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The Role of Leucine and Branched Chain Amino Acids in Regulating Protein Synthesis in Myotubes

Abstract

Leucine, isoleucine, and valine make up a distinct class of amino acids known as branched chain amino acids (BCAAs). BCAAs, especially leucine, have been shown to increase protein synthesis in skeletal muscle cells by activating the mammalian target of rapamycin (mTOR), a protein kinase. Leucine has also been show to directly increase the concentration of key initiation factors for protein synthesis, such as eIF4G. In addition to stimulating protein synthesis in muscle cells, leucine also slows protein degradation by binding to ubiquitin (Ub) proteasomes and preventing proteolysis. While leucine appears to be the main BCAA involved in increasing skeletal muscle protein synthesis, studies have determined that the addition of leucine without the other two BCAAs has a potential negative impact. The cellular presence of any of the three BCAAs activates branched chain ketoacid dehydrogenase (BCKD), an enzyme breaks down all three of the BCAAs. Due to the activity of BCKD it is generally believed that leucine should be administered in a proper ratio with the other BCAAs to avoid an imbalance. Muscle cells in a starved or exhausted state respond the most to the addition of BCAAs. While much has been learned about BCAAs' impact on skeletal muscle cells, many questions remain unanswered. Using C2C12 and L6 cells, two cultured cell lines that are widely studied models of normal skeletal muscle, we will determine what ratio of leucine to other BCAAs best stimulates protein synthesis. C2C12 and L6 myotubes will be tested in multiple trials involving the addition of BCAAs in different concentration ratios. Whether or not insulin synergistically works with BCAAs to increase protein synthesis will also be tested. We will also determine if leucine has any effect on the level of the myogenic factor MRF4 protein, because increased MRF4 has previously been found to be associated with muscle growth. If MRF4 protein is increased, the possibility that these treatments increase levels of MRF4 gene transcripts will be examined.

Aim #1: Compare how C2C12 (mouse) and L6 (rat) muscle cell lines respond to the administration of leucine/BCAAs

Administration of leucine alone increases BCKD activity, potentially causing an amino acid imbalance and negatively impacting the cell. We plan to test how total cellular protein levels vary depending on the ratio of BCAAs administered to C2C12 and L6 cells. Insulin’s effects in conjunction with leucine will also be examined. Protein levels will be measured via spectro-photometric protein assay.

Aim #2: Determine if MRF-4 protein level increases with leucine/BCAA concentration

After the best culture conditions and cell line with the best protein response to leucine is determined, we will see if MRR-4 protein level increases in response to the addition of leucine. Western blot with a commercial anti-MRF4 antibody will be used to determine levels of MRF-4.

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Aim #3: Determine if BCAAs increase MRF4 mRNA

If MRF-4 protein increases in response to BCAAs, a RT-PCR will be performed to see if there is an increase in MRF-4 mRNA. This would demonstrate a link from the known translational regulation effect of BCAAs to transcriptional regulation of MRF-4 (and likely other genes as well).

Project Goals:

The goal of this research project is to further understand how leucine/BCAAs impact skeletal muscle cells. A more precise understanding of how leucine/BCAAs impact protein synthesis could greatly benefit athletes, trauma victims, and the elderly by speeding muscle recovery and preventing muscle wasting. By determining a specific ratio of nutrients to optimize protein synthesis with leucine, many citizens experiencing muscle loss due to trauma, disease, or aging could benefit greatly. Also, any evidence linking BCAAs to MRF-4 expression could help us better understand the specifics of how leucine is able to increase protein in skeletal muscle.

Introduction

Protein synthesis is the process of a cell building new proteins from amino acids. This involves DNA being transcribed to mRNA, which leaves the nucleus to enter the cytoplasm. Ribosomes, with the help of translational RNA (tRNA), synthesize the peptide by reading the mRNA and linking specific amino acids together. The faster this process occurs, the quicker skeletal muscle cells can recover from various types of trauma. This process essential for all life is especially important in skeletal muscle cells due their activity level. Many proteins work to regulate protein synthesis by binding to a gene or ribosome and regulating its activity.

The essential amino acid leucine plays several major roles in muscle cells, such as preserving glycogen, maintaining muscle tissue, and regulating protein synthesis. It is also a branched chain amino acid (BCAA) along with isoleucine and valine. Human skeletal muscle is composed of approximately 33% BCAAs. Leucine has been shown in several studies to increase protein synthesis in skeletal muscle by activating the mTOR protein kinase and directly increasing the concentration of eIF4E. Protein synthesis increase via leucine is believed to be greatest in starved or fatigued muscle tissue. While leucine has been shown to do this independently of isoleucine and valine, branched chain ketoacid dehydrogenase (BCKD) activity increases whenever BCAAs are administered. This suggests BCAAs should be administered in an optimal ratio to avoid an amino acid imbalance. In addition to increasing protein synthesis, leucine has been shown to reduce protein breakdown in muscle by inhibiting the ubiquitin (Ub) proteolytic pathway (Wilson, 2006).

A number of studies have been done involving leucine and exercise. Exercised rats experienced an increase in BCAA oxidation, directly lowering leucine levels in muscle cells (Norton, 2006). This suggests that administering leucine/BCAAs during or after exercise could benefit the cell. The same study found that leucine increased protein synthesis 15-30% more in starved rats than fed rats. Research by Kobayahsi (2006) determined that leucine administration by itself is not as effective as when leucine is added with other essential amino acids (EAAs) (2006). Rats receiving the full spectrum of EAAs + leucine experienced increased protein synthesis for 8 times as long as the leucine only group. As a result of these studies, leucine and BCAA products have become very popular among athletes. BCAAs are also sometimes used to help trauma victims recover, and to help the elderly retain muscle mass as they age.

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While current research suggests leucine increases skeletal muscle protein synthesis, there are several things unknown about how it accomplishes this. It is likely that leucine also increases protein synthesis by some other means besides mTOR or eIF4E, such as increasing the concentration of MRF-4 or similar myogenic factors related to muscle growth. Dr. Hinterberger at the University of Alaska Anchorage has been conducting extensive research on MRF-4 in *Xenopus.* MRF-4 works to increase protein synthesis and has been shown to be present at higher levels after exercise (Psilander et al 2003). While no current work is being done with BCAAs or leucine in his lab, I plan to begin this new project with Dr. Hinterberge's help, and determine if leucine administration to C2C12/L6 myotubes has any effect on MRF-4 levels. I will also attempt to determine which nutrient ratios best enables leucine to increase protein synthesis. This includes things such as the ratio of the BCAAs administered, and the effect of insulin with BCAAs.

An extensive understanding of leucine's role as a protein synthesis modulator in skeletal muscle will greatly benefit athletes, trauma victims, the elderly, and possibly those with degenerative diseases. By determining what nutrients best support leucine in muscle cells we will be able to develop more effective treatments for those with injuries. It will also enable people to retain more muscle mass as they age, ultimately supporting their long term health.

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Research and Design Methods:

Aim #1: Compare how C2C12 (mouse) and L6 (rat) muscle cell lines respond to the administration of leucine/BCAA

Both C2C12 and L6 cell cultures will be grown according to the procedures in use in Dr. Hinterberger's lab. The control groups will receive the amino acid concentrations in standard Dulbecco's minimal essential medium (DMEM). There will be many experimental groups, each receiving specific amounts of leucine, isoleucine, valine, and insulin in addition to DMEM. For example, some cell cultures will receive only leucine, while others may receive a 2:1:1 ratio of leucine: valine: isovaline along with insulin. Many trials will be used in order to find which ratio of nutrients best increases protein synthesis. Protein levels will be measured by scraping and homogenizing the cells, and then using a Bradford spectrophotometric protein assay. In order to demonstrate that protein levels are specifically elevated rather than simply due to increased cell proliferation, DNA concentrations in each homogenate will be measured fluorometrically and the ratio of protein to DNA will be determined.

Aim #2: Determine if MRF-4 protein level increases with leucine/BCAA concentration

After we determine which condition best elevates total protein synthesis in the L6 and C2C12 myotube cultures, we will determine if MRF4 protein levels increase specifically. A western blot with an anti-MRF4 antibody will be performed to detect levels of MRF-4 in the cells, compared to cultures receiving standard BCAAs and no insulin. Samples taken from cell cultures will be homogenized and samples will be run on SDS-PAGE gels. The proteins will then be transferred to a nitrocellulose membrane and immunochemical detection with an MRF4 antibody will be performed according to standard procedures. By comparing MRF4 levels in the control vs. the protein-optimized condition, we will be able to determine if nutrients play a role in increasing cellular MRF4 protein levels. If this is shown, we will then test to see if the MRF4 mRNA is also increased.

Aim #3: Determine if BCAAs increase MRF-4 mRNA

If Aim #2 shows a direct correlation between BCAA administration and an increase in MRF4 protein levels in the cell, we will test for an increase in MRF4 mRNA. This will be done by performing RT-PCR. Total RNA will be prepared from cell cultures according to standard protocols in Dr. Hinterberger's lab. Complementary DNA will be prepared with a commercial kit. The levels of various transcripts, including both muscle-specific and other genes, will be determined with endpoint PCR. Primers for specific detection of these transcripts are either available in Dr. Hinterberger's lab or will be orderd, based on published DNA sequences.

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Anticipated Results:

Previous research suggests that skeletal muscle cells respond better to a blend of BCAAs rather than leucine alone (Wilson 2006). This is primarily due to BCKD activity. This leads me to believe that the C2C12 and L6 myotubes will respond best to a nutrient ratio consisting of leucine, isoleucine, and valine. Past studies suggest that "starved" cultures will respond the most to BCAA supplementation (Norton 2006). Since leucine is more prominent in cells and is the only BCAA directly linked to triggering protein kinases such as the mTOR, I hypothesize that a 2:1:1 leucine to valine to isoleucine ratio will best increase protein synthesis in the myotubes. While it has been shown that leucine alone can increase protein synthesis, the presence of the other 2 BCAAs may increase the extent of this protein synthesis increase experienced. In addition to this, I believe adding insulin to this mixture will further increase protein synthesis since insulin helps facilitate amino acid transport into the cell. I believe the spectro-photometric protein assays will confirm this hypothesis. To ensure consistency between all trials, we will look at the results in terms of protein to DNA ratio, rather than protein level alone. Our initial results will also guide us in experimenting with other various administered BCAA/insulin ratios throughout the research. Also, I predict that both L6 and C2C12 myotubes will respond best to similar conditions based on their genetic similarity. Once the ideal nutrient ratio for increasing protein synthesis is determined, future research should include oral administration of such a ratio to rats or mice. Muscle biopsies could then be taken and the level or protein synthesis increase could be compared to those levels experienced in the L6 and C2C12 myotubes. It would also be beneficial to experiment with such a BCAA ratio in human muscle, specifically in trauma victims and the elderly (or anyone else prone to muscle loss). Determining the optimum ratio of BCAAs in myotubes would open many doors for improving the treatment of citizens prone to muscle loss.

There is reason to believe that MRF-4 protein levels may be correlated with intracellular leucine and BCAA concentration. MRF-4, a myogenic factor, plays an important role in muscle hypertrophy and has been found to be present in higher concentrations in skeletal muscle after resistance exercise (Psilander et al 2003). Since leucine directly increases muscle protein synthesis through several mechanisms which are not entirely understood, it would make sense if leucine somehow up-regulated the level of MRF-4 in order to increase protein synthesis. I predict the western blot will show increased MRF-4 levels as leucine concentration is increased. Evidence of this would raise several questions. Future research would be aimed at determining if leucine also up-regulates other related myogenic factors such as MyoD, Myf5, and Myogenin. With evidence of increased MRF-4 levels via leucine, I would expect the RT-PCR to show an increase in MRF-4 mRNA. If proven, future experiments would need to explore what other genes leucine possibly initiates.

The potential of leucine and BCAAs as a protein synthesis modulator is very exciting. Whether citizens are recovering from various types of muscular trauma, or are experiencing muscular atrophy with aging, there is a wide array of patients who would greatly benefit from BCAAs. I believe this research project will advance leucine research several steps further by determining which nutrients best support its regulation of protein synthesis and by further understanding the cellular mechanisms it uses to increase protein synthesis.

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Proposed Budget:

-RT-PCR Kits: $600

-Antibodies: $1000 [Santa Cruz Biochemical MRF4 antibody retailer]

-Western Blot Kits: $300

-DMEM (growth medium): $1500

- L6 and C2C12: $400

- Sterile Plastic ware, filters, plates: $500

- Insulin/BCAA/Other Supplies: $200

-Assay kits for DNA: $500

Total= $5000

Budget Justification:
All of the above materials are essential to the experiments. For what we have in mind this should be sufficient in funding our project. Several antibodies will be needed, including one for MRF-4 and one for Myosin (to be used as the control).

Literature Cited

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Norton L and Layman D 2006. Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. Journal of Nutrition136: 533S-537S.

Psilander et al 2006. Resistance exercise alters MRF and IFG-1 mRNA content in human skeletal muscle. The Journal of Applied Physiology 1152. 21.

Wilson JM 2006. Leucine's general effects on muscle growth and protein balance. Journal of Hyperplasia Research 6(3): 1-13.

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Timeline:

March-April 2008: Enter lab and practice growing C2C12 and L6 cultures. Continue to plan other aspects of the project.

May-August 2008: Carry out Aim #1 of the project. This time frame will vary depending on the results obtained and further trials that may be needed

September 2008 – January 2009: Carry out Aim's 2-3. Analyze data from Aim #1

February –March 2009: Analyze data and work on completing my final report of the project. Prepare presentation for Undergraduate Research Symposium.

April 2009: Present research at Undergraduate Research Symposium and also AHI members.