INFLUENCE OF INCREASED EXTRACELLULAR LEUCINE ON THE PROTEIN METABOLIC RESPONSES DURING OSMOTIC STRESS IN SKELETAL MUSCLE

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ABSTRACT

The purpose of this study was to examine the effects of increased extracellular leucine concentration on protein metabolism in skeletal muscle cells when exposed to 3 different osmotic stresses. L6 skeletal muscle cells were incubated in either a normal or supplemental leucine (1.5mM) medium set to hypo-osmotic (230 ± 10 Osm), iso-osmotic (330 ± 10 Osm) or hyper-osmotic (440 ± 10 Osm) conditions. 3H-tyrosine was used to quantify protein synthesis. Western blotting analysis was performed to determine the activation of mTOR, p70S6k, ubiquitin, actin, and µ-calpain. Hypo-osmotic stress resulted in the greatest increase in protein synthesis rate under the normal-leucine condition while iso-osmotic stress has the greatest increase under the elevated-leucine condition. Elevated-leucine condition had a decreased rate in protein degradation over the normal condition within the ubiquitin proteasome pathway (p<0.05). Leucine and hypo-osmotic stress therefore creates a favourable environment for anabolic events to occur.

Keywords: Protein Synthesis, Protein Degradation, Leucine, Osmolality, Skeletal Muscle
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List of Abbreviations

AA – Amino Acid
Akt – Protein Kinase B
AQP4 – Aquaporin 4
BCAA – Branched Chain Amino Acids
BCAT – Branched Chain Aminotransferase
BCKA – Branched Chain Keto Acids
BCKDH – Branched Chain Keto Acid Dehydrogenase
BSA – Bovine Serum Albumin
Ca^{2+} - Calcium
Cl^- - Chloride
CON – Control Condition
CV – Cell Volume
DHPR - dihydropyridine receptors
dH2O – distilled water
EMEM – Eagle’s Modified Media
ESS – Essential Amino Acid
FBS – Fetal Bovine Serum
H^+ - Hydrogen Ion
HCl – Hydrocholic Acid
HYPER – Hyper-Osmolality
HYPO – Hypo-Osmolality
INS – Insulin
ISO – Isotonic Osmolality

K⁺ - Potassium

KCC – K⁺-Cl⁻ Cotransporter

mRNA – messenger RNA

mTOR – Mammalian Target of Rapamyacin

Na⁺ - Sodium

NaOH – Sodium Hydroxide

NHE – Sodium/Hydrogen Exchanger

NKCC – Na⁺-K⁻-2Cl⁻ Cotransporter

NMPB - Net Metabolic Protein Balance

PBS – Phosphate Buffer Saline

PD – Protein Degradation

PVDF – Polyvinylidene Fluoride Membrane

PS – Protein Synthesis

SDS – Sodium Dodecyl Sulfate

RNA – Ribonucleic Acid

RVD – Regulatory Volume Decrease

RVI – Regulatory Volume Increase

TBST – Tris-Buffered Saline

TCA – Trichloroacetic Acid
Chapter 1: Water as a medium

1.1.2. Introduction

Human skeletal muscle comprises upwards of 30-40% of an individual’s total body mass (Usher-Smith, Huang, & Fraser, 2009), and is often thought of as being primarily important for locomotion. However, skeletal muscle tissue also represents a critical fluid reservoir for humans and other mammals. In humans tissue contains ~50% of total body water content, and as such represents the largest reservoir of intracellular fluid (~75%) and interstitial fluid (~33%) within the human body compared to all other tissues (Frigeri, Nicchia, Balena, Nico, & Svelto, 2004; Hamilton, Ward, & Watson, 1993; Usher-Smith et al., 2009). Minor changes in the osmotic state of the extracellular fluid can lead to significant alterations in intracellular compartmental volumes, especially muscle cell volume (CV) (Sjogaard & Saltin, 1982). Extracellular hyper-osmolality results in an acute decrease in muscle CV, which appears to contribute to protein catabolism, while extracellular hypo-osmolality leads to an increase in CV, and consequently protein anabolism (Darling, 2011; Lang et al., 1998; Stoll, Gerok, Lang, & Haussinger, 1992; Vandommele, 2012). Previous work from our lab using an isolated rat skeletal muscle model has demonstrated that such acute changes in the cellular osmotic state lead to catabolic and anabolic conditions in extensor digitorum longus muscle (Darling, 2011; Vandommele, 2012). We have subsequently observed similar trends with a cell culture model using L-6 muscle cells (Roy, 2009).

Within the skeletal muscle system, there are highly regulated mechanisms that control protein metabolism. More specifically, protein synthesis (PS) and protein degradation (PD) collectively work to maintain the net protein balance. In general, PS
and PD are tightly regulated however, under certain conditions where PD becomes more active than PS, muscular atrophy will occur if the net balance is not returned to a neutral or more positive state. Muscle atrophy or muscle wasting is a common characteristic of many conditions and is termed sarcopenia. Apoptosis as a result of PD has been considered to contribute to sarcopenia in the older population (Dirks & Leeuwenburgh, 2002). Sarcopenia is often further observed with diseases such as cancer (cachexia), severe burns, sepsis, chronic kidney disease, heart failure, and can occur during aging (Evans, 1996; Gordon, Kelleher, & Kimball, 2013; Schiaffino & Mammucari, 2011). Interestingly, it has been observed that the basal rate of PS appears to be lower in many of these muscle wasting disorders (Little & Phillips, 2009). In an older population, muscular atrophy is partially due to an anabolic resistance to nutrients leading to the inability of skeletal muscle to maintain or increase protein mass despite adequate nutrition (Nicastro et al., 2012). In particular a decrease in muscle mass is partially a result of an increase in amino acid (AA) resistance within skeletal muscle, causing a reduction in overall net PS (Nicastro et al., 2012). It should further be noted that some of the conditions previously listed could lead to significant physical inactivity, which can also be another major contributor to increased muscle wasting. Although it may appear that we should solely focus on promoting PS to achieve muscle hypertrophy, net protein balance is a function of both synthesis and degradation. As discussed in the Literature Review, attenuating PD is very important in attempting to maintain or increase muscle mass.

One critical factor that appears to dramatically influence protein metabolism in skeletal muscle is AA availability. Under most conditions, increased availability of AAs
can promote PS, while decreased availability supresses PS (Drummond et al., 2011; Wolfe, 2002). The type of available AAs has additionally shown to influence PS responses in skeletal muscle (Anthony et al., 2000; Liu et al., 2001; K. Smith, Reynolds, Downie, Patel, & Rennie, 1998). More specifically, increased availability of branched-chain amino acids (BCAA) (leucine, isoleucine, and valine) appears to have a stimulatory effect on skeletal muscle PS. Of the three BCAAs, leucine has demonstrated to have the greatest anabolic effect in promoting PS (Apro & Blomstrand, 2010; Bajotto, Sato, Kitaura, & Shimomura, 2011; Li, Yin, Tan, Kong, & Wu, 2011; Phillips, 2011; Stipanuk, 2007). The importance of the BCAAs will be discussed in detail in later sections of this literature review but, in general, the type of available AAs can directly influence protein metabolism within skeletal muscle cells. But, what remains unclear is if alterations in the intramuscular availability of AAs result from acute change in CV.

1.1.3. Osmolality and osmolarity

There are two primary terms to describe the amount of solutes dissolved within the fluid compartments in the body: osmolality and osmolarity. While these terms are often used interchangeably, the units and cofounding variables are what make them distinctly different. Osmolality is measured as solute osmotic concentration per mass (Osm/kg) and is the result of a total amount dissolved in a solvent, while osmolarity is osmotic concentration per unit of solvent (Osm/L) (Sareen S. Gropper, 2005). Osmolality is not affected by temperature, pressure or the addition of other molecules, making it a very reliable and consistent measuring technique. There are four different laboratory methods that can be utilized for determining osmolality: osmotic pressure, boiling point, freezing point, and vapour pressure. The most common technique used to measure osmolality is
the vapour pressure technique, which is known to be fast and reliable (Sweeney & Beuchat, 1993). This technique involves loading a sample into a sealed chamber. The temperature of the chamber then drops below the dew point allowing the formation of water droplets. The droplets are sensed by a thermocouple and the dew point of the solution is compared to a pure solvent dew point (Sweeney & Beuchat, 1993).

As stated previously, osmolarity is measured relative to the amount of fluid (Osm/L) and is highly affected by the environmental conditions (Sareen S. Gropper, 2005). Due to the greater variance in osmolarity, osmolality is more often the favoured terminology and technique, and will be referred to throughout this paper.

1.1.4. Movement of water throughout the body

The permeable nature and presence of specialized proteins contained within cell membranes allows for water to move relatively freely across membranes. This is vitally important to the physiological well-being of all cells (Conner et al., 2012). How water moves through different compartments of the human body is partially dependent on the specific cell type. There are three different ways in which water moves between body compartments: simple diffusion, co-transport, and facilitated diffusion via aquaporins (Borgnia, Nielsen, Engel, & Agre, 1999; Day et al., 2014). Simple diffusion is a relatively slow process allowing water to cross through a lipid bi-layer (Borgnia et al., 1999). At first, simple diffusion was thought to be the only method in which water moved across the membrane but it was later discovered to be untrue (Agre et al., 2002; Borgnia et al., 1999). Movement of water via the co-transport method involves water moving across the cell membrane with other ions or solutes (Loo, Zeuthen, Chandy, & Wright, 1996). While this mechanism is able to move large volumes of water, it does rely on other molecules.
Some examples of co-transporter systems include: the Na+/glucose transporter and the Na⁺-K⁺-2Cl⁻ cotransporter (Loo et al., 1996; Okada, 2004). The latter will be discussed in detail when we take into consideration different mechanisms that have been implicated in regulating skeletal muscle cell CV. The final mechanism listed that mediates water movement is the aquaporin channel, which consist of proteins and is known to be the fastest method for water diffusion (Agre et al., 2002; Borgnia et al., 1999). Due to the nature of aquaporins and the dominant presence within cells, they will be the primary focus of this section.

Aquaporins are found in abundance in various cell membranes and allow for water to flow bi-directionally (Agre et al., 2002). The aquaporin channels have exceptional specificity for water, allowing rapid movement of water in response to changes in tonicity (Day et al., 2014). Cell membrane permeability to water is partially due to the number of aquaporins available on the membrane and the physical properties of the aquaporins (Conner et al., 2012). To date, 13 different aquaporins have been identified that work collectively to maintain fluid homeostasis (Wakayama, 2010). The 13 aquaporins are subdivided into 2 groups: aquaporins that are exclusive channels for water and aquaglyceroporins that allow the passage of water, glycerol and urea across the cell membrane (Conner et al., 2012; Wakayama, 2010). Aquaporins enhance membrane permeability and appear to be important contributors to the regulation of CV. When aquaporins are present, there is an increase in membrane water permeability by up to ~50 fold (van Hoek & Verkman, 1992; Yang & Verkman, 1997). Therefore, these proteins represent a mechanism that lead to an increase in the rate at which water moves into or out of cells.
When water moves between body compartments, simple diffusion, co-transport, and facilitated diffusion all contribute to facilitate the shift in fluid volume into or out of the cell. Furthermore, these mechanisms work collectively with the fluid volume regulatory mechanisms found within cell membranes and assist in restoring volumes back to a more homeostatic state.

1.1.5. Fluid levels and regulation

Under normal conditions, serum osmolality in the human body is within 285 to 295 mmol/kg of water and is referred to as an isotonic osmotic state (ISO) (Antolic et al., 2007; Kraft, 2000). During ISO, there is equilibrium of osmotic pressure between the inside and outside of the cell along with an equal number of water molecules moving in and out of the cell (Kraft, 2000). Changes in CV occur when water moves across the cell membrane driven by hydrostatic or osmotic pressure (Lang et al., 1998). Hydrostatic pressure is the overall force of fluid based on volume, while the osmotic pressure results from the diffusion of fluid across a membrane based on osmosis. The regulation of CV is essential for every cell as it can lead to cell metabolic dysfunction, alterations in cell shape, proliferation, differentiation and migration, and even apoptosis (Akita & Okada, 2014). A loss or gain of cellular water of only a few percent can have profound effects on protein and cell function, thus it is an important variable for cells and tissues (Friedrich, Matskevich, & Lang, 2006). When osmotic levels move away from the ISO state, cells rely on volume-regulatory mechanisms and transporters to return their volume to homeostasis. These auto-regulating mechanisms generally increase or decrease fluid volume within minutes of CV moving away from its normal set point. These regulatory
mechanisms create an efficient environment where the mechanisms are only active when required, leading to a reduction in unnecessary energy expenditure (O’Neill, 1999).

There are two terms to describe the extracellular conditions that lead to fluid shifts that occur within cells: extracellular hypertonic- (HYPER) and hypotonic- (HYPO) osmotic states (figure 1). During HYPER, there is a net loss of fluid from the cell causing the cell to shrink, which in turn results in macromolecular crowding (Lang et al., 1998). Excessive fluid loss resulting from extremely high extracellular osmolality (greater than 500 mmol-kg) can lead to cellular apoptosis (Lang et al., 2000; Michea et al., 2000). In contrast, HYPO leads to an increase in cell fluid volume as there are less solutes in the extracellular space compared to the intracellular compartment and therefore causing fluid to shift into the cell, resulting in swelling (Friedrich et al., 2006). Please see figure one for a generalized description of how a cell reacts to extracellular HYPER- and HYPO-osmolality.

There are multiple mechanisms that work cooperatively in an attempt to achieve ISO-osmolality when fluid levels move away from a homeostatic state. There are two primary CV regulatory mechanisms that are stimulated under such circumstances. During extracellular HYPER conditions, the cell regulatory volume increase (RVI) is activated and with extracellular HYPO conditions the cell regulatory volume decrease (RVD) is activated, both of which will be discussed in proceeding sections of the paper (Lang et al., 1998). Alterations of CV are partially accomplished by transport of ions and solutes across the cell membrane with the activation or inhibition of Cl⁻ channels, K⁺ channels, Ca²⁺ channels, Na⁺/H⁻ exchanger, Na⁺, K⁺, 2Cl⁻ cotransport and the Na⁺/K⁺...
ATPase (Lang et al., 2006). In general, fluid moves in the same direction as the flow of ions between the intracellular and extracellular compartments.

In cells, RVI uses a combination of the ions listed above to facilitate the two primary transport systems: \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) cotransporter (NKCC) and the \( \text{Na}^+/\text{H}^+ \) antiporter (NHE) (Demaurex & Grinstein, 1994; O'Neill, 1999). The NKCC system involves the process of transporting 1 \( \text{Na}^+ \), 1 \( \text{K}^+ \) and 2 \( \text{Cl}^- \) ions across and into the cell membrane whilst promoting water to flow into the cell (Demaurex & Grinstein, 1994; Haas & Forbush, 1998; Lindinger, Leung, Trajcevski, & Hawke, 2011). In terms of the \( \text{Na}^+ \) ion, it is known to be the most rapid and thermodynamically effective ions for inducing RVI (Mongin & Orlov, 2001). \( \text{Na}^+/\text{H}^+ \) antiporter works like an antiporter-system as molecules leave the cell in exchange for a different molecule to enter. In order for cells exposed to HYPER to return to a steady volume state, the NHE system transports \( \text{Na}^+ \) out the cell and \( \text{H}^+ \) in (Demaurex & Grinstein, 1994). Like the first system explained, NHE will carry water into the cell until CV is restored. Please refer to figure 1A for a simple diagram depicting this system. The effectiveness of RVI has been studied in the TE671Rd human skeletal muscle cell line (Wibberley, Staunton, Feetham, Vereninov, & Barrett-Jolley, 2015). Following incubations of 30 min or 2 hrs with HYPER medium, cell shrinkage was observed, however, following a 4 hr incubation there was an increase in CV and thus an increase in cell size (Wibberley et al., 2015). It is thought that RVI was triggered to a greater extent in the 4 hr incubation under HYPER, thus allowing the cell to regain CV and stop shrinking.

In terms of the RVD regulator, ions are often released and upon shrinkage (of the cell) they are taken up by their respective transport system (Friedrich et al., 2006).
Restoring fluid levels to ISO primarily involves the K⁺Cl⁻ cotransporter (KCC) (O'Neill, 1999). The hypotonic activation of K⁺ selective channels appears to be one of the most common principles of RVD, while Cl⁻ is one of the most abundant anion channels in skeletal muscle (Okada, 2004; Wehner, 2006). Upon activation of RVD, K⁺ and Cl⁻ are released out of the cell with the addition of water to restore fluid levels (Friedrich et al., 2006; Okada, 2004). In organs such as the liver, under extreme cell hypo-osmotic situations, extracellular Ca²⁺ will be called upon to enter the cell to help activate the K⁺Cl⁻ cotransporter system (Bear & Petersen, 1987). The stimulation of Ca²⁺ comes from the inside of the cell membrane to open the channel and then allow the passage of water in the outward direction (figure 1B). Finally, there are a vast number of hormonal and their physiological triggers that can induce cell swelling. These include, but are not limited to amino acids, insulin, growth factors, adenosine, vasopressin, extracellular ATP, oxidative stress, and urea (Ritz et al., 2003). Such bodily functions happen on a regular basis and typically impose no extreme threat to the system due to its ability to rapidly overcome the fluid level changes.

Overall, profound modifications in CV can interfere with the integrity of the cell membrane and the cytoskeletal structure as proteins are ultimately affected by the fluctuations (Friedrich et al., 2006). In closing, cellular osmolytes play a vital role in keeping the RVI and RVD systems in check.
Figure 1: CV regulation under extracellular HYPER and HYPO conditions. Additionally, the respective primary mechanisms for RVI and RVD are depicted. The cell remains at an ISO condition until a shift in extracellular fluid occurs. (A) The individual loses fluid via sweat. Fluid is drawn out of the cell into the extracellular space, causing a sudden decrease in CV leading to cellular shrinkage and an increase in plasma concentration. The decrease in CV stimulates RVI mechanisms, leading the H+/NA\(^+\) antiporter and/or the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter to respond. Fluid molecules will follow and restore extracellular CV levels back to the ISO condition. (B) The individual is hydrating, causing a sudden increase in CV and a decrease in plasma concentration. RVD mechanisms respond via the K\(^+\)-Cl\(^-\) cotransport and/or the activation of K\(^+\) via the inward flow of Ca\(^{2+}\), thus allowing K\(^+\) to exit the cell. Fluid molecules will follow and restore extracellular cell volume back to ISO condition.

1.1.6. Cellular osmolytes

It appears that cells also exploit the properties of organic osmolytes, which are used in an attempt to maintain normal CV and as such act as another regulatory mechanism.
Osmolytes are classified into three groups: polyols (mannitol, glycerol, sorbitol), free amino acids (glycine, glutamine, alanine), and methylalamines (betatines, glycerophosphorylcholine, sarcosine) (Lang et al., 1998; Singh et al.). In skeletal muscle, AAs appear to be the primary group called upon as they are readily available during long-term adjustments of CV (Low, Rennie, & Taylor, 1997). Additionally, they have defining characteristics making them unique at the cellular level. These include their ability to stabilize proteins against denaturing stresses and that their presence in the cell promotes and creates osmolality without compromising other cell functions (Lang et al., 1998). Put simply, osmolytes are used in an attempt to counteract external osmotic forces. Such forces can include the change in pH levels that can arise when a shift in fluid volume occurs or to re-stabilize macromolecules (Lang et al., 1998). In a cell, the loss of cellular osmolytes leads to cell shrinkage, and as such, cell swelling generally triggers the release of osmolytes, resulting in a loss of water (Friedrich et al., 2006; Lang et al., 2006).

It is clear that fluid dynamics within the body can impact skeletal muscle function and physiology. In response to extracellular osmolality levels, cells respond through the two regulatory mechanisms (RVI and RVD) that were discussed previously. These mechanisms occur rapidly, despite the influence of environmental factors. Osmolytes, specifically free AAs, are another contributing factor as previously described. The importance of these osmolytes will be further explored in the following sections with emphasis on the BCAAs and their role in regards to protein metabolism.

1.2. Protein metabolism in skeletal muscle

The skeletal muscle system is comprised of a series of unique cells that are required to respond to a vast array of functional demands and external environmental conditions.
Taking a brief look at the skeletal muscle structure, the two primary contractile proteins within muscle are myosin and actin. These proteins are critical in the performance of muscle contractions via cross-bridge cycling (Bottinelli & Reggiani, 2000). The protein myosin forms the main part of the thick filament and part of this protein binds to actin. Actin comprises the thin filament. Working collectively, these two proteins bind, moving the thin filaments closer together, creating a muscular contraction and force production. Together, these proteins along with many others contribute to skeletal muscle protein. They represent a total of about 50-75% of total body protein, thus having a protein turnover rate equalling roughly 30-45% of whole body protein metabolism (Rennie & Tipton, 2000).

Daily protein intake and physical activity strongly influences the amount of muscle mass in an individual (Wolfe, 2002). Ingestion of protein-containing meals stimulates the synthesis of new muscle proteins (Bennet, Connacher, Scrimgeour, Smith, & Rennie, 1989; Bohe, Low, Wolfe, & Rennie, 2003; Bohe, Low, Wolfe, & Rennie, 2001; Fujita et al., 2007). Furthermore, the promotion of PS helps offset the activation of PD. Thus, the maintenance of skeletal muscle mass relies heavily on the metabolic basis and regulation of PS and PD (Tipton & Phillips, 2013). PS and PD are critical during early life to facilitate growth and muscle development. Small increases in muscle mass can further be attained into adolescence and adulthood with the inclusion of resistance exercise and are complimented with good nutritional habits, which include the ingestion of quality proteins based on the protein digestibility corrected amino acid score (Phillips, 2012).

Generally, PS activity is approximately 3-5 times greater than PD during any given day when adequate amounts of protein are ingested (Tyler A. Churchward-Venne, Burd,
attain a net muscle protein balance (NMPB) to reach homeostasis, meaning that the rates of synthesis are at least equal to the rates of degradation. This equation can be described as: \( \text{NMPB} = \text{PS} - \text{PD} \) (Tipton & Phillips, 2013). For a net increase to occur, muscle PS must surpass muscle PD. A chronic imbalance between PS and PD occurs when an increase in muscle mass or muscle hypertrophy occurs (Apro & Blomstrand, 2010). While a negative NMPB value may occur, for ongoing normal functionality, it is best when avoided for prolonged periods, as significant muscle loss would occur.

### 1.2.1. Protein synthesis

Protein synthesis is extremely important in the maintenance of body structure and function. Chronic increases in skeletal muscle PS can lead to skeletal muscle hypertrophy (Goodman, Mayhew, & Hornberger, 2011), the replacement of proteins that are old or no longer healthy, and developmental growth (Volpi, Ferrando, Yeckel, Tipton, & Wolfe, 1998). This is despite the uncontrollable loss in muscle mass seen beyond the fifth decade of life (Phillips, 2011; Volpi, Ferrando, Yeckel, Tipton, & Wolfe, 1998). There are a number of different signalling pathways that control the messenger RNA translation and therefore the rate of PS (Scot R. Kimball, 2014). Some important promoters of PS in skeletal muscle are insulin, specific AAs, and physical activity (Scot R. Kimball, 2014). While insulin and physical activity are extremely important for optimal growth and human functioning, the role of AAs will be the focus and further discussed due to their strong presence in enhancing PS metabolism.

Current literature suggests that the primary signalling mechanism underlying activation of PS is a three system-pathway comprised of Akt, mTOR and p70S6K (Bodine
et al., 2001). Collectively, they work downstream of Akt, which stimulates mammalian target of rapamycin (mTOR), and in turn mTOR stimulates p70S6K, ultimately resulting in an increase in skeletal muscle hypertrophy. The AA leucine is a key stimulator of PS and how it stimulates this signalling pathway is depicted in figure 2. Beginning with Akt, it is a serine/threonine kinase that regulates general physiological responses to changes in metabolism, cell proliferation and cell survival, (Lai, Liu, Jacobs, & Rider, 2012; Manning & Cantley, 2007). Akt is recognized as being one of the most important and versatile protein kinases within human cells (Manning & Cantley, 2007). Akt signals an increase in the rates of PS via activation of the mTOR system, although the mechanism is not completely understood (Manning & Cantley, 2007; Sekulic et al., 2000). Akt also inhibits the expression of FOXO transcription factors. FOXO transcription factors are activators of the ubiquitin proteasome system, which in turn results in apoptosis and PD within muscle (Manning & Cantley, 2007; Schiaffino & Mammucari, 2011).

The next major component of the signalling cascade is mTOR. mTOR plays a prominent regulatory role in skeletal muscle hypertrophy and overall cellular growth versus cellular starvation and wasting (Drummond et al., 2009; Holz, Ballif, Gygi, & Blenis, 2005; Zoncu, Efeyan, & Sabatini, 2011). mTOR is a large protein kinase that functions as a serine/threonine kinase (Holz et al., 2005; Stipanuk, 2007). The activity of mTOR is regulated by many components including growth factors, nutrients, stress, and mechanical loading (Aguirre, van Loon, & Baar, 2013; Zoncu et al., 2011). mTOR is a critical nutritional and cellular checkpoint sensor, and regulator of cell growth in humans, thus altering the phosphorylation status and activity of several proteins within the cells (Gingras, Raught, & Sonenberg, 2001; Nicastro et al., 2012; Richardson, Schalm, &
mTOR is the catalytic subunit of two complexes, mTORC1 and mTORC2. mTORC1 is rapamycin-sensitive (rapamycin inhibits mTOR activity) and phosphorylates the proteins p70S6K and eukaryotic initiation factor 4E binding protein (4E-BP1). mTORC2 is rapamycin-insensitive and phosphorylates Akt and the serum/glucocorticoid regulated kinase 1 (Scot R. Kimball, 2014; Zoncu et al., 2011). Moreover, mTOR1 acts as a signal-housing complex from four regulatory inputs: nutrients, growth factors, energy and stress (Zoncu et al., 2011). Whether or not these two complexes work together throughout the body is yet to be determined (Zoncu et al., 2011). However, the signals are created and allow the downstream effect to continue and to stimulate PS.

The second last step in PS activation is the phosphorylation of p70S6K. Like Akt and mTOR, this kinase is another serine/threonine kinase (Baar & Esser, 1999). Currently, there are two isoforms associated with this kinase. These are a 70 kDa cytoplasmic form and a 85 kDa nuclear form (Berven & Crouch, 2000). Despite the two isoforms, they share similar molecular sequences and are referred to as p70S6K (Berven & Crouch, 2000). Upon activation of p70S6K, phosphorylation of the S6 ribosomal protein occurs at the level of the ribosomes in skeletal muscle cells (Baar & Esser, 1999; Berven & Crouch, 2000). The S6 ribosomal protein will then enhance the translation of specific mRNAs required for PS (Blomstrand, Eliasson, Karlsson, & Kohnke, 2006). It has been determined by multiple researchers that if there is a decrease in gene expression in any of these protein kinases, the outcome is a decrease in overall cell size (Bohni et al., 1999; Leevers, Weinkove, MacDougall, Hafen, & Waterfield, 1996; Montagne et al., 1999; Zhang, Stallock, Ng, Reinhard, & Neufeld, 2000). Inactivation, or decreased expressions, can
come from internal or external sources. For example, inactivation of the p70\textsuperscript{66k} by dephosphorylation can be attributable to either rapamycin treatment or AA deprivation that can affect the mTOR signalling pathway (Berven & Crouch, 2000; Dufner & Thomas, 1999). Ideally Akt, mTOR and p70\textsuperscript{66k} are continually working collectively through the signalling cascade to promote proper growth and function of skeletal muscle.
**Figure 2:** Intracellular signalling upon the stimulating effects of leucine leading to protein synthesis. Arrows indicate activation; solid lines indicate inhibition. Leucine enters muscle tissue undergoing aminotransferase via BCAT isoymes (BCATm) producing the branched-chain keto acid (BCKA) $\alpha$-ketoisocaproic (KIC) for leucine. Oxidative decarboxylation catalyzes the keto acid (BCKD), activating mTOR and p70S6k, while 4E-BP1 is inhibited turning in eIF4F. This stimulant activates initiation and elongation, and increase protein synthesis.
1.2.2. Protein degradation

Skeletal muscle PD is equally as important in the maintenance of cellular homeostasis as PS. PD has an essential role in the maintenance of intracellular AA levels and maintaining muscle protein quality by removing abnormal or damaged proteins produced by mutations (Tyler A. Churchward-Venne et al., 2012; A. Hershko & Ciechanover, 1982; Lecker et al., 1999). Such proteins are recognized and rapidly degraded and an opportunity is created for the free AAs to assist in the building of new muscle proteins. Unlike most regulatory mechanisms, PD is completely irreversible (Lecker et al., 1999). Thus, mechanisms responsible for PD are important in maintaining cellular homeostasis given that PS will generally follow and replace degraded proteins with new healthy ones. There are a number of different multi-step systems that contribute to the process of PD, two of which include; the ubiquitin proteasome system and the calpain proteolytic system.

The ubiquitin proteasome system is one of the primary systems in skeletal muscle responsible for PD. This system is a multi-unit complex comprised of 26 subunits, known as the 26S proteasome. It uses ATP to drive the transformation and eventual degradation of the targeted protein (Pickart & Cohen, 2004). Furthermore, the system is involved in endocytosis and down regulation of receptors and transporters in addition to its role in degrading intracellular proteins (Avram Hershko & Ciechanover, 1998). In this system, PD begins to occur once the targeted protein is tagged with a series of linked ubiquitin molecules (Pickart, 2004). The ubiquitin-proteasome pathway involves three different enzymes corresponding to three different steps leading to the degradation of the protein.
The first step (and enzyme) involves the enzyme E1. This is an ATP-dependent step responsible for forming a thiolester bond between the E1 enzyme and ubiquitin (A. Hershko & Ciechanover, 1992). The E1 step is viewed as a preparation step for the proteasome to act on the protein to be degraded. The second step involves E2 and another thiolester bond is necessary as the activated ubiquitin from E1 transfers onto an E2 (carrier protein) cysteine residue (A. Hershko & Ciechanover, 1992). The E2 step can also be referred to as the ‘conjugating step’ due to the ubiquitin protein bonding the E1 cysteine to an E2 cysteine (Pickart & Eddins, 2004). The final step involves the E2 cysteine transferring the ubiquitin to the E3 ligase. The E3 ligase is involved in the process of completing the linkage between the specified targeted protein and ubiquitin molecule (A. Hershko & Ciechanover, 1992). In order for the protein to be fully degraded, the three-enzymatic steps must occur four times. This repetition of the three-step process is referred to as poly-ubiquitination. Once the targeted protein is tagged with 4 ubiquitin molecules, the 26S proteasome will degrade the protein. This is completed by utilizing a specific ATP-dependent protease and converting the poly-ubiquitinated protein conjugate into free amino acids (Ciechanover, 1993b). The ubiquitin molecules are released and recycled during this process to be used for other PD cycles (Ciechanover, 1993a; Pickart & Eddins, 2004).

In some forms of cancer, the ubiquitin proteasome pathway is targeted by the tumour necrosis factor alpha, an inflammatory cytokine that has a direct catabolic effect on skeletal muscle (Nicastro et al., 2012). This is one example of a system that up regulates PD through another pathway. As mentioned, the ubiquitin proteasome system is highly specific and the pathway will not be active unless necessary. The specificity of PD
makes for an advantageous mechanism as it assists in the maintenance of bodily functions. For example, at times where individuals have inadequate caloric intake, breakdown of skeletal muscle proteins for substrate metabolism is necessary as these proteins are more easily sacrificed (Lecker et al., 1999). This serves to provide the more important organs with additional AAs such as the heart or liver.

Another important PD system within skeletal muscle is the calpain proteolytic system. Calpains are proteins that are often found in high concentrations within skeletal muscle in patients with muscular dystrophy and wasting conditions associated with muscle disuse (Kemp, Oliver, Wheeler, Chishti, & Koohmarai, 2013). These proteins belong to the calcium-dependent, non-lysosomal cysteine protease family and are found throughout skeletal muscle, typically around the Z-disk region (Goll, Thompson, Li, Wei, & Cong, 2003; Koohmarai & Geesink, 2006). There are three primary isoforms of calpain found within the body, µ-calpain, m-calpain, and calpain 3. µ-calpain and m-calpain require 1 – 100 µmol and 0.1 – 1 mmol concentrations of Ca$^{2+}$ for activation, respectfully, whereas calpain 3 requires little to no Ca$^{2+}$ for activation (Berchtold, Brinkmeier, & Muntener, 2000; Pandurangan & Hwang, 2012). Of the three isoforms, µ- and m-calpains are more predominant and therefore will be the focus of this section. They are heterodimers composed of two subunits of about 80 and 30 kDa, with the larger unit containing the catalytic domain and the smaller containing regulatory functions (I. J. Smith, Lecker, & Hasselgren, 2008). Within skeletal muscle, both these calpains are found in relatively even quantities (Goll et al., 2003). Additionally, if µ-calpain is in poor supply, m-calpain is able to continue with the proteolytic activity. This shows that these molecules cleave the same substrates when activated (Goll et al., 2003).
Under normal basal conditions calpains generally remain inactive. During such conditions, calpains are distributed closer to the cell membrane (Todd et al., 2003). Typically within skeletal muscle myofibers, Ca\(^{2+}\) concentrations are \(~50\) nM which is not enough to activate these systems. Hence, a rise in Ca\(^{2+}\) is required for activation (Berchtold et al., 2000). Calpains rely on an increase in intracellular Ca\(^{2+}\) concentration to undergo a conformational change within the two subunits that ultimately leads to autolysis (Berchtold et al., 2000; I. J. Smith et al., 2008). Autolysis will reduce the molecular mass of the large subunit of 80 kDa to 78 kDa (Pandurangan & Hwang, 2012). Ca\(^{2+}\) will then bind to the regulatory site on the calpain and convert it to an active enzyme (Moldoveanu et al., 2002). Once the calpain is activated, it is ready to bind to a protein and initiate degradation of that protein through cleavage of peptide bonds. To degrade the protein in its entirety, two steps are involved: 1) the removal of the protein from the myofibrillar structure and 2) degradation of the protein to small peptides and free amino acids (Pandurangan & Hwang, 2012; Suzuki, Hata, Kawabata, & Sorimachi, 2004). It should also be noted an increased abundance of calpains inhibits the activation of Akt (I. J. Smith et al., 2008). When Akt activation is inhibited, there is a reduction in mTOR activation and a decrease in rates of PS. It would therefore be detrimental if calpain activity were to be elevated for prolonged periods, as the increase in PD exceeds PS, causing a net loss of protein.

Calpain is partially regulated through the inhibitory protein calpastatin. Calpastatin is a protein with a molecular mass of 110 kDa that specifically inhibits the proteolytic activity of both \(\mu\)-calpain and m-calpain (Berchtold et al., 2000; Goll et al., 2003). When calpain activity is elevated for prolonged periods, due to persistently high levels of Ca\(^{2+}\),
activation of calpastatin occurs. This protein intervenes to help prevent uncontrolled and unspecific degradation of proteins (Todd et al., 2003). Calpastatin contains four inhibitory domains, and each domain is capable of inhibiting a single calpain molecule (Wendt, Thompson, & Goll, 2004). Furthermore, each of the four domains has 3 conservative binding regions (A, B, and C) and will bind to a separate domain of the targeted calpain (Todd et al., 2003). A and C allow for calpastatin to secure to the calpain molecule, while B loops interact with the active site to inhibit calpain activity (Todd et al., 2003). In order for this inhibitor to be effective, Ca\(^{2+}\) must be present, but in concentrations lower than what is needed to stimulate calpain activity (Goll et al., 2003; Otsuka & Goll, 1987). Calpastatin undergoes a conformational change in the presence of Ca\(^{2+}\) leading to a hydrophobic pocket opening on the surface. This opens the area for a calpain molecule to bind to (Otsuka & Goll, 1987). In the absence of Ca\(^{2+}\), calpastatin is dephosphorylated via a phosphoprotein phosphatase and is redistributed throughout the cytoplasm (Todd et al., 2003).

Hyper-osmotic extracellular stress leads to an increase in calpain activity within skeletal muscle (Darling, 2011; Vandommele, 2012). This stress induces an environment that results in macromolecular crowding and an increase in Ca\(^{2+}\) concentration (Apostol, Ursu, Lehmann-Horn, & Melzer, 2009; Moldoveanu et al., 2002). HYPER extracellular environments have also been linked to the presence of Ca\(^{2+}\) sparks. Ca\(^{2+}\) sparks refer to acute repeated increases in intracellular Ca\(^{2+}\) concentrations in skeletal muscle where fluid volume is reduced due to a hypertonic state in the absence of an action potential (Martin et al., 2003; Wang et al., 2005). These spontaneous releases of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) occur when there is a disruption in the structural interaction
of the cell due to cellular shrinkage between the dihydropyridine receptors (DHPR) and
the coupling ryanodine receptors that normally control Ca\textsuperscript{2+} release from the SR (Wang et
al., 2005). As discussed earlier, calpain requires an increase in intracellular Ca\textsuperscript{2+}
concentration for activation. Based on HYPER inducing cell shrinkage and Ca\textsuperscript{2+} sparks, it
is possible that the previously observed increase in calpain activity with HYPER is due to
the associated Ca\textsuperscript{2+} sparks that also occur. Thus, it appears that HYPER may lead to an
increase in calpain activity, leading to a more catabolic environment and ultimately a
higher rate of PD occurring in skeletal muscle (Goll et al., 2003).

PS and PD work together at the molecular level to facilitate skeletal function and
adaptation. PD should not always be viewed as a negative outcome, but rather as an
important step in facilitating adaptation. This is especially true due to the necessary
removal of damaged or unwanted proteins as the resulting AAs would then be available
for protein synthetic processes. Within skeletal muscle, total net protein balance is often
in a positive state as PS is activated to a greater extent than PD. In healthy young adults,
muscular atrophy may occur but it often results from a decrease in basal muscle PS with
no increase in the rate of PD, indicating the PD is not always elevated with muscle
atrophy (Ferrando, Lane, Stuart, Davis-Street, & Wolfe, 1996; Paddon-Jones, Sheffield-
Moore, Katsanos, Zhang, & Wolfe, 2006). Understanding that inadequate protein
consumption can decrease PS rates, it is the individual’s best interest to stimulate PS
metabolic markers, through diet, to reverse the affects of muscle atrophy. Overall, a
delicate balance is accomplished between PS and PD while external factors can also
influence this balance, including nutritional intake and physical activity.

1.2.3. The effects of osmolality on protein metabolism
Whether directly or indirectly, changes in CV can modify or control a number of cellular metabolic functions, and on the contrary, cellular metabolism can alter the consistency of cell volume although this is more difficult to quantify (Haussinger & Lang, 1991). Previous work has demonstrated that serum osmolality can vary between individuals based on their current hydration state and environmental conditions (Cornish, Wotton, Ward, Thomas, & Hills, 2001).

The liver is another tissue that represents a good example of a tightly regulated system where protein metabolism can be directly influenced by extracellular osmolality. Upon cell swelling, the liver stimulates anabolic events such as PS while cell shrinkage stimulates catabolic events (Haussinger, Roth, Lang, & Gerok, 1993). By incubating isolated rat hepatocyte samples in twice the physiological concentration of leucine for 2 hours, Stoll et al. (1992) observed that PS was inhibited by ~60% in the HYPER osmotic incubation where liver cell shrinkage occurred. The reduction in PS was restored upon re-establishing normo-osmotic conditions. In contrast, HYPO osmotic induced cell swelling did not show any significant changes in liver cells like those seen in skeletal muscle, suggesting that PS is possibly already at its maximum activity level in the ISO state within these cells (Stoll et al., 1992).

1.3. Dietary amino acids and skeletal muscle growth/metabolism

1.3.1. Essential amino acids

Dietary intake of the nine essential amino acids (EAA) is critical in helping to fuel protein metabolism in humans (T. A. Churchward-Venne et al., 2012; Mitsuhashi, 2014). It appears that the availability of EAAs is an important factor that drives PS. In skeletal muscle, feeding appears to double the rate of muscle PS with much of this change
attributed to the actions that come from ingesting AAs (Bennet et al., 1989; Rennie et al., 1982). If there is an insufficient supply, this could ultimately limit PS and promote PD (Wolfe, 2002; Zhang et al., 2000). The consequences from a brief period of dietary restriction of AA can easily be reversed once the individual has replenished their stores to meet healthy standards (Hara et al., 1998). In relation to physical activity, individuals that consume AAs prior to or immediately following exercise facilitates an even greater increase in PS occurring during and following the activity session as compared to the exercise alone (Wolfe, 2002). In another study that measured the fractional PS rate after exercise alone and after exercise with the addition of AA infusion, muscle protein metabolism and AA transport were both increased with the infusion of AAs (Biolo, Maggi, Williams, Tipton, & Wolfe, 1995; Nicastro et al., 2012). Thus, there is a definite interaction between the provision of AAs and the response in PS, especially following exercise.

1.3.2. Branched-chain amino acids

Of the EAAs, it appears that a subgroup of AAs known as the branched-chain amino acids (BCAA) are potent stimulators of PS and therefore are important for skeletal muscle protein metabolism. BCAAs provide the building blocks for PS with their availability influencing PS (Bohe et al., 2003; Mitsuhashi, 2014). The BCAAs include leucine, isoleucine and valine represent the most abundant AAs of the nine EAAs within dietary protein (Li et al., 2011). Approximately 35% of the EAAs and 18% of all AAs located within skeletal muscle are BCAA (Riazi, Wykes, Ball, & Pencharz, 2003; Shimomura, Murakami, Nakai, Nagasaki, & Harris, 2004). Unlike most EAAs that are oxidized in the liver, BCAAs are primarily oxidized within skeletal muscle (Li et al.,
2011; Shimomura et al., 2004). This is because the liver contains low levels of mitochondrial branched-chain aminotransferase that is the first enzyme in the catabolism of BCAA (Wahren, Felig, & Hagenfeldt, 1976). There are three main methods for transporting these AAs into cells to facilitate protein metabolism: (1) a uniport system where AAs come in on their own; (2) a co-transport where the AA will bind to ions/AAs already located near the cell or bind to other AAs to enter; or (3) the antiport system where the cell must release an ion to the extracellular environment in exchange for the AA to enter (Taylor, 2014). When BCAAs (especially leucine) are supplemented to one’s diet with the addition of resistance training, the total net muscle protein balance increases to a greater extent than if the participants were given no BCAAs with training (Apro & Blomstrand, 2010; Nicastro et al., 2012). An exercise intervention in combination with consumption of a BCAA drink showed a 65-160% increase in phosphorylation of mTOR (Apro & Blomstrand, 2010). An increase in p70<sup>S6k</sup> phosphorylation by 5-11-fold in both legs immediately and one hour post exercise was also observed (Apro & Blomstrand, 2010). In this study, participants were provided with a 150 mL beverage containing either a mix of the BCAAs (45% leucine, 30% valine and 25% isoleucine) or a placebo to consume prior to warm up, immediately before performing resistance exercise, immediately after exercise and then another following a 15- and 45-minute recovery before measurements were taken. For those consuming the BCAA beverage, a total of 85 mg of BCAA/kg of body weight was ingested. BCAAs are not only important for increasing PS, but also been suggested to have the ability to decrease muscle wasting by interacting with the ubiquitin proteasome system, lower or prevent excess fat deposition, and possibly treat diabetes (Aguirre et al., 2013; Wu, 2009). Dietary habits can
significantly alter BCAA concentrations as intake varies based on dietary consumption. How these AAs are detected becomes equally important to help facilitate positive adaptations in protein metabolism.

Human cells respond to a dietary deficiency of BCAA through nutrient sensors contained within AA transporters (Taylor, 2014). These sensors control the response signals from the intracellular and extracellular space and assist in determining the oxidation rates of these AAs (Taylor, 2014). Postprandial intake of BCAAs induces hyperaminoacidemia, due to digestion and absorption of the AAs, which also stimulates PS (Bajotto et al., 2011). Once consumed, the path of oxidization for all three BCAAs start out similar before taking their individual paths for further oxidization. The entire catabolic pathway is located in the mitochondria and the first BCAA reaction is catalyzed by branched-chain aminotransferase (BCAT) (Shimomura et al., 2006). It is a reversible reaction of transamination of the AAs to form the respective branched-chain keto acid (BCKA) (Nicastro et al., 2012; Shimomura et al., 2006). The keto acids are as follows: α-ketoisocaproic acid for leucine, α-keto-β-methylvaleric acid for isoleucine, and α-ketoisovaleric acid for valine (Nicastro et al., 2012).

The second reaction for BCAAs is irreversible as the BCKA is catalyzed by the branched chain keto acid dehydrogenase (BCKDH) complex, and involves the oxidative decarboxylation of the keto acids (Hatazawa et al., 2014; Shimomura et al., 2006). This is often considered to be the rate-limiting step of the whole oxidative process of BCAAs. BCKDH is highly regulated through phosphorylation (inactivation) and dephosphorylation (activation) mechanisms, and is dependent upon the concentration of available BCKA (Harper, Miller, & Block, 1984). There are two proteins associated with
this step: a tightly bound protein kinase and a loosely bound phosphatase (Harper et al., 1984; Shimomura, Obayashi, Murakami, & Harris, 2001). The former is responsible for the inactivation of the complex and the latter is responsible for activating the complex (Shimomura et al., 2001). It has been observed in rat liver that inactivation of the BCKDH complex can be the result of a low-protein (8%) diet and that the opposite will occur upon feeding rats a high-protein (30%) diet (Kobayashi et al., 1999). These observations are consistent with previous research recorded where different cell culture cell models (1 rat- C9, 2 human- DG75 and lymphoblast, EM280) were used, and observed a decrease in BCKDH activity following the 5th day of utilizing BCAA deprived media (Doering, Williams, & Danner, 2000). These results are important to our research because it can attest to possible mechanisms for the inactivation of PS.

With the phosphorylation of the BCKA to BCKDH activation in check, the downstream effect of the anabolic pathway leads phosphorylation of mTOR signalling in skeletal muscle (Bajotto et al., 2011; Nicastro et al., 2012). Little AA availability will ultimately lead to a decrease in mTOR activation (Blomstrand et al., 2006). On the contrary, an abundance of BCAAs (with emphasis on leucine) induces hyper-phosphorylation of p70S6 kinase and 4E-BP1, and promoting PS accordingly (Schliess, Reissmann, Reinehr, vom Dahl, & Haussinger, 2004). The physiological, nutritional and pathogenic state of the organism has a contributable role in the timing and frequency of these reactions (Harper et al., 1984). It is through these reactions, however, that we are able to see an increase in net protein metabolism. The role and characteristics of each BCAA, leucine, isoleucine and valine, will be discussed in the proceeding section.

1.3.3. Specificity of the BCAAs
1.3.3.a. Leucine

Leucine is one of the most important EAA our body needs. It has been described as the most potent AA in promoting and stimulating muscle PS (Apro & Blomstrand, 2010; Stipanuk, 2007). When leucine is in high availability it is an excellent reservoir for PS (Dangin, Boirie, Guillet, & Beaufreere, 2002) This BCAA can be found in approximately 20% of the total dietary protein humans eat and has been associated with satiety, body weight control, and whole body energy expenditure (Li et al., 2011). Individuals who are overall well-nourished should seek to consume ~ 40 mg/ kg of body weight per day of this AA (Kurpad, Regan, Raj, & Gnanou, 2006). These requirements could change base on individual needs due to an illness or disease. Food sources that contain the greatest amount of leucine include soybeans, beef, nuts, eggs and fish. When protein sources contain high levels of leucine, stimulation of muscle PS have been found to be greater compared to protein sources with little to no leucine (Phillips, Tang, & Moore, 2009). Post-exercise, it is recommended that BCAAs, in particular leucine, be ingested due to its anabolic effect on protein metabolism (Blomstrand et al., 2006). It is therefore possible to speculate that leucine and physical activity have an additive effect to enhance PS.

From a mechanistic perspective, it has been suggested that the powerful anabolic effect of leucine is due to its ability to enhance mTOR signalling and further inhibit PD (Shimomura et al., 2006). The stimulatory effect of leucine on PS occurs at the level of translation initiation through to mTOR activation (Doi et al., 2005). Postprandial consumption of leucine has been shown to be the primary stimulator for PS, demonstrating its robust effects on the skeletal muscle system (T. A. Churchward-Venne et al., 2012; Shimomura et al., 2004).
Whether leucine is administered orally or intravenously, the outcome is still a positive net protein balance. In an experiment where participants were provided with one of three drinks to consume after performing an acute bout of resistance exercise, the drink containing the greatest leucine concentration (as opposed to a standard EAA or whey protein drink) resulted in the quickest postprandial increase in PS (T. A. Churchward-Venne et al., 2012). Additional work has been completed using cardiac muscle from pigs. Leucine, compared to other BCAAs was shown to phosphorylate p70\textsuperscript{S6k} and 4E-PB1 to the greatest extent (Jeffery Escobar et al., 2006). This caused PS to increase in the left ventricular wall by 26% compared to the saline infused group (Jeffery Escobar et al., 2006). Similar findings were also observed using rat liver where leucine strongly induced hyper-phosphorylation of p70\textsuperscript{S6K} and 4E-BP1 as shown by Western Blot analysis (Schliess et al., 2004). Leucine consumption has further been shown to effectively stimulate PS even under food-deprived situations, demonstrating why this is a unique AA (Anthony et al., 2000; Li et al., 2011). More specifically, administration of food under one of four dietary treatments: control or 1.35 /kg body weight of valine, isoleucine or leucine has been researched in rats (Anthony et al., 2000). Only the leucine treated group had greater PS response compared to the control group with 1.55 and 1.09 mg of protein per hour, respectfully. A similar response was also observed in rats when rapamycin was administered 2 hours prior to the administration of either high leucine or control chow with values of 1.12 and 0.83 mg of protein per hour respectfully. These findings on leucine can play a pivotal role for individuals who struggle to ingest a well-balanced meal high in protein for reasons such as an illness leading to limited food choices or an individual’s dietary requirements. Instead they could focus on having protein sources
with high leucine concentrations or take leucine supplements helping to optimize protein synthetic responses.

**1.3.3.b. Isoleucine**

The remaining two BCAAs are isoleucine and valine. The daily recommended nutritional intakes of isoleucine and valine for a healthy adult is 19mg/kg of body weight and 17-20mg/kg of body weight, respectfully (Kurpad et al., 2006). Unlike leucine, neither of these other BCAAs have the ability to solely stimulate mTOR signalling. Each BCAA has been individually researched in skeletal muscle and found that both isoleucine and valine were ineffective at stimulating mTOR when solely infused into rats (J. Escobar et al., 2006). This does not mean they are invaluable at the cellular level in the skeletal muscle system. Like leucine, isoleucine assumes a role in the signalling processes involving glucose metabolism (Doi et al., 2005). In fact, isoleucine is able to stimulate glucose metabolism in skeletal muscle at a quicker rate than leucine, thus decreasing circulating glucose levels at a faster rate postprandial (Doi et al., 2005). The mechanism of this action appears to be related to the stimulation of insulin- and mTOR-independent glucose transport (Doi et al., 2005). We can conclude that isoleucine plays a supporting role in the possible stimulation of PS.

**1.3.3.c. Valine**

Whole-body oxidation and turnover of valine occurs at slower rates compared to leucine. This could explain why there are lower concentrations of valine in body proteins (Staten, Bier, & Matthews, 1984). Even flooding the system with excess does not enhance rates of PS in either fast-twitch or slow-twitch skeletal muscle (C. B. Smith & Sun, 1995). It is difficult to attest the significance of this particular BCAA in being a
prime contributor in enhancing PS but rather plays a minor role in initiating a signal to mTOR (Atherton, Smith, Etheridge, Rankin, & Rennie, 2010; Jeffery Escobar et al., 2006).

All three BCAAs are important for improving cellular functioning, however through numerous studies, leucine has repetitively demonstrated why it is recorded to be the most important amongst the three in regards to regulation of protein metabolism. In conclusion, ingesting adequate amounts of BCAA, with an emphasis on leucine, leads positive skeletal muscle protein balance.

1.4. Osmolality and its effects on amino acid concentration

It is well established that changes in extracellular osmolality can have positive and negative effects on muscle protein metabolism (Haussinger, Hallbrucker, vom Dahl, Lang, & Gerok, 1990; Waldegger et al., 1997). Similar results have also previously been observed in our lab regarding HYPER and HYPO-osmotic stress and PS/PD (Darling, 2011; Vandommele, 2012). Furthermore, it has been established that the intracellular concentration of AAs is a driving mechanism to stimulate PS, whereas reduced AA availability could affect bodily functions including PS, osmotic regulation, regulation of hormone secretion, gene expression and cell signaling (Wu, 2009). While all these functions are important, we are primarily focused on the activity of protein metabolism and osmotic regulation, and their interdependency on available AAs. We are interested to see if increased availability of these BCAAs, more specifically leucine, could help to restore protein metabolism with osmotic stress. From an osmolality perspective, hyperosmotic cell shrinkage stimulates proteolysis in liver and skeletal muscle cells, thus reducing the net overall protein balance (Hallbrucker, vom Dahl, Lang, & Haussinger,
We suggest that the role of AAs could be twofold, as they could aid in restoring cell volume as they are an established osmolytes, and they could also promote PS.

**1.4.1. Findings from our research group**

Within our lab, it has been shown that both HYPER and HYPO extracellular conditions can acutely affect skeletal muscle protein metabolism, but do so differently. Utilizing an isolated skeletal muscle model, exposure to extracellular osmolality resulted in acute, yet reversible changes in cellular metabolism (Antolic et al., 2007). Early work demonstrated that cross sectional area of type II skeletal muscle fibres decreased when exposed to HYPER conditions and increased when exposed to HYPO conditions (Antolic et al., 2007). In regards to protein metabolism, further work investigated the relationship of PS and PD in whole skeletal muscle. Specifically, HYPER has been observed to result in a 50% reduction in PS and a 25% increase in PD within isolated skeletal muscle (Vandommele, 2012), indicating that HYPER results in a more catabolic environment within skeletal muscle. In contrast, HYPO demonstrated no significant changes in PD, but lead to a 65% increase in the rate of PS (Darling et al., 2011), suggesting HYPO leads to a more anabolic state. This is visually depicted in figure 3. Such responses were observed in fast twitch muscle fibres, as they appear more responsive to extracellular osmotic influences (Antolic et al., 2007).
Figure 3: Depicting the results shown when skeletal muscle protein is induced in a HYPER, ISO, and HYPO-osmolality situation. From an ISO state, HYPER leads to muscular atrophy by activating PD, while HYPO leads to muscular hypertrophy with no change in PD. This is a result from a decrease and increase in protein synthesis, respectfully and the addition of protein degradation occurring under hyper-osmotic stress.

Similar results have been achieved using a cell culture model with the L-6 muscle line. L-6 myoblasts are to be differentiated into myotubes prior to experimental testing. This model is found to be more robust than those using animals or humans due to a more controlled environment. Using a 24 and 48 hour incubation period for HYPER, ISO, HYPO, there was a significant decrease in the rates of PS when cells were stressed in a HYPER solution (Roy, 2009). The rate of PD within the cell culture model has yet to be determined.

1.4.2. Practical implications

In summary, given the information through previous literature increased skeletal muscle CV due to HYPO osmotic stress may help to facilitate a positive net protein balance within skeletal muscle cells. If this is the case, from a clinical perspective, hydration state should be taken into consideration when working with patients who have disorders characterized by muscular atrophy as extracellular hypo-osmotic cell swelling promotes a protein-anabolic situation (Haussinger et al., 1993). Additionally, patients
who have experienced a severe burn could possibly benefit from increased hydration, or provision of hypotonic saline to help reduce the effects of hyper-metabolism that is often correlated to muscular catabolism as a hypotonic saline infusion could possibly help attenuate a catabolic environment (Pereira, Murphy, Jeschke, & Herndon, 2005). Increased provision of AAs should additionally be considered as patients with severe burns oxidize AAs at a rate ~50% faster than healthy individuals (Pereira et al., 2005).

From a movement perspective, with the assistance of various intracellular proteins, our muscles are able to function consistently and optimally from day to day. The nutritional intake and digestion of protein, and thus AAs, contribute to the postprandial protein gains seen following digestion (Dangin et al., 2002). It can therefore be stated that the skeletal muscle improvements from PS and decreasing the rate of PD is partially dietary-dependent (Luiking, Engelen, Soeters, Boirie, & Deutz, 2011). As such, daily consumption of both solid foods and liquids become vitally important to enhance protein metabolism and muscle protein turnover. Lastly, fluid intake with meals might be another factor to consider when seeking to gain the most from a high protein meal and we hope to draw some conclusions to gain a better understanding of the connection between AAs, precisely the BCAAs, and extracellular osmolality.

Whether it is a healthy individual, an elderly patient with sarcopenia, a patient with a burn, or an individual suffering from a chronic illness, it appears that proper hydration may play a vital role in attenuating loss of muscle protein and enhance PS metabolism. Specifically, inducing a hypo-osmotic extracellular environment might potentially represent a complementary form of treatment. This treatment could possibly aid in
slowing and/or reduce the loss of skeletal muscle mass and further have the opportunity to slow the deterioration of muscle function, whilst facilitating recovery.

1.4.3. Summary

Mammalian skeletal muscle cells are very dynamic and can readily adapt to various environmental factors, with one such factor being the osmotic state. With osmotic changes to the extracellular environment, there can be dramatic down-stream effects on protein metabolism. It has been well established how cellular osmotic stress levels influence PS and PD via HYPER or HYPO changes, with HYPER leading to decreases in PS and increases in PD, while HYPO leads to increases in PS and no changes in PD. However, another factor that needs to be considered is the availability of EAAs, as these can also have a dramatic influence on protein metabolism. For example, leucine can clearly stimulate the mechanisms responsible for promoting PS within skeletal muscle cells. What remains unclear is whether an increase in the availability of leucine can overcome the effects of extracellular hyper-osmotic stress on protein metabolism within skeletal muscle cells. Second, does provision of increased concentrations of leucine with the addition of a HYPO-osmotic environment lead to a greater increase in PS within skeletal muscle cells. These questions are the primary focus of this research study and will continually be addressed upon the completion of each experiment.
Chapter 2: Statement of the Problem

2.1. Statement of the problem

Current literature suggests that different extracellular osmotic conditions influence net protein metabolism in skeletal muscle. We are aware that extracellular HYPO creates a favourable environment for protein synthesis to take place. On the contrary, extracellular HYPER decreases total net protein in skeletal muscle due to the activation of protein degradation. Furthermore, certain AAs may initiate the phosphorylation of the Akt/mTOR/p70S6K system-pathway, thus promoting PS (Bajotto et al., 2011). Current research has demonstrated that the presence of the BCAAs, specifically leucine, enhances PS (Apro & Blomstrand, 2010). This has been demonstrated through research observing circulating plasma levels of leucine and protein synthesis markers such as mTOR activation and measures of PS within humans, animals and skeletal muscle cells (C2C12). BCAAs, specifically leucine, appear to be important for skeletal muscle growth and functionality but it is currently unclear how altered concentrations of leucine influence protein metabolism under differing osmotic environments (hyper and hypo).

2.2. Purpose

The purpose of this experiment was to:

1. Investigate if the concentration of leucine influences the protein synthetic response and markers of protein synthesis and protein breakdown under varying osmotic conditions.

2. Investigate if the increase in leucine concentration can alter the protein degradation response as previously observed under HYPER osmotic stress.

2.3. Hypotheses
It was hypothesized that:

1. By exposing the skeletal muscle cells to an increase in leucine concentration, PS would increase in all three osmotic conditions (HYPO, ISO, HYPER). Furthermore, the HYPO with leucine would elicit an even greater increase in PS compared to muscle cells exposed to HYPER.

2. An increase in leucine concentration would decrease the amount of PD within skeletal muscle cells under HYPER osmotic stress. Additionally, the elevated leucine would further attenuate PD in the remaining two osmotic conditions (HYPO and ISO).
Chapter 3: Methodology

3.1. Cell culture

Rat L6 myotubes were utilized for the completion of all experiments. Cells were stored at -80°C and were then thawed at 37°C in a dry bead bath. Cells were then transferred with media drop-wise to a 15 mL falcon tube, inverted gently multiple times and centrifuged at 200 G for 5 minutes, forming a pellet. Excess media was removed and fresh media was added to the falcon tube. Cells were then seeded in a cell culture flask (Diamed, Mississauga, ON, No: SPL70075) with Eagle’s Modified Medium EMEM (Gibco, Life Technologies, Burlington, ON; No: 11095-098) (See Appendix I for media content), containing 10% (vol/vol) fetal bovine serum (FBS; Gibco, Life Technologies, Burlington, ON; No: 12483-020), and antibiotic-antimycotic of 1% (vol/vol), penicillin-streptomycin (Sigma-Aldrich, Oakville, ON, No: P0781). All flasks were maintained in an incubator at 37°C and 5% CO₂. The proliferation medium was changed 24 hr after the initial thawing phase and every 48 hr thereafter. As cultures approached 70% confluence, cells underwent another passage. Cells from a given cryo-tube underwent 3 passages to ensure viability. Figure 5 demonstrates the growth/passages that cells from a cryo tube underwent and its use for experimental data collection.
Figure 4: In the top left corner of this figure is a simple timeline of cell passages. A passage will happen every 4 days. Cells from all 4 flasks are mixed into one falcon tube before seeding to a 6-well plate. The number of flasks allocated for each experiment is simply a number to run 1 set of data collection. This cycle will be repeated multiple times to attain a respectable amount of data for further analysis.

Upon the sixth passage, 100,000 cells were seeded in each well, of 6-well plates (Diamed, Mississauga, ON, No: SPL30006). To count cells a hemacytometer (Hemacytometer Counting Chamber #3120, Hausser Scientific, Horsham, PA) was used to determine the number of viable cells available to be seeded, per well. Proliferation media was then replaced after 24 hr with 2mL of differentiation media, EMEM supplemented with 2% adult horse serum (Gibco, Life Technologies, Burlington, ON, No: 16050-122), while maintaining all other variables constant. Differentiation medium was then replaced every 48 hr. Myoblasts were allowed to differentiate for 10-12 days to form myotubes and were then used for experimentation.
3.2. Experimental design

The current investigation involved 2 different experimental groups exposed to three different extracellular osmotic states (conditions). The 2 experimental groups included: control group with a normal concentration of leucine (N-LEU), and a group with an elevated concentration of leucine (E-LEU). Each experimental group was also exposed to 3 different extracellular osmotic states; hypo-osmotic (HYPO) iso-osmotic (ISO), and hyper-osmotic (HYPER). In an attempt to stimulate protein synthesis via leucine signalling, cells were incubated in Eagles Minimum Essential Medium (EMEM) with an additional 1.5 mM of leucine. This amount of leucine has been previously demonstrated to elicit an increase in protein synthesis (Areta, Hawley, Ye, Chan, & Coffey, 2014). The total leucine content of the E-LEU condition was approximately three times of the N-LEU condition. Please refer to table 1 for intervention groups.

<table>
<thead>
<tr>
<th>LEUCINE CONDITION</th>
<th>OSMOTIC EXTRACELLULAR FLUID CONDITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (0.4 nM)</td>
<td>HYPO (230 ± 10 mmol/kg)</td>
</tr>
<tr>
<td></td>
<td>ISO (330 ± 10 mmol/kg)</td>
</tr>
<tr>
<td></td>
<td>HYPER (430 ± 10 mmol/kg)</td>
</tr>
<tr>
<td>Elevated (1.9 nM total)</td>
<td>HYPO (230 ± 10 mmol/kg)</td>
</tr>
<tr>
<td></td>
<td>ISO (330 ± 10 mmol/kg)</td>
</tr>
<tr>
<td></td>
<td>HYPER (430 ± 10 mmol/kg)</td>
</tr>
</tbody>
</table>

Table 1: Experimental design depicting media manipulation and osmolality levels.

3.3. Osmotic conditions

To manipulate the osmotic state of our control EMEM, medium was supplemented with either mannitol or distilled water (dH2O) to reach the experimental osmolalities: HYPER (430 ± 10 mmol/kg), 25.6 mg/mL of mannitol per mL of media, ISO (ISO, 330 ±
10 mmol/kg), 7.8 mg of mannitol per mL of media and HYPO (230 ± 10 mmol/kg), 240 mL of dH2O per mL of media (Table 1). Mannitol was used due to its ability to quickly equilibrate in a solvent without disrupting intracellular compartments (Stuart, Torres, Fletcher, Crocker, & Moore, 1970). An osmometer (5520 VAPRO Vapor pressure osmometer, Wescor, Logan, UT) was used to determine and verify the osmolality of each condition. This technique has been previously used by our lab with incubations of whole muscles from Long Evans rats (Antolic et al., 2007; Darling, 2011; Vandommele, 2012). Lastly, media conditions were all set to a pH of 7.4.

3.4. Cell Culture Treatment

L6 cell myotubes were cleared of media and washed 3X with ice-cold PBS. Immediately following, cells incubated in 2 mL of serum-free medium for 1 hr. In separate experiments, myotubes were pre-incubated with appropriate media content as previously described in the CO₂ incubator at 37°C for 4 hr. For cells utilized for protein synthesis, cells were treated additionally with EMEM containing 1.0 µCi of [³⁵S]tyrosine/ml and 2 mM of cold L-tryosine during the 4 hr incubation period. Following incubation, cells were re-washed with ice cold PBS (3x) and collected for protein metabolism analysis.

3.5. Protein turnover measures

3.5.1. Protein synthesis

Each well was treated with 2 mL of 10% trichloroacetic acid (TCA) at a temperature of 4°C and then scrapped. Cells and TCA were then collected and incubated
on ice for 1 hr. The cells were then centrifuged at 20,000 G for 10 minutes at a temperature of 4°C. The resulting pellet was then washed with 1 mL of cold TCA and re-
 centrifuged using the previous settings. The pellet was then left overnight at room temperature dissolving in 0.5 mL of 1 M NaOH and triton X-100. The dissolved pellet was then vortexed and 4 µL will be combined with scintillation fluid. Samples were then counted on an LS Multi Purpose Scintillation Counter (Beckman Coulter, Mississauga, ON).

3.6. Protein extraction and Western blot

Total protein was extracted from another cohort of cells incubated as previously described. Cells were extracted on ice using a RIPA buffer solution containing one phosphatase inhibitor cocktail tablet (PhosStop, Roche Diagnostics, Laval, QU) and one protease inhibitor tablet (Protease Inhibitor Cocktail, Roche Diagnostics, Laval, QU) dissolved in 10 mL dH2O. Following a 1 hr incubation period at 4°C, cells were centrifuged for 15 minutes at 13,000 rpm. The supernatant was then collected and total protein content was determined using the Bradford Assay method (Biotek Instruments, Winooski, VT, USA). Based on the protein values obtained, samples were diluted accordingly with dH2O and sample buffer (0.5M Tris-HCl, 2mL glycerol, 10% SDS, 1% bromophenol blue, 1mL β-mercaptoethanol, and 0.3 mL dH2O).

SDS-PAG electrophoresis was performed using a 7.5% (mTOR and phosphor-
mTOR, ubiquitin, actin and µ-calpain) or 10% (p70s6k and phospho- p70s6k), separating gel and a 4% stacking gel, then immersed in a buffer containing 250 mM Tris-HCL, 1.92 M glycine and 1% SDS. Proteins were transferred onto 0.45 or 0.2 µm polyvinylidene
fluoride membrane (PVDF) using a method previously optimized in our lab. Binding of nonspecific proteins to membranes were blocked by incubating in a blocking buffer of 1 - 2.5 % non-fat dairy milk-TBST solution (TBST buffer: 20mM tris base, 137mM NaCl, and 0.1% (v/v) tween 20, pH 7.5), for 1 hr at room temperature. Membranes were then incubated overnight at 4°C with the following primary antibodies: mTOR - rabbit polyclonal, phosphor-mTOR Ser 2448 - rabbit monoclonal, p70s6k - rabbit polyclonal, phosphor- p70s6k Thr 389– goat monoclonal, ubiquitin- mouse monoclonal, actin goat-polyclonal, and µ-calpain rabbit-polyclonal in milk-TBST solution using a dilution previously optimized in our lab. Following overnight primary antibody incubation, membranes were washed with TBST (4 x 5 min) and subsequently incubated for 1 hr at room temperature with conjugated secondary antibody diluted in milk-TBST or BSA-TBST, again, using a dilution ratio previously optimized for the protein of interest. After another series of washes with TBST (4 X 5 min), proteins were detected with chemiluminescence using a FluroChem 5500 imaging system (Alpha-Innotec, Santa Clara, CA) and bands will be quantified by densitometry.

3.7. Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) to compare N-LEU and E-LEU. When a significant interaction (p<0.05) was observed, a Bonferroni post-hoc analysis was conducted to determine which of the pair wise comparisons are significantly different (HYPO, ISO, HYPER osmolality; N-LEU, E-LEU). Values are reported as the means ± standard mean error. All statistical analyses were completed using GraphPad Prism (version 6.0).
Chapter 4: Results

4.1. Media osmolality

Media osmolality was adjusted immediately prior to skeletal muscle cell incubation to attain the correct osmolalities for HYPO (230 ± 10 mmol/kg), ISO (330 ± 10 mmol/kg) and HYPER (430 ± 10 mmol/kg) (figure 5).

![Figure 5: Osmolality of the 3 different media levels. * Significantly different from ISO (p<0.05).]

4.2. Protein synthesis

Following the 4 hr incubation period with the $^3$H-tyrosine, no statistically significant interactions were observed for protein synthesis. However, a significant main effect for osmolality was observed (p<0.05) (figure 6) such that PS was significantly decreased in HYPER as compared to HYPO and ISO. No differences were observed between the two different leucine conditions.
**Figure 6**: Rate of protein synthesis based on 4hrs of $^{3}$H-tyrosine uptake with each of the 6 conditions. * Significant main effect for osmotic condition (HYPER > HYPO and ISO) (P<0.05).

### 4.3. Western blotting analysis

Comparison of phosphorylated mTOR to total mTOR content revealed no significant differences for leucine concentration groups (N-LEU vs E-LEU) or the 3-osmotic conditions (HYPO, ISO, HYPER). Furthermore, there were additionally no significant interactions observed between any conditions or any main effects (figure 7).

No differences were observed for the ratio of phosphorylated p70S6 kinase content to total p70s6 kinase across the 2 groups (N-LEU vs E-LEU) and the 3 osmotic conditions (HYPO, ISO, HYPER). Additionally no significant interactions or main effects were observed (figure 8).

A significant main effect for leucine was observed for ubiquitinated protein content. The elevated leucine concentration resulted in lower total amounts of ubiquitinated proteins as compared to the low leucine concentration group (Figure 9). Levels of ubiquitinated proteins were not affected by the different osmotic conditions.
Lastly, Western blotting for μ-calpain resulted in no significant interactions or main effects within or between all of the experimental conditions (figure 10), suggesting no affect on calpain activity with the different experimental conditions.

Figure 7: mTOR phosphorylation normalized to total mTOR in skeletal muscle cells exposed to 6 experimental conditions with representative Western blots. Values are expressed in means ± SEM. No significant differences.
Figure 8: Phosphorylated p70S6 kinase normalized to total p70S6 kinase in skeletal muscle cells exposed to 6 experimental conditions with representative Western blots. Values are expressed in means ± SEM. No significant differences.
Figure 9: Total ubiquitinated protein normalized to total actin in skeletal muscle cells exposed to 6 experimental conditions with representative Western blots. Values are expressed in means ± SEM. * Significant from control condition.
Figure 10: Representative Western blot for μ-calpain in skeletal muscle cells exposed to 6 experimental. Unable to produce autolysis form of μ-calpain therefore insufficient data.
Chapter 5: Discussion

In the present study it was investigated if extracellular leucine concentration affects protein metabolism within skeletal muscle cells under various osmotic conditions. This was addressed using the L6 rat cell culture model. Using this model allowed me to study the regulation of muscle protein metabolism within a contained and well-controlled environment (Haegens, Schols, van Essen, van Loon, & Langen, 2012). To our knowledge, this is the first study to investigate the effects of leucine concentration on skeletal muscle protein metabolism during extracellular osmotic stress within L6 skeletal muscle cells.

5.1 Protein synthesis

5.1.2. Osmotic conditions

It has been previously demonstrated that extracellular osmolality can have a metabolic influence in most cells within the human body (Akita & Okada, 2014). Within the current study, estimates of PS, as measured by $^{3}H$-tyrosine uptake were affected by the different osmotic conditions (HYPO, ISO, and HYPER) with a main effect for osmolality observed. HYPO conditions (~230 mmol/kg) resulted in increases in PS compared to HYPER conditions (~430 mmol/kg). PS appeared to be lowest when cells were exposed to the hypertonic media. Compared to ISO, HYPER lead to a 35% decrease in PS. When observing HYPO to ISO, there were no significant differences in PS. These results are consistent with data from researchers within our laboratory and researchers of other laboratories who have previously investigated the influence of extracellular osmotic stress on PS utilizing a variety of different models (Darling, 2011; Roy, 2009; Stoll et al.,
In isolated intact skeletal muscle, HYPO led to an increase in PS of approximately 65% while HYPER lead to a decrease in PS by approximately 50%, compared to the ISO condition (Darling, 2011; Vandommele, 2012). Previous results demonstrated a greater difference in PS between the different osmotic states than what was observed in the current study. The implication of differing osmotic states on PS in isolated rat hepatocytes during hyperaminoacidemia has also previously been researched (Stoll et al., 1992). They observed that HYPER inhibited PS by approximately 60% compared to the ISO condition (Stoll et al., 1992). It is worthy to note that Stoll et al. did not observe any significant differences between HYPO and ISO, which is similar to what was observed in the current study. In another study using a similar model and cell line to the current study, HYPO conditions lead to a non-significant increase in PS of 4% and 2% following an incubation period of 24 hr and 48 hr, respectfully (Roy, 2009). In contrast, HYPER led a 13% and 15% decrease in PS following incubations of 24 and 48 hrs, respectfully (Roy, 2009). These results are similar to what was observed in the current study. Based on our observations and others, it appears that HYPO does not result in an increase in PS in skeletal muscle cells in culture as it does in isolated intact skeletal muscles. This could be the result of the inherent differences in the arrangement and amount of the contractile proteins within these distinctive models. However, HYPER osmotic stress appears to suppress PS in skeletal muscle cells in culture and isolated intact skeletal muscles. These findings support the concept that HYPER osmotic stress leads to declines in PS activity (Lang et al., 1998), however, HYPO does not appear to stimulate PS in L6 skeletal muscle cells.

The decrease in PS with HYPER could have resulted from a number of different
mechanisms. Firstly, it is well established that cells use AAs as a mechanism to counteract changes in extracellular osmolality (Hyde, Taylor, & Hundal, 2003; Lang et al., 1998) AAs accumulate over the longer term to increase the osmolality inside the cell in an attempt to preserve cellular fluid levels. For example, glutamate, glutamine, glycine, taurine and aspartate have all been shown to exert a significant influence on cell volume (Lang, Busch et al. 1998). Interestingly, HYPER has been established as a stimulus for increased AA transporter expression (System A transporters) (Hyde et al., 2003). Theoretically, a decrease in PS could lead to an expansion of the free AA pool in the cells, leading to an accumulation of AAs and a greater osmolality, in an attempt to counter the external hyperosmotic state.

A second possible mechanism that might contribute to the reduction in PS with HYPER could be the macromolecular crowding that would have taken place inside the cells. Such crowding has been suggested to have a role in the detection of cell volume as well as the altered metabolic signalling responses commonly seen with HYPER (Haussinger, 1996). The loss in cellular fluid that occurs with HYPER alters the concentration of cytosolic proteins (e.g. protein kinases and phosphatases) and consequently alters their activity by altering their tendency to associate with substrates. These changes are thought to eventually lead to volume-regulatory responses as well as changes in gene expression and metabolism. Limited research has suggested a link with this crowding, mitogen protein kinase (MAP) regulation and HYPER in hepatocytes. It has been suggested that there is a possible link between osmotic stress and PS, but additional research is necessary, especially in skeletal muscle cells (Haussinger, 1996).
5.1.3. Leucine concentration

It is well established that leucine plays a pivotal role in the regulation skeletal muscle PS. It has been established as a key stimulator of mTOR signaling, thus assisting in the regulation of PS within skeletal muscle (T. A. Churchward-Venne et al., 2014). Within the current study, cells were exposed to an elevated concentration of leucine in the media and it had been hypothesized that an increase in PS stimulation would occur upon exposure to a higher concentration of leucine. This hypothesis was based on previous research, where the dose response of leucine was observed to influence mTOR signaling within skeletal muscle cells (Areta et al., 2014). When comparing the rate of PS between the two groups, N-LEU versus E-LEU, there were no differences observed. It is well established that leucine is a potent simulator of protein synthesis in humans (Churchward-Venne et al. 2014), but mixed results have been observed in muscle cell culture with some reporting increases in PS while others do not (Deldicque et al., 2008; Du, Shen, Zhu, & Ford, 2007; Haegens et al., 2012; S. R. Kimball, Shantz, Horetsky, & Jefferson, 1999). It is possible that a higher dose of leucine is required to stimulate PS in myotubes (Areta et al. 2014) where as myoblasts may be more sensitive to lower doses of leucine (Talvas, Obled, Fafournoux, & Mordier, 2006). Future work should differentiate the responses observed in myoblasts versus myotubes in response to varying doses of leucine. The primary question of the current work was could a higher leucine concentration overcome the effects of HYPER on protein synthesis. This will be addressed in the proceeding section.
5.1.4. Combining the effects of osmolality and leucine levels

To the author’s knowledge, this was the first study to try to utilize an increased leucine concentration to overcome the suppression in PS that is observed with HYPER. The protein synthetic responses were similar in all of the different osmotic groups when they were exposed to the two different leucine conditions (N-LEU and E-LEU). Therefore, despite adding 1.5 mM of leucine to the media, the PS responses to the osmotic conditions remained similar. As discussed earlier, leucine is a potent stimulator of PS signaling (Areta et al., 2014), and PS in myoblasts (Talvas et al., 2006) but mixed results observed in myotubes (Deldicque et al., 2008; Du et al., 2007; Haegens et al., 2012; S. R. Kimball et al., 1999). The influence of osmotic stress in myoblasts remains unclear, however, it is likely that it HYPO and ISO would have little influence on PS, while HYPER would lead to suppression of PS.

It could be argued that possibly having used an even greater concentration of leucine within the extracellular environment, ( > 1.5 mM) could possibly elicit more significant results. The concentration of leucine used in the current experiment was chosen based on a previous study using C2C12 skeletal muscle cells (Areta et al., 2014). They observed that a leucine concentration of 1.5 mM had a significant stimulatory effect on PS signaling, however, they did not have an effect on measures of PS (Areta et al., 2014). They used the uptake of $^3$H-phenylalanine as an estimate of PS rates across 5 conditions (control, and leucine levels of 1.5 mM, 3.2 mM, 5 mM and 16.5 mM) and no differences in PS were observed between any of the conditions (Areta et al., 2014). One difference between the two studies was the incubation time as our study had an incubation time of 4 hrs compared to 1 hr in the study by Areta et al. (Areta et al., 2014).
Despite the increase in incubation time of our study, we still saw no increases in PS with the leucine. Therefore, perhaps an even longer incubation time may be required for leucine to have an influence on PS in myotubes in culture. The influence of the extracellular concentration of leucine has also been studied in human skeletal muscle samples (Glynn et al., 2010). Blood samples and muscle biopsies were taken from the vastus lateralis of healthy subjects before and following the consumption of either a control or high leucine (3.5g) beverage. Interestingly, increases in arterial leucine concentrations were observed with consumption of the high leucine-dose beverage, however no differences were observed for the intracellular availability of leucine in skeletal muscle regardless of the beverage consumed (Glynn et al., 2010). This observation was also consistent with previous research (Bohe et al., 2001). Given that our research used cell culture, it is possible that there may have been a reduction in the activity or availability of AA transporters to move leucine from the extracellular to the intracellular space as suggested by previous researchers (Biolo et al., 1995; Glynn et al., 2010). A reduction or impairment in leucine uptake would have blocked any effect on PS and possibly PS signaling processes. Clearly, more work is required to look at AA transporter activity and availability during osmotic stress.

5.1.5. Protein synthesis cellular signaling

It is well established that mTOR activity within skeletal muscle cells is critical in the regulation of PS. Phosphorylation of this kinase at Ser 2448 has been well documented to contribute to the activation of PS within skeletal muscle cells (Bodine et al., 2001; Schmelzle & Hall, 2000). Beginning with the differing osmotic states, the level of mTOR phosphorylation was found to be similar across all three osmotic conditions. It
has been previously suggested that HYPO would have been a favourable environment for mTOR activation, while HYPER would lead to inactivation of this signal (Haussinger, Kubitz, Reinehr, Bode, & Schliess, 2004; Schliess, Richter, vom Dahl, & Häussinger, 2006). Previous work using incubated isolated skeletal muscles looked at the influence of extracellular osmotic states on phosphorylation of mTOR. Phosphorylation of mTOR in HYPO and HYPER were shown to be significantly higher than ISO (Darling, 2011; Vandommele, 2012). It was interesting that HYPER activated mTOR phosphorylation to a greater extent than ISO, as this contradicted previous literature (Schliess et al., 2006). Regardless, the different osmotic states used in the current study did not impact mTOR signaling in myotubes.

It has been previously suggested that leucine can stimulate mTOR signaling (Atherton et al., 2010; Meijer, 2003; Shimomura et al., 2006; Yoshizawa, Mochizuki, & Sugahara, 2013). In the current study, we did not observe any significant differences between the control condition and condition with the added leucine. Instead, mTOR signaling remained relatively steady compared to the control condition. Further investigation of previous literature suggests that leucine may not have as robust of an effect on mTOR signaling as originally suggested. Contradictory results have been reported using a variety of different experimental models (Apro et al., 2015; Haegens et al., 2012). Recent work in a human model demonstrated that leucine appears not to have the ability to stimulate mTOR (Apro et al., 2015). Participants in this study underwent a 6 week customized heavy leg resistance exercise regime followed by experimental resistance testing at 60%, 75%, 80% and 90% of their 1RM and consumption of a drink with a specific concentration of leucine, which was dependent on the condition they were
assigned to. Muscle biopsies were taken and it was observed that despite the higher muscle concentration of leucine, intracellular signaling of mTOR was not greater when the participant’s drink contained solely leucine compared to those who drank a blend of EAAs (Apro et al., 2015). Therefore, it is possible that a mix of EAAs may be more influential than leucine alone. This could be a possible reason as to why we did not see any significant changes with the added leucine in the current study. However, there are reports in the literature using C2C12 myotubes that support the notion that leucine enhances mTOR signaling (Atherton et al., 2010; Deldicque et al., 2008). Both research studies elicited a significant difference once cells were incubated in greater leucine levels. Studies incubated the cells in 5 mM or 2 mM for 30 minutes and were able to detect a stronger signaling result for the mTOR kinase (Atherton et al., 2010; Deldicque et al., 2008). However, the myotubes used in one of the studies were AA starved prior to exposure to leucine (Atherton et al., 2010), while in the other study the cells used were myoblasts, not myotubes (Deldicque et al., 2008), which have been seen to have differing responses in regards to PS as compared to myotubes. Given that there are mixed results from different research designs, it is difficult to understand exactly why we were not able to see a trend with the added leucine concentrated conditions. More work is needed to differentiate the response in mTOR signaling in myoblasts as compared to myotubes under different osmotic conditions.

One of the downstream targets of mTOR is p70S6 kinase. This kinase has been implicated in the regular cell growth of mammalian cells (Baar & Esser, 1999; Berven & Crouch, 2000). Again, our data was inconclusive and as such did not support previous findings from our lab. It has been demonstrated previously that the phosphorylation of
p70\textsuperscript{S6k} at Thr 389 decreased as extracellular osmotic levels decrease in the cell (Darling, 2011; Vandommele, 2012). When we observe the results for this kinase from our study, we observed no main effects or significant differences for any of the osmotic conditions or different leucine concentrations.

Previous studies have observed that leucine increased p70\textsuperscript{S6k} signaling (Areta et al., 2014; Atherton et al., 2010; Deldicque et al., 2008; Greiwe, Kwon, McDaniel, & Semenkovich, 2001; Haegens et al., 2012). In the current study when leucine was added to the medium, p70\textsuperscript{S6k} phosphorylation did not increase when compared to the control condition. Previous work from the literature looked at the effects of a flooding dose of leucine (5 mM) in human primary skeletal muscles cells (Gran & Cameron-Smith, 2011). They found a significant increase in p70\textsuperscript{S6k} phosphorylation compared to the cells treated with standard media (Gran & Cameron-Smith, 2011). Others have had similar results in C2C12 cells, where cell exposure to leucine at concentrations of 5 mM and 2 mM (for 30 minutes) imposed a significant increase in the activation of this kinase by approximately 70% and 80%, respectfully (Atherton et al., 2010; Deldicque et al., 2008). Due to the higher dose in the studies indicated above, it is possible that our dose of leucine was not high enough to elicit a response or the incubation period was too long for optimal phosphorylation.

5.2. Protein degradation

Protein degradation is an important part of cellular life and function (Lecker et al., 1999). In the present study, we investigated markers for two of the primary protein degradation pathways and how they were affected by the concentration of extracellular
leucine content and extracellular osmotic states. First, looking at the ubiquitin proteasome pathway we were able to detect a significant main effect such that added leucine decreased the amount of total ubiquitinated proteins. The ubiquitin proteasome pathway is a major proteolytic pathway well known for its role in degrading intracellular proteins and releasing free AAs as a result (Ciechanover, 1993a; A. Hershko & Ciechanover, 1992; Nakashima, Ishida, Yamazaki, & Abe, 2005). In the current study the additional leucine appeared to have a positive influence on this pathway due to the ability to reduce the total amount of ubiquitinated proteins. These results were conclusive across all three osmotic conditions when comparing N-LEU/HYPO and E-LEU/HYPO, N-LEU/ISO and E-LEU/ISO, and finally N-LEU/HYPER and E-LEU/HYPER. These data indicate a possible role for leucine in decreasing the activity of this pathway. It was hypothesized that an increase in leucine concentration should decrease the amount of PD, and the current data support this hypothesis. To our knowledge, this is the first study to observe the effects of extracellular osmotic stress and differing leucine concentrations on this PD pathway as many research studies solely focus on PS. One research study examined the effects of leucine (1 mM) on the ubiquitin pathway and that the BCAA was able to significantly attenuate the total of ubiquinated proteins within the cultured chick myotubes (Nakashima et al., 2005). It should also be noted that all three BCAA’s were measured, and as suspected, leucine remained to be the most potent in preventing activation of this pathway. Another study observing the effects of a leucine-supplemented diet had similar effects. Using 8 month and 22 month-old rats, the experimental group was supplemented with 5% leucine in their diet for either one or 10 days. While one day of leucine-supplementation had no effect, 10 days lead to a down-regulation of the
ubiquitin proteasome pathway (Combaret et al., 2005). It is interesting to note that the 8 month-old rats down-regulated this pathway to a greater extent than the 22 month-old rats, indicating that age is a factor in trying to attenuate the total amount of ubiquitin proteins (Combaret et al., 2005). The current work suggests that leucine supplementation can attenuate ubiquitin proteasome activity similarly across different types of osmotic stress.

Lastly we were also interested in the calpain protein degradation system. There are two calpain systems found within skeletal muscle- μ-calpain and m-calpain (Kemp et al., 2013). Due to the nature of this study and resources available, we were solely able to probe for μ-calpain. Typically, it was anticipated that μ-calpain would be in its autolyzed and un-autolyzed state at 76 kDa and 80 kDa, respectfully (Edmunds, Nagainis, Sathe, Thompson, & Goll, 1991). The available calcium concentration required for these two states differ, with the autolyzed state requiring a lower concentration for activation (Edmunds et al., 1991). In the present study, we did not add any additional calcium to the media. It should be noted that the structure of myotubes is not identical to muscle tissue found in mammals. Taking a looking at the structure of the L6 cell line, as cellular passaging and differentiation progresses, the cells are fully capable in forming into myoblasts. However, the nucleus does not continue to form as multiplication continues (Richler & Yaffe, 1970). This understanding provides insight as a possibility for why we were only able to observe μ-calpain in its un-autolyzed state when viewing the Western blot. Calpain requires calcium for activation and calcium is required for various cellular functions, one of them including cell survival, influencing PS and PD, and, to some extent programmed cell death (Berchtold et al., 2000; Leite et al., 2003; Santella &
Carafoli, 1997). Much of this programming occurs in the nuclei. While calcium may have been present in the cell, it is possible that there was simply not enough to stimulate activation of the autolysis of μ-calpain. There appears to be some disconnect for when u-calpain becomes un-autolyzed and in turn, lowering the calcium required for the autolyzed form of u-calpain (Edmunds et al., 1991). It is therefore challenging to determine the effects of leucine concentration and osmolality levels on the calpain system due to the possible limitation of the model used for experimentation. In conclusion we cannot comment on any differences observed in any of the conditions. Despite inconclusive observations of this PD system, there are general conclusions in research that implies AA’s and as such, leucine, simply help suppress PD overall in skeletal muscle (Bajotto et al., 2011; Wolfe, 2002). The following is an illustration the data observed in PS and PD signaling pathways in figure 11 and 12.

Figure 11: The primary finding when cells were exposed to one of three osmotic conditions were: HYPO led to an increase PS activity and HYPER led to a decrease in PS activity.
Figure 12: The primary finding when leucine was added to the medium was a decrease in ubiquitin proteins due to a decrease in the ubiquitin proteasome pathway. This was observed across all three osmotic conditions. mTOR, p70S6k, and µ-calpain did not have any significant data, therefore their role remains questionable based on the current study.
Chapter 6: Conclusion and Future Directions

6.1. Conclusion

It is well established that leucine has an important role in skeletal muscle metabolism. In general, PS in skeletal muscle cells was stimulated in extracellular HYPO to a greater extent than extracellular HYPER. It was hypothesized that leucine would decrease the amount of PD under HYPER stress. The study revealed that PD was down regulated with the addition of leucine treatment to the L6 cells in the ubiquitin proteasome pathway. This was not only observed in HYPER, but also in the HYPO and ISO conditions. These observations suggest that the BCAA leucine remains an excellent possibility for reducing muscular atrophy. What we cannot confidently conclude from this study is the influences of leucine on PS during period of extracellular osmotic stress. It was hypothesized that an elevated leucine concentration would elicit and increase in PS. However, as a result of our inconclusive data, further questions remain as to the role of leucine in promoting PS during osmotic stress. The current study is the first to investigate the relationship of extracellular leucine concentration and differing cellular osmotic stresses. Furthermore, we sought to observe how one component influences the other in skeletal muscle cells. Previous research determined the importance of both leucine and cellular osmotic shifts separately within skeletal muscle (Apro & Blomstrand, 2010; Lang et al., 1998; Sjogaard & Saltin, 1982; Stipanuk, 2007). In conclusion, leucine and hypo-osmotic stress contribute to a favourable environment for anabolic events to occur.

6.2. Future directions
After completing the study, the additive effects of leucine and osmolality remain unclear when considering PS and PD. In the future, it would be best to look at changing the dose of leucine and/or the length of incubation time. The dose of added leucine may not be strong enough to stimulate significant changes from the control media content. Additionally, it is possible that the 4-hour cell incubation with each condition was not long enough for changes to occur given that some of the mechanistic pathways for PS and PD are multi-step systems. Additionally, the responses to these different conditions should be investigated in myoblasts and myotubes. More specifically, they should be investigated during the differentiation process of myoblasts into myotubes. During this transition, higher rates of protein turnover occur potentially leading to a greater impact, similar to how larger difference in protein turnover in human skeletal muscle is observed following resistance exercise. The next step would be to take the experiment from a cell culture model to perhaps animal or human model. This would detect if we can truly overcome muscular atrophy when leucine is added to a hypotonic saline solution and administered to humans. If positive results were observed using an animal model, this would then create implications for real word situations where PS and PD are extremely important for life.
References


water channel. *J Biol Chem*, 287(14), 11516-11525. doi: 10.1074/jbc.M111.329219


Darling, R. (2011). *Protein turnover and regulation in rat skeletal muscle during in vitro hypo-osmotic stress* Brock University


Vandommele, C. (2012). *REGULATION OF PROTEIN TURNOVER DURING HYPER-OSMOTIC STRESS IN SKELETAL MUSCLE*. (Masters of Science), Brock University


APPENDIX A: Eagle’s Modified Medium

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine hydrochloride</td>
<td>0.6</td>
</tr>
<tr>
<td>L-Cystine 2HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Histidine hydrochloride-H2O</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.2</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Tyrosine disodium salt dehydrate</td>
<td>0.2</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline chloride</td>
<td>0.007</td>
</tr>
<tr>
<td>D-Calcium pantothenate</td>
<td>0.002</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0.002</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>0.008</td>
</tr>
<tr>
<td>Pyridoxal hydrochloride</td>
<td>0.005</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>2.695</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>0.003</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>0.011</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inorganic Salts</th>
<th>nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Chloride (CaC12)</td>
<td>1.8</td>
</tr>
<tr>
<td>Magnesium Sulfate (MgSO4)</td>
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</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>5.3</td>
</tr>
<tr>
<td>Sodium Bicarbonate (NaHCO3)</td>
<td>26.2</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>117.2</td>
</tr>
<tr>
<td>Sodium Phosphate monobasic (NaH2Po4-H2O)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Components</th>
<th>nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose (Dextrose)</td>
<td>5.6</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.03</td>
</tr>
</tbody>
</table>
APPENDIX B: Laboratory procedures

B.1. Protein synthesis protocol

Media and cell preparation
1. Prepare media with appropriate condition (see chart below), pH to 7.4 and place in labeled falcon tube.

<table>
<thead>
<tr>
<th>Control</th>
<th>[High] LEU</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYPO (220 mmol/kg)</td>
<td>HYPO (220 mmol/kg) + 1.5 mM Leucine</td>
</tr>
<tr>
<td>ISO (330 mmol/kg)</td>
<td>ISO (330 mmol/kg) + 1.5 mM Leucine</td>
</tr>
<tr>
<td>HYPER (430 mmol/kg)</td>
<td>HYPER (430 mmol/kg) + 1.5 mM Leucine</td>
</tr>
</tbody>
</table>

2. Aspirate off current media on cells.
3. Wash 3x with cold PBS.
4. Add 2mL of serum free media to each well and incubate at 37°C for 1 hour.
5. Add 1.0 µCi of $[^{3}\text{H}]$-tyrosine/ml and 2 mM of cold L-tryosine to each media condition. Allow falcon tubes to sit in bead bath to mix.
6. Discard media and wash 3x with cold PBS.
7. Add 2mL of a media to each well ensuring each well (or plate) represents a condition. Number of plates required in dependent on experiment.
8. Incubate at 37°C for 4 hours.
9. Quickly aspirate off media and wash 3x with cold PBS.

Protein extraction
1. Add 2mL of 10% TCA (4°C) to each well and sit on ice for 10 minutes.
2. Scrape cells and transfer, with TCA, to 2mL eppendorfs.
3. Place eppendorfs on ice for 1 hour.
4. Spin down samples at 20,000g for 10 minutes at 4°C.
5. Remove TCA and add 1mL of fresh-cold TCA.
6. Spin down samples at 20,000g for 10 minutes at 4°C.
7. Remove TCA.
8. Add 0.5mL of 0.1M NaOH/1% triton x-100 and let sit overnight at room temperature in dark location.

Scintillation counter preparation
1. Vortex samples after sitting overnight.
2. Add 4mL of scintillation fluid to scintillation vials.
3. Add 0.4mL of sample into the vial
4. Quick vortex
5. Allow scintillation counter to read samples. Complete readings 5 times.
6. Calculate the cpm mean from the 5 scintillation readings.
**Protein concentration- Bradford assay**

1. Prepare all samples by loading 60µL of protein samples and 300µL of dH₂O into individually labeled eppendorfs.
2. Create standard curve following table C1 below.
3. Using a 96-well plate, add 10uL standard curve and samples to each well in triplicate.
5. Let incubate for 5 minutes and open KC4 software.
6. Set reading parameters to absorbance at wavelength 595nm, shaking intensity level 3, and shaking duration of 5 seconds.
7. Copy the absorbance readings into a Microsoft Excel spreadsheet and generate a standard curve by plotting known protein concentrations (from BSA standard solutions) against their average absorbance readings.
8. Use the curve equation to determine the y-intercept.
9. Set protein equation to multiply by 6 (to represent the dilution factor).
10. In a new column, provide the total protein within a sample by multiplying protein concentration by 4.

Protein Synthesis = mr (cmp) / mg protein

**B.2. Protein extraction procedure**

RIPA Solution
- 9mL dH₂O
- 1mL RIPA
- 1 Protease Inhibitor Tablet
- 1 Phosphatase Inhibitor Tablet
- Mix on stir plate

1. Aspirate media (with condition) off.
2. Wash 2 x with cold (4°C) PBS and aspirate off.
3. Add 80µL RIPA solution per well/6-well plate.
4. Scrape on ice and pipette protein into 1.5mL eppendorf.
5. Quickly vortex samples.
6. Put on turning table at 4°C for at least 1hr at medium speed.
7. Centrifuge for 15 minutes at 13,000 rpm at 4°C.
8. Remove supernatant and put into newly labeled eppendorf.
9. Store protein at -80°C until ready for use.

**B.3. Western blotting**

**Reagents**

Laemmli Buffer (2x Sample Buffer)
- 2.5mL 0.5M Tris-HCl pH 6.8
- 2mL glycerol
- 2mL 10% SDS
- 0.2mL 1% bromophenol blue
- 1mL β-mercaptoethanol
- 0.3mL dH₂O

**Running Gel Buffer**
- 1.5 Tris-HCl, pH 8.8

**Stacking Gel Buffer**
- 0.5M Tris-HCl, pH 6.8

**10x Running Buffer**, pH 8.3
- 250mM Tris base (15.15g)
- 1.92M glycine (72.05g)
- 1% SDS (50mL)
- 450mL dH₂O
- Store at 4°C

**10x Transfer Buffer** (semi-dry)
- 312.5mM Tris base (3.03g)
- 2.4M glycine (14.4g)
- fill to 100mL with dH₂O
- Before use, dilute 80mL in 720mL dH₂O and add 200mL methanol

**10X TBS**
- 200mM Tris base (24.2g/L)
- 1.37M NaCl (80.1g/L)
- 38mL 1M HCl/L of TBS made
- adjust to pH 7.5

**TBST**
- dilute 100mL of 10x TBS in 900mL dH₂O
- add 4mL of 25% Tween 20 while stirring (polyoxyethylenesorbitan monolaurate)

**Protein determination (Bradford assay)**

**Standard Curve Preparation**
1. Retrieve all samples from -80°C storage and place on ice.
2. Retrieve one aliquoted sample of 1mg/mL stock BSA solution from -20°C storage.
3. Prepare all BSA standard solution in eppendorf labelled tubes.

**Table C1: Volumes of stock BSA solution and dH₂O required for Bradford protein assay standard solutions.**
<table>
<thead>
<tr>
<th>Protein Concentration (mg/mL)</th>
<th>Stock BSA Solution (µL)</th>
<th>dH₂O (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>350</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>250 (from 1 mg/mL)</td>
<td>250</td>
</tr>
<tr>
<td>0.25</td>
<td>250 (from 0.5 mg/mL)</td>
<td>250</td>
</tr>
<tr>
<td>0.125</td>
<td>250 (from 0.25 mg/mL)</td>
<td>250</td>
</tr>
<tr>
<td>0.05</td>
<td>50 (from 0.5 mg/mL)</td>
<td>450</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>250</td>
</tr>
</tbody>
</table>

4. Prepare all samples by loading 10µL of protein samples and 90µL of dH₂O into individually labeled eppendorfs.
5. Pipette in triplicate 10µL of each sample (standard and diluted samples) into 86-well plate.
7. Let incubate for 5 minutes and open KC4 software.
8. Set reading parameters to absorbance at wavelength 595nm, shaking intensity level 3, and shaking duration of 5 seconds.
9. Copy the absorbance readings into a Microsoft Excel spreadsheet and generate a standard curve by plotting known protein concentrations (from BSA standard solutions) against their average absorbance readings.
10. Use the curve equation to determine the y-intercept and multiply sample concentrations by 10 to determine the protein concentration of each sample.

**Sample preparation for Western blotting**

1. To prepare samples for blotting first determine the sample concentration needed (done through previous optimization with the protein you are probing for). Using the results from the Bradford assay, calculate the sample volume, sample buffer volume, and water volume. Follow table below:

<table>
<thead>
<tr>
<th>Total Volume (TV)</th>
<th>50 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample Volume (SV)</strong></td>
<td>SV = TV - Desired Final Protein/Protein</td>
</tr>
<tr>
<td><strong>Sample (Laemelli) Buffer (SB)</strong></td>
<td>SV = TV - TV/5 (i.e. For 5x SB)</td>
</tr>
<tr>
<td><strong>Water (dH₂O)</strong></td>
<td>dH₂O = TV - SV - SB</td>
</tr>
</tbody>
</table>

2. Boil prepared samples for 5 minutes and subsequently place on ice for 5 minutes.

**Electrophoresis preparation**

1. Wipe glass plates with methanol and a kimwipe.
2. Assemble the glass holding apparatus with the short plate facing the front.
3. Ensure plates are flush and level before locking and inserting onto the gel casting stand.
4. Prepare gels (makes 2):
### Table

<table>
<thead>
<tr>
<th>Percent Gel</th>
<th>dH₂O (mL)</th>
<th>Protogel (mL) (30% acrylamide)</th>
<th>Gel Buffer (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (stacking)</td>
<td>6.1</td>
<td>1.3</td>
<td>2.5 Stacking</td>
</tr>
<tr>
<td>6</td>
<td>5.3</td>
<td>2.0</td>
<td>2.5 Resolving</td>
</tr>
<tr>
<td>7.5 (Running)</td>
<td>4.85</td>
<td>2.5</td>
<td>2.5 Resolving</td>
</tr>
<tr>
<td>8</td>
<td>4.62</td>
<td>2.67</td>
<td>2.5 Resolving</td>
</tr>
<tr>
<td>10 (Running)</td>
<td>3.96</td>
<td>3.33</td>
<td>2.5 Resolving</td>
</tr>
<tr>
<td>12</td>
<td>3.29</td>
<td>4.0</td>
<td>2.5 Resolving</td>
</tr>
</tbody>
</table>

5. Before loading the **running gel**, add 100µL APS (50mg/500µL) and 10µL of TEMED. Swirl and load until reaching the depth of the comb. Use methanol to remove any bubbles. Dump once hardened.

6. Before loading the **stacking gel**, add 100µL of APS and 20µL TEMED. Swirl and load until the top of the short plate is reached. Insert combs and let sit till hardened.

7. Prepare running buffer (50mL 10x running buffer + 450mL dH₂O).


9. Fill the inner chamber of the apparatus with diluted running buffer and check of any leaks. Continue to add ~200mL to the outside chamber.

10. Load the standard (5µL) and the appropriate amount of sample into each well. Determine the appropriate amount of sample to load by performing separate optimizations with varying amounts of protein.

11. Run electrophoresis at a pre-determined voltage and length of time. (Note: see Protein Specific Western Blotting chart for more information). If tiny bubbles are visible, electrophoresis is working.

**Transfer**

1. Prepare transfer buffer (See above for buffer solution reagent).

2. Soak all transfer material in transfer buffer to equilibrate in plastic tray.

3. Create the “transfer sandwich” in one of the filled trays as followed:
   - Black side of the holder
   - Black sponge pad
   - White fibre pad
   - Filter paper
   - Gel
   - Membrane (PVDF pre-soaked in methanol for 5-10 minutes then rinsed in transfer buffer)
   - Filter paper
   - White fibre pad
   - Black sponge
   - White/clear side of the holder

4. Place the sandwich into the holders with black side facing the black side of the holder and clear/white facing the red.

5. Fill the tank with transfer buffer, adding an ice pack and stir bar.
6. Place the tank in a larger container and surround with ice. This will prevent from cooking the gel. Place on stir plate.
7. Run the transfer at a pre-determined voltage and length of time. (Note: see Protein Specific Western Blotting chart for more information).

**Antibodies**

1. Discard filter papers and gel and transfer membranes to petri dish. Block membranes with 20mL milk-TBST solution for 1hr. Milk % is dependent on protein of interest. (Note: see Protein Specific Western Blotting chart for more information).
2. Discard blocking solution and wash membrane 4 times for 5 minutes with TBST.
3. Add 1° antibody in appropriate solution (milk- or BSA-TBST) and dilution. Sit overnight on shaker at 4°C.
4. The next day, wash membrane 4 times for 5 minutes with TBST.
5. Add 2° antibody (dependent on 1° antibody) in appropriate solution and dilution for 1hr.

**Enhanced chemiluminescence**

1. Wash membrane 4 times for 5 minutes with TBST.
2. Combine 1mL of each chemiluminescent HPR substrate (peroxide solution + luminal reagent) over membrane. Continue to pipette over membrane for 5 minutes.
3. Blot membrane dry and place between two transparency sheets, ensuring no air bubbles.
4. Expose the membrane using the FluroChem imaging system. Exposure time is dependent on the protein of interest. (Note: see Protein Specific Western Blotting chart for more information).
5. Determine the density of the bands using “Image J” software.
Appendix C: Protein specific Western blotting information

<table>
<thead>
<tr>
<th></th>
<th>Anabolic Markers</th>
<th>Catabolic Markers</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td>mTOR</td>
<td>p-mTOR</td>
<td>p70S6K</td>
</tr>
<tr>
<td><strong>Sample Load</strong></td>
<td>5 µg</td>
<td>5 µg</td>
<td>10 µg</td>
</tr>
<tr>
<td><strong>Stacking Gel</strong></td>
<td>4%</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td><strong>Running Gel</strong></td>
<td>7.5%</td>
<td>7.5%</td>
<td>10%</td>
</tr>
<tr>
<td><strong>Electrophoresis</strong></td>
<td>150min @ 100V (on ice)</td>
<td>150min @ 100V (on ice)</td>
<td>90min @ 120V</td>
</tr>
<tr>
<td><strong>Membrane</strong></td>
<td>0.45µm PVDF</td>
<td>0.45µm PVDF</td>
<td>0.2µm PVDF</td>
</tr>
<tr>
<td><strong>Transfer</strong></td>
<td>120min @ 100V</td>
<td>120min @ 100V</td>
<td>90min @ 100V</td>
</tr>
<tr>
<td><strong>Blocking</strong></td>
<td>5% milk/TBST for 1hr</td>
<td>5% milk/TBST for 1hr</td>
<td>2.5% milk/TBST for 1hr</td>
</tr>
<tr>
<td><strong>1° Antibody</strong></td>
<td>1:1000 rabbit polyclonal in 5%BSA/TBST overnight</td>
<td>1:1000 rabbit polyclonal in 5%BSA/TBST overnight</td>
<td>1:1000 rabbit polyclonal in 5%BSA/TBST overnight</td>
</tr>
<tr>
<td><strong>2° Antibody</strong></td>
<td>1:5000 anti-rabbit in 5%milk/TBST for 1 hr</td>
<td>1:5000 anti-rabbit in 5%milk/TBST for 1 hr</td>
<td>1:5000 anti-rabbit in 5%milk/TBST for 1 hr</td>
</tr>
<tr>
<td><strong>Exposure</strong></td>
<td>2 min</td>
<td>1 min</td>
<td>2 min</td>
</tr>
</tbody>
</table>

88
Appendix C: Representative blots

1. Total mTOR

2. Phospho-mTOR

3. Total p70s6 kinase

4. Phospho-p70s6 kinase

5. Ubiquitin

6. Actin

7. µ-Calpain