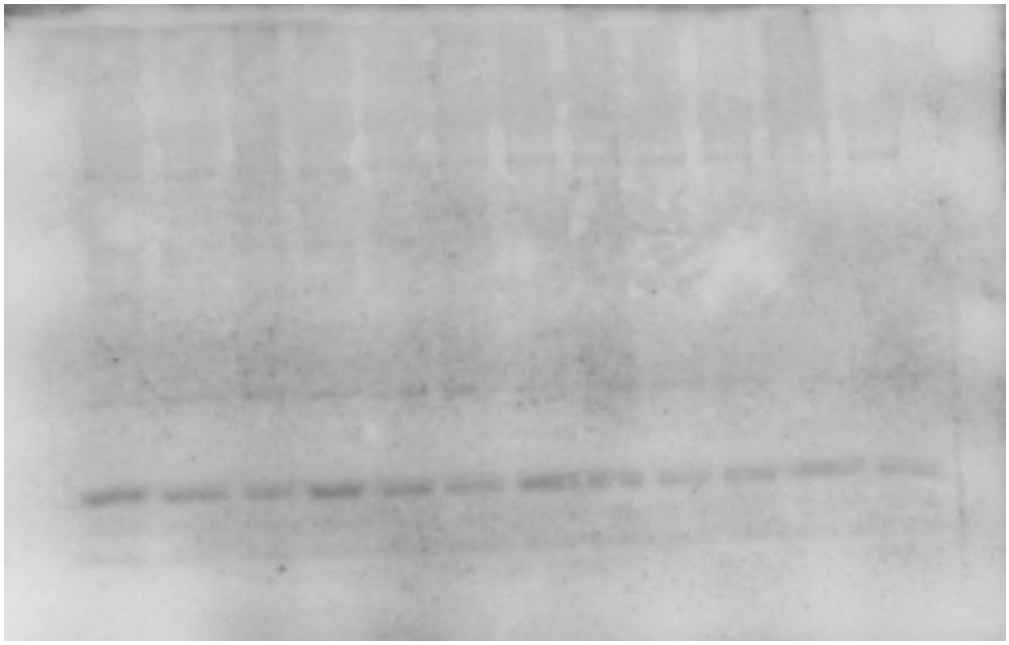




4) Phospho-p70s6 kinase (Samples are loaded HYPO, ISO, HYPER)

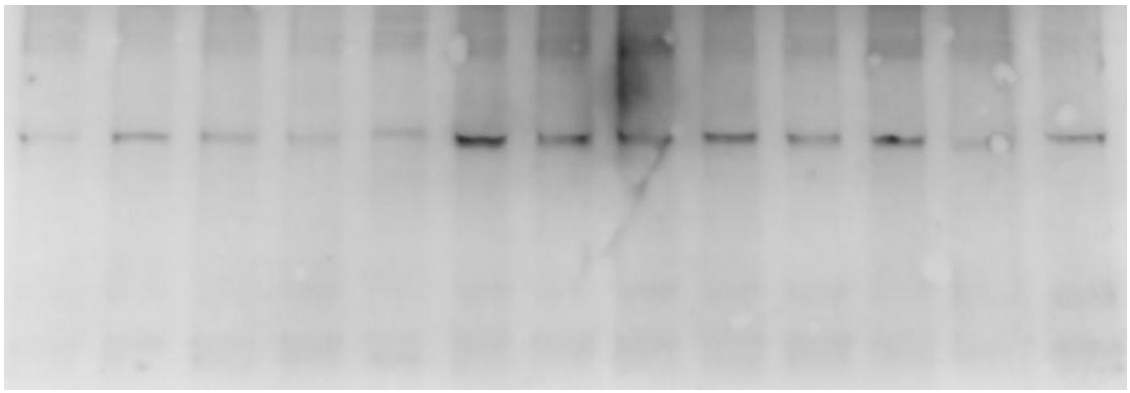


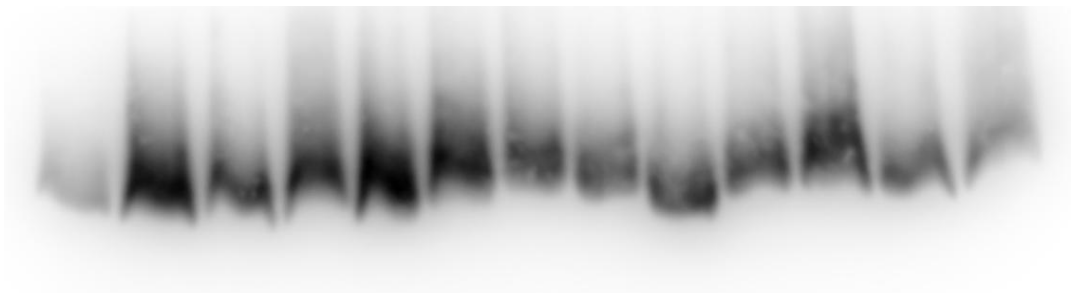
Sample loading same as above



5) Ubiquitin Conjugates and Actin

Samples loaded (HYPO, ISO, HYPER)





5) μ-Calpain (Samples loaded HYPO, ISO, HYPER)

Chemical Reagents:

Homogenization Buffer

- 250mM sucrose (21.4g)
- 100mM KCL (1.865g)
- 5mM EDTA (0.4695g)
- Add 225 ml dH_2O, fix pH to 6.8, then top up to 250ml with water

Laemelli Buffer (2 x sample buffer)

- 2.5ml 0.5M Tris-HCL ph 6.8
- 2ml glycerol
- 2ml 10% SDS
- 1 ml Beta-mercaptoethanol
- 0.3ml dH_2O

Running Gel Buffer

- 1.5M Tris-HCL, ph 6.8

Stacking Gel Buffer

- 0.5M Tris-HCL, ph 6.8

10x Running Buffer, ph 8.3

- 250mM Tris base
- 1.92M Glycine
- 1% SDS
- Store at 4 C. If precipitation occurs, warm to room temp before use

10x Transfer Buffer (semi-dry)

- 312.5mM Tris base (3.786g)

- 2.4M glycine (18g)
- 12.5g/L SDS (1.25g)
- Fill to 100ml with dH_2O
- Before use, dilute 80ml into 720ml of water and add 200ml methanol

10x TBS

- 200mM Tris base
- 1.37M NaCl
- 38ml of 1M HCl/L of TBS made
- Adjust ph to 7.5

TBST

- Dilute 100ml of 10x TBS in 900ml dH_2O
- Add 4ml of 25% Tween 20 while stirring (polyoxyethylenesorbitan monolaurate)

Protein Specific Protocols:

Ubiquitin-protein conjugates (Enzo sciences: BML-UG9510)

Storage: @ -20 degree freezer.

Running gel pore size: 7.5%

Loading: 10ug protein

Electrophoreisis: 75mins @ 100V

Membrane: PVDF

Transfer: 75 mins @ 100V

Blocking: 60 mins @ room temp. In 5% milk-TBST

Primary Ab: 1:2000 in 5% milk-TBST in fridge overnight

Wash: 3 x 5 mins in TBST

Secondary Ab: 60 mins, 1:6000 in 5% milk-TBST @ room temp.

Wash: 3 x 5 mins

ECL

Total p70 S6 Kinase (Santa Cruz Biotechnology) 70 kDa

Storage: @ 4 degrees *DO NOT FREEZE

Running gel pore size: 7.5%

Loading: 10ug

Electrophoreisis: 90 mins @ 100V

Membrane: PVDF

Transfer: 90 mins @ 100V

Blocking: 60 mins in 2.5% milk-TBST @ room temp.

Wash: 3 x 5 mins in TBST

Primary Ab: 1:1000 in 5% milk-TBST in fridge overnight

Wash: 3 x 5 mins

Secondary Ab: 60 mins in 1:6000 in 5% BSA-TBST @ room temp.

Wash: 3 x 5 mins

ECL

Phospho-p70 S6 Kinase (Thr389) (Santa Cruz Biotech: sc-11759)70kDa

Storage: @ 4 degrees *DO NOT FREEZE

Running gel pore size: 7.5%

Loading: 10ug protein

Electrophoreisis: 90 mins @ 100V

Membrane: PVDF

Transfer: 90 mins @ 100V

Blocking: 60 mins @ room temp. in 2.5% milk-TBST

Wash: 3 x 5 mins in TBST

Primary Ab: 1:1000 in 5% milk-TBST in fridge overnight

Wash: 3 x 5 mins in TBST

Secondary Ab: 60 mins, 1:6000 in 5% BSA-TBST @ room temp.

Wash: 3 x 5 mins

ECL

mTOR (Cell Signalling Technology: #2972) 289 kDa

Storage: @ -20 freezer.

Running gel pore size: 7.5%

Loading: 20ug

Electrophoreisis: 120 mins @ 120V

Membrane: PVDF

Transfer: 120 mins @ 100V *Put lots of ice and stir bar to offset heat

Blocking: 120 mins @ room temp. in 1% milk-TBST

Primary Ab: 1:1000 in 1% milk-TBST in fridge overnight

Wash: 3 x 5 mins in TBST

Secondary Ab: 120 mins @ 1:3000 @ room temp. in 1% milk-TBST

Wash: 3 x 5 mins

ECL

Phospho-mTOR (Cell Signalling Technology: #2971)

Storage: @ -20 freezer.

Running gel pore size: 7.5%

Loading: 20ug

Electrophoreisis: 120 mins @ 120V

Membrane: PVDF

Transfer: 120 mins @ 100V *Put lots of ice and stir bar to offset heat

Blocking: 120 mins @ room temp. in 1% milk-TBST

Primary Ab: 1:1000 in 1% milk-TBST in fridge overnight

Wash: 3 x 5 mins in TBST

Secondary Ab: 120 mins @ 1:3000 @ room temp.

Wash: 3 x 5mins

ECL

Calpain 1 large subunit (u-type) (Cell Signalling Technology: #2556) 80, 78, 76 kDa

Storage: @ -20 in freezer.

Running gel pore size: 7.5%

Loading: 10ug

Electrophoreisis: 15 mins @ 100V then 60 mins @ 160V

Membrane: NC

Transfer: 90 mins @ 100V *No SDS in transfer buffer, ph to 8.3

Blocking: 60 mins in 5% milk-TBST at room temp.

Wash: 3 x 5 mins

Primary Ab: 1:1000 in 1% BSA in PBST in fridge overnight

Wash: 5 x 5 mins in TBST

Secondary Ab: 60 mins in 1:5000 in 5% milk-TBST @ room temp.

Wash: 5 x 5 mins in TBST

ECL

Metabolites

Metabolite Extraction:

Because metabolites within skeletal muscle are highly reactive, sample preparation for their investigation is the key step in the process. The time between excision and removal of the muscle from blood flow to the time that enzyme activity is stopped by flash freezing in liquid nitrogen is usually where the greatest variability can be introduced. Therefore, extremely rapid freezing post excision is required to get a true representation of muscle metabolites in your muscle of interest. Because enzymes and proteins can cause changes in the quantities of metabolites (ATP, PCr), they are most commonly assayed in protein free extracts prepared with perchloric acid ($HClO_4$). This acid is preferred as after extraction is finished most of it can be removed as a precipitated potassium salt. All of the following analyses are performed on freeze-dried tissue, this assures differences in tissue water content and continued oxidation processes are not occurring as you continue to prepare the samples for extraction.

Metabolite assays are performed with protein-free extractions prepared with percholoric acid ($HClO_4$). $HClO_4$ is preferred because in combination with $KHCO_3$, most of it can be removed as a potassium salt through centrifugation.

I. Reagents

0.5M PCA	21.5mL of 70% PCA
	Bring to 500mL with dH_2O; Store in 0-4°C for 1 month
2.3 M $KHCO_3$	2.3g $KHCO_3$
	Add 10.0mL dH_2O; Make Fresh Daily

II. Extraction Procedure

1. Freeze dry tissue (~24 hours to ensure all water is removed)
2. Store with dry rite in freezer until powdering
3. Tease out connective tissue and powder
4. Place in pre-weighed microcentrifuge tube and weight (3-5mg)
5. Place tubes in an ice-bucket (make sure tubes remain cold)
6. Add 600uL of pre-cooled 0.5M PCA
7. Extract for 10 minutes, vortexing several times
8. Centrifuge for 10 minutes at 15,000G (spinning helps remove some of the enzymes that can influence concentration)
9. Remove 540uL and place in freezer (-20°C) for 10 minutes
10. To the frozen supernatant add 135uL of 2.3M $KHCO_3$ and vortex until liquid (addition of $KHCO_3$ to a frozen supernatant prevents foaming over)
11. Centrifuge for 10 minutes, 0°C at 15,000G. Remove supernatant to assay metabolites.
12. **Assay Procedure** *(Note: run everything in triplicate)*
13. Prepare substrates (reagents 1-5 in section IV) without G-6-P-DH enzyme and bring to volume
14. Prepare ATP (Solid in -20) and PCr (aliquots found in -80) standards
15. Vortex each concentration before pipetting
    a. Fill the next 5 wells with 10.00µL of varying concentrations of ATP
16. Vortex each concentration before pipetting
    a. Fill the next 5 wells with 10.00µL of varying concentrations of PCr

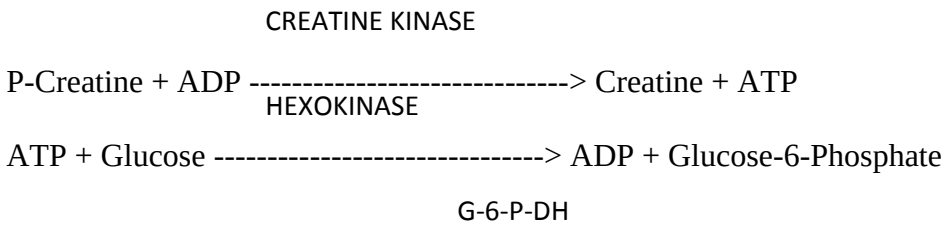
17. Add G-6-P-DH enzyme to substrates and add 185µL of this buffer (pH 8.1) to each well
18. Incubate in a dark environment for 20 minutes, set up the fluorometer and read at 25 minutes

Part 2:

19. Mix 2.5µL of Hexokinase (HK) with 1mL of buffer. Mix by inversion.
20. Add 6µL of dilute HK to all of the wells
21. Incubate in a dark environment for 80 Minutes
22. Read the plate (excitation setting 340, emission setting 460)
23. R2-R1 = reflects [ATP] in extract

Part 3:

24. Mix ~1.5mg of phosphocreatine kinase and 5mg of ADP into 5mL of buffer. Mix by inversion
25. Add 6µL of dilute Creatine Kinase to all the wells
26. Place in a dark environment for 120 Minutes
27. Read the plate (excitation setting 340, emission setting 460)
28. R3-R2 = reflects [PCr] in extract
29. **Muscle Adenosine Triphosphate (ATP) and Phosphocreatine (PCr) Assay for Plate Reader Flourometer**



Glucose-6-Phosphate + NADP ----------> Glucanolactone + NADPH

Reagent	[Stock]	[Final]	Volume – 25mL	Volume – 50 mL	Volume – 100 mL
1. Tris (on shelf; pH 8.1) *stored in fridge*	1.00M	50mM	1.25mL	2.5mL	5.00mL
2. $MgCl^2$ (on shelf; fresh) (.2033g/mL)	1.00M	1.0mM	25.00µL	50.00µL	100.00µL
3. D.T.T. (found in -20) *aliquots -80*	0.5M	0.5mM	25.00µL	50.00µL	100.00µL
4. Glucose (on shelf) *aliquots -80*	100.0 mM	100.0 µM	25.00µL	50.00µL	100.00µL
5. NADP (found in -20) *aliquots -80*	50.0 mM	50.0 µM	25.00µL	50.00µL	100.00µL
6. G-6-P-DH (liquid found in +4) (Sigma G-5760)	2660 U/mL	0.02 U/mL	1.00 µL	2.00 µL	4.00 µL
7. ADP (found in -20) (Sigma A-2754)	Solid – Refer to Section V				
8. Creatine Kinase (found in -20) (Sigma C-3755)	324 U/mL				

Note: Mix reagents 1-5 together. Bring to volume (23.649mL for 25mL) with distilled water and **adjust** pH to 8.1. Then add reagent 6. Mix by inversion with enzymes.

V. Standard Curves

ATP (Sigma A-7699)

Make fresh 5.51mg in 5 mL dH_2O

Concentration (mM)	**Stock (µL)**	**dH_2O (µL)**
0.05	25	975
0.1	50	950
0.2	100	900
0.3	150	850
0.4	200	800

Phosphocreatine (Sigma P-7936)

Stored in 5mM aliquots in the -80°C; (1.2755 g/L)

Concentration (mM)	**Stock (µL)**	**dH_2O (µL)**
0.1	20	980
0.2	40	960
0.4	80	920
0.8	160	840
1.0	200	800

Modified Medium M199 – M4530 (Gibco)

Component	*M4530 (1X)* g/L
Inorganic Salts	
CaCl2 • 2H2	0.2
Fe(NO3)3 • 9H2	0.00072
$MgSO_4$ (anhydrous)	0.09767
KCl	0.4
Na • Acetate (anhydrous)	0.05
$NaHCO_3$	2.2
NaCl	6.8
NaH_2PO_4 (anhydrous)	0.122
Amino Acids	
L-Alanine	0.025
L-Arginine • HCl	0.07
L-Aspartic Acid	0.03
L-Cystine • HCl • H_2O	0.00011
L-Cysteine • 2HCl	0.026
L-Glutamic Acid	0.0668
L-Glutamine	0.1
Glycine	0.05
L-Histidine • HCl • H_2O	0.02188
Hydroxy-L-Proline	0.01
L-Isoleucine	0.02
L-Leucine	0.06
L-Lysine • HCl	0.07
L-Methionine	0.015
L-Phenylalanine	0.025
L-Proline	0.04
L-Serine	0.025
L-Threonine	0.03
L-Tryptophan	0.01
L-Valine	0.025
Vitamins	
Ascorbic Acid • Na	0.0000566
D-Biotin	0.00001
Calciferol	0.0001
Choline Chloride	0.0005
Folic Acid	0.00001
Menadione (sodium bisulphate)	0.000016
Myo-Inositol	0.0005
Niacinamide	0.000025
Nicotinic Acid	0.000025
p-Amino Benzoic Acid	0.00005

D-Pantothenic Acid • ½Ca	0.00001
Pyridoxal • HCl	0.000025
Pyridoxine • HCl	0.000025
Retinol Acetate	0.00014
Riboflavin	0.00001
DL-alpha-Tocopherol Phosphate • Na	0.00001
Thiamine • HCl	0.00001
Other	
Adenine Sulfate	0.01
Adenosine Triphosphate • 2Na	0.001
Adenosine Monophosphate • Na	0.0002385
Cholesterol	0.0002
Deoxyribose	0.0005
Glucose	1.0
Glutathione (reduced)	0.00005
Guanine • HCl	0.0003
Hypoxanthine	0.0003
TWEEN 80	0.02
Ribose	0.0005
Thymine	0.0003
Uracil	0.0003
Xanthine • Na	0.000344