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Aaron's paper received a *Quaesitum* outstanding paper award.

Aaron Persinger
The Effect of Leucine Supplementation on Liver Protein Synthesis in a Cancer Cachexia Model

Faculty Sponsor
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Abstract

Cancer-induced cachexia plays a major role in the interruption of protein synthesis pathways in both the skeletal muscle and the liver. Diminishing protein synthesis leads to muscle atrophy and loss of quality of life. Branched chain amino acids, specifically leucine, have been shown to increase protein synthesis in both diseased and regular populations. We supplemented leucine into the food of mice to see if leucine could counteract the effect of cachexia. We wanted to see how exactly this happened, so we looked at 6 different proteins run on Western Blots to analyze the protein pathway. Our results seem to suggest that our dose of leucine does not impact liver protein synthesis in a cancer cachexia model. There may be a different pathway the liver uses to promote protein synthesis.

Introduction

Cachexia is a metabolic disease where the patient involuntarily loses body mass that occurs alongside another illness or disease. (Von Haehling & Anker, 2017). Cachexia differs from regular weight loss because in a cachectic model, a disproportionate amount of skeletal muscle atrophies (Evans et al., 2008). Metabolically, cachexia reduces protein synthesis and interferes with the protein messenger chain within the muscle and the liver. In cancer patients, 30% of all deaths can be attributed to cachexia and when patients with cancer die, approximately 50% of them have cachexia present (Von Haehling & Anker, 2010). Cachexia is diagnosed when, alongside skeletal muscle loss, weight loss exceeds 5% in the previous year, abnormalities in the biochemistry of the body are present, and common characteristics of cachexia such as fatigue are found. Cachexia is associated with increased mortality and a very poor quality of life (Von Haehling & Anker, 2010).

Cachexia causes inflammation to spread throughout the entire system (Vaughan, Martin, & Lewandowski, 2013). The protein STAT3, as well as pro-inflammatory cytokines such as IL1 and IL2, are increased. (Morley, Thomas, & Wilson, 2006). These inflammatory markers are associated with an increased metabolism, leading to some of the weight loss associated with cachexia. The presence of these cytokines leads to an increase in the number of adrenal gland hormones in the body. Resting metabolism is increased due to catecholamines circulating, which increases the energy demands of the body. Another cause of low quality of life is a decrease in available energy. One process that causes a decrease in available energy is futile metabolic cycling, during which the tumor gives off lactic acid. The body sends this lactic acid to the liver, where it enters the Cori Cycle. In the Cori Cycle, lactic acid becomes pyruvate, which becomes glucose. The body then sends this glucose circulating throughout the body. Specifically, it goes to the tumor to be converted to lactic acid again. This high energy process may even use the glucose to feed the tumor and help it grow (Morley et al., 2006).

The liver plays an important role in meeting the energy demands of the entire body by regulating both lipid and glucose metabolism. Depending on the energy demands placed on the body, the liver will send different substrates to different areas to meet the imposed demands. Within the liver, a process called deamination occurs. Deamination is the process

by which the body converts amino acids into useable components. The liver is also a location for the Urea cycle. The Urea cycle is the process by which ammonia, which is highly toxic, is removed from the body. The liver regulates lipid and glucose metabolism, supplying energy to the entire body based upon imposed demands. Further, the liver is shown to play a role in the synthesis of protein (Narsale et al., 2015).

In a cachectic model, liver function is diminished. The glycogen stores in the liver and glycolytic enzymes are reduced (Narsale et al., 2015). Due to this decrease in glycolytic enzymes, the body is unable to use glucose as energy and must resort to other sources of fuel. Also, cachectic progression increased PSTAT3 (Narsale et al., 2015). STAT3 progression is significant in that STAT3 interacts with the body-tumor interaction by promoting tumor growth and inhibiting the body's tumor suppressants (Yu, Pardoll, & Jove, 2017). For protein synthesis, cachectic signaling induced PMTOR and suppressed S6 and AKT. Suppressing AKT directly influences protein synthesis because AKT signals to MTOR in the protein synthesis pathway. Impeding that signal directly impedes the protein synthesis process. STAT3 also contributes to cancer cachexia through enhancing tumorigenesis, metastasis and immune suppression, particularly in tumors associated with high prevalence of cachexia (Zimmers, Fischel, & Bonetto, 2016). This means that the tumor is assisted in growing and spreading.

Thus, while we know that cachexia impairs liver function in multiple ways (Narsale et al., 2015) we do not know exactly how the process works. MTOR is the starting point in two protein synthesis complexes. Does cachexia target the liver more than muscles? By what protein pathway does cachexia inhibit protein synthesis? Are there specific proteins in the muscle synthesis process that are more affected or less affected by cachexia? If cachexia increases PSTAT3 and STAT3 levels, does it also alter the way STAT transcribes with the nucleus?

Cachexia has no cure. At its core, cachexia is dangerous because it inhibits protein synthesis and accelerates muscle atrophy. Therefore, we look for a way to promote protein synthesis and inhibit the muscle atrophy process. One way to do this is by supplementing the branch chain amino acid (BCAA) leucine in the diet. Leucine is of vital importance in the process of protein synthesis. (Tamanna & Mahmood, 2014). In fact, leucine is shown to promote protein synthesis in humans and in rats.

Branched chain amino acids play a vital role in the protein synthesis pathway and in protein turnover. They have been shown to promote protein synthesis in humans in skeletal muscle (Shimomura et al., 2017). The three branched chain amino acids are leucine, isoleucine, and valine. They cannot be synthesized in the body, and therefore must be ingested. Branched chain amino acids, unlike other amino acids, do not experience their first catabolic step in the liver. They are unique in the fact that they go through catabolism in the skeletal muscle (Rajendram, Preedy, & Patel, 2015). In fact, the enzyme that begins the process of breaking down BCAA is not present at all in the liver. Interestingly though, the enzyme for the second step is found in high levels in hepatocytes (Rajendram et al., 2015). Therefore, while the liver cannot begin the process of breaking down BCAAs, it can continue the process once it has begun.

In a disease state, some research shows that high levels of leucine can inhibit protein degradation in both the muscle and the liver (Tamanna & Mahmood, 2014). Knowing that cachexia inhibits protein synthesis and also knowing that supplemental leucine can facilitate protein synthesis and inhibit protein degradation, we wanted to know if leucine can diminish or even counteract the effects of cachexia on protein synthesis. There is some proof to back up these thoughts. In a study with mice with the tumor MAC16, researchers supplemented leucine and valine and found an increase in protein synthesis and a decrease in protein degradation (Eley, Russell, & Tisdale, 2017). Further encouraging the support of leucine as compared to other BCAAs, Anthony et al. (2017) found that leucine was unique in its ability to promote protein synthesis in rats, increasing it by as much as 65%. Due to this, we decided to supplement leucine in our mice to see how it affects the protein synthesis in the liver.

Methods

Mice

All use of animals was approved by the University of Memphis Institutional Animal Care and Use Committee. Twenty male mice, approximately 7 weeks old, were purchased from ENVIGO and were housed in the University of Memphis animal facility. Their room was climate-controlled and on a 12-hour day, 12-hour night cycle. After two weeks of adjusting to their facility, they were placed in four separate groups of five. During this adjustment period, all mice had the same diet. After their adjustment peri-

od, the mice were given one of two injections. The control groups (Chow1 and Leu1) were given a PBS injection, while the cancer groups (Llc1 and Llc+Leu1) received an injection of 1x 106Lewis Lung Carcinoma (LLC) cells suspended in PBS. After injection, the separate groups began receiving their special diets. Groups 1 and 3 received a regular (Chow) diet. Groups 2 and 4 received a Leucine supplemented (Leu) diet.

Over the course of 28 days, the mice were monitored and checked daily. Their food intake and body weight were measured every 48 hours. If the mice were not eating, had a movement pattern that was severely impacted, had a tumor greater than 2.5 cm in diameter, were unresponsive to our actions, lost more than 20% of their body weight since the last check, or otherwise seemed to be severely deteriorating, they were immediately euthanized. Mice were fasted for 5 hours prior to euthanasia. Thirty minutes before the collection and removal of tissues, the mice were injected with 0.04 µM/g BW of puromycin for use in protein synthesis measurement assays. Isoflurane was used to anesthetize the mice before euthanasia. The tissues were subsequently harvested and frozen in liquid nitrogen for temporary storage. Then, they were played in a negative 80 degree centigrade freezer for long-term storage and use.

Western Blots

A portion of the liver was homogenized on ice with Meuller Buffer that contained protease and phosphatase inhibitors. These samples were placed in the centrifuge for 10 minutes at 10,000g. Then, the supernatant was removed and placed in a new tube. A Bradford assay was used to quantify the protein in each sample. Afterwards, the samples were diluted to 5ug/ul with diluent buffer and 5X Lane Marking Reducing Sample Buffer and were heated at 95°C for 5 minutes. The samples were then loaded into a Western Blot apparatus where they ran through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-15% gradient gel and subsequently transferred onto a PVDF membrane. Afterwards, the transfer was checked with Ponceau stain. After removal of the Ponceau stain, membranes were blocked in 5% Milk in Tris-buffered saline 0.1% Tween 20 (TBST), then placed in their primary antibody overnight (Puromycin 12D10, Millipore; PGC1α 3G6, Cell Signaling Technology; 4EBP1; AMPK D5A2; PAMPK T172; MTOR 7C10, PMTOR D9C7; STAT3, D3Z2G; PSTAT3 T705). The next day, they were washed three times for five minutes each in TBST. Then, they were placed into the

appropriate animal-based secondary for 2 hours. After washing again in TBST, the blots were photographed using a chemiluminescent agent and imaged using a Fotodyne® 60-7020 bench top imager. The bands were then quantified using ImageJ software.

Protein synthesis was quantified with a western blot adaptation of the SUrface SEnsing of Translation (SUnSET) technique (Schmidt, Clavarino, Ceppi, & Pierre, 2009). The puromycin injection prior to euthanasia allows for a quantification of overall protein synthesis.

The blots were stained with Ponceau in order to have a means of normalizing and controlling for any loading variances. Two blots were generated for each protein. All data were averaged across both blots and centered around 1.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 7® software. All data are presented as means + Standard Error of Mean (SEM). All graphs are based on IOD (Integrated Optical Density). Statistical significance was set at $\alpha = 0.05$. Western blot data were analyzed via two-way analysis of variance (ANOVA).

Results

To understand how liver protein synthesis is altered by leucine and cancer, we measured several markers of protein synthesis. MTOR activation through phosphorylation was unaltered by the presence of cancer. Leucine by itself did not change MTOR phosphorylation; however when both leucine and cancer were present, MTOR phosphorylation was decreased (Figure 1A). Next, we measured puromycin incorporation to get a more direct measure of protein synthesis. Neither cancer nor leucine had an effect on puromycin incorporation in liver protein synthesis (Figure 1B). P4EBP1 is a marker for protein synthesis translation. In both cases, cancer trended to decrease P4EBP1 phosphorylation (p=.07) while leucine by itself had no impact (Figure 1C). These data suggest that in our model of the early stage of cancer, there is no alteration in the liver protein synthesis.
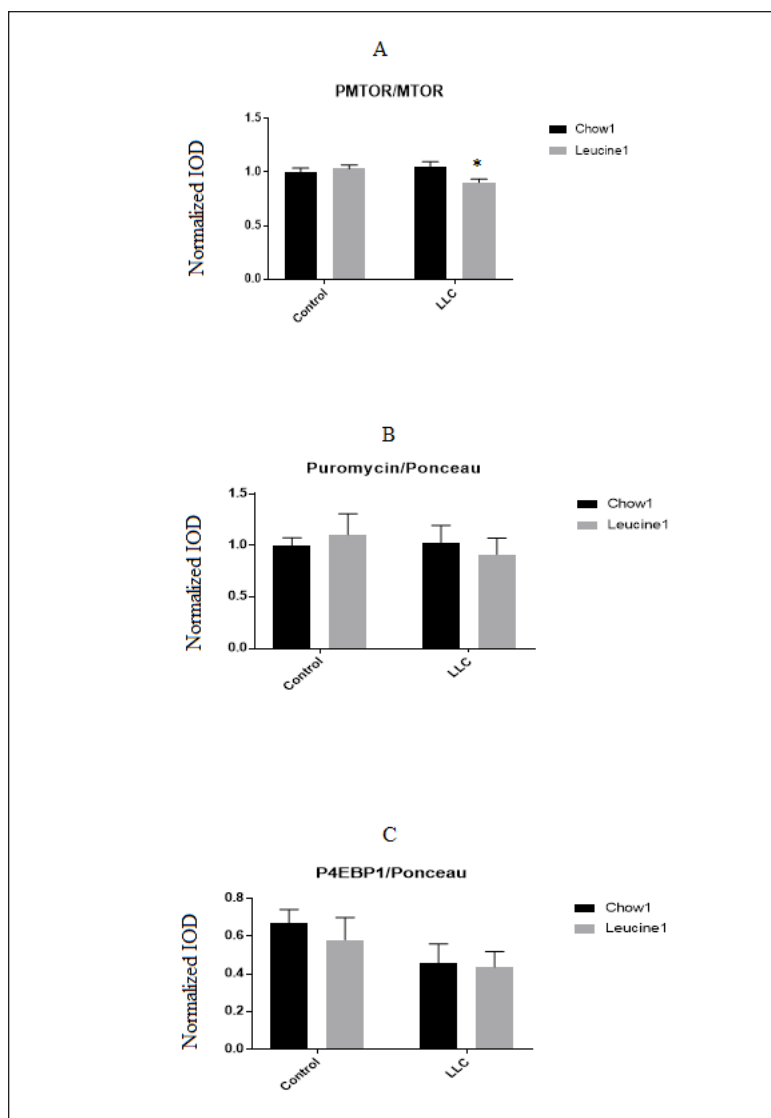
Figure 1. Markers of Protein Synthesis

A) The ratio of pMTOR and total mTOR was taken from western blot analysis. B) Incorporation of puromycin into liver was measured by western blot analysis C) Phosphorylation of 4EBP1 was measured by western blot analysis. Data were normalized to total protein quantified by Ponceau stain. All data are presented as mean ± SEM.
* Represents a difference from all other groups $p<0.05$.

Next we looked at STAT3 activation as a marker of inflammation. With cancer, STAT3 levels were decreased. Supplementing leucine had no effect on STAT3 phosphorylation. When both cancer and leucine were present, STAT3 levels were diminished. This indicates that there was not significant inflammation in the liver of the mice with cancer (Figure 2).
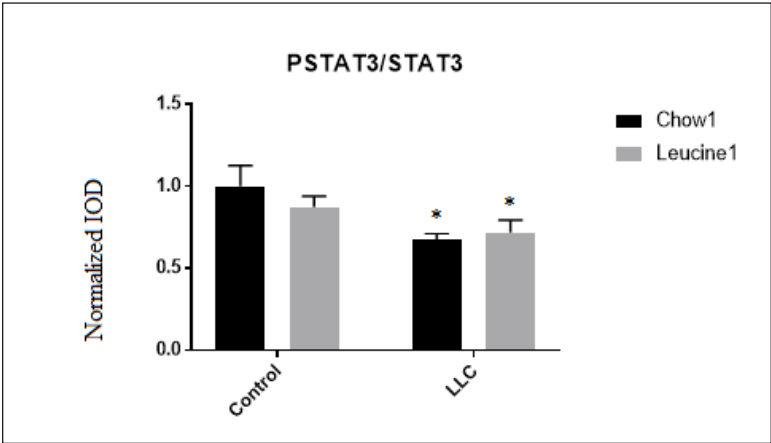

Figure 2. PSTAT3/STAT3

The ratio of PSTAT3 to STAT3 was taken from western blot analysis. Data were normalized to total protein quantified by Ponceau stain. All data are presented as mean ± SEM.
*Represents a difference from all other groups $p < 0.05$.

AMPK negatively regulates protein synthesis and is a potent sensor of energy levels. In the group with cancer, AMPK levels were increased. The group receiving just leucine did not have altered AMPK levels, and the group with both cancer and leucine had increased AMPK phosphorylation (Figure 3). Because protein synthesis was not decreased by the increase in AMPK, AMPK may be acting in an alternative manner.

Finally, we looked at PGC1, a known marker for mitochondrial biogenesis. PGC1 alpha can be regulated by AMPK. With cancer, PGC1 levels were unaffected. Adding leucine into the diet also did not alter PGC1 levels. With both cancer and leucine, PGC1 levels were not changed (Figure 4). This tells us that our cachexia progression and leucine dosage may not have been high enough to alter mitochondrial biogenesis in the liver; however, more research needs to be done to confirm if dosage was the issue.
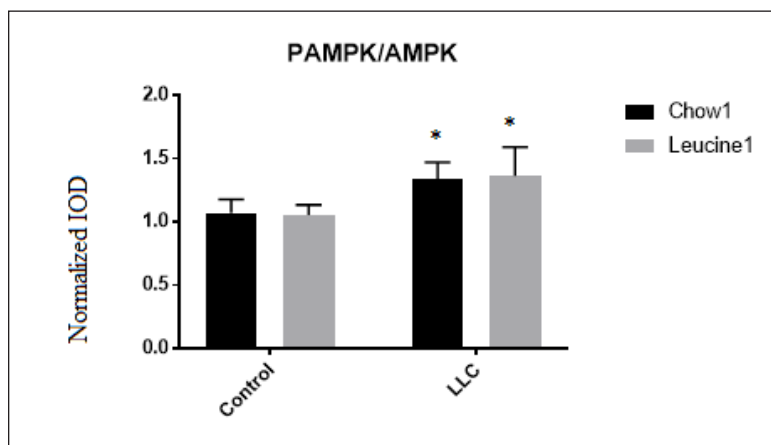
Figure 3. PAMPK/AMPK

The ratio of PAMPK to total AMPK was taken from western blot analysis. All data are presented as mean ± SEM.
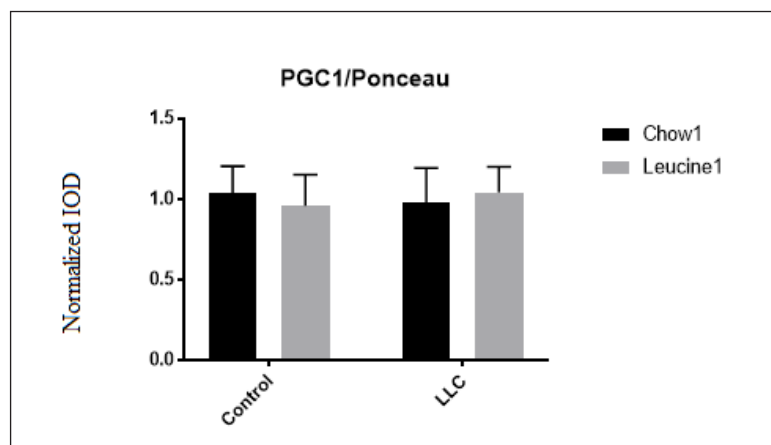*Represents a difference from all other groups p<0.05.

Figure 4. PGC1/Ponceau

Phosphorylation of PGC1 was measured by western blot analysis. Data were normalized to total protein quantified by Ponceau stain. All data are presented as mean ± SEM.
*Represents a difference from all other groups p<0.05.

Discussion

Cancer cachexia causes a decreased quality of life in part due to the negative impact on protein synthesis. Leucine has been shown to increase protein synthesis, therefore, we wanted to see if supplementing leucine in a cancer cachexia model could diminish or even counteract the negative impact of cancer cachexia on liver protein synthesis. After supplementing leucine in our mice, we expected to see a decreased effect of cachexia on liver protein synthesis as compared to the mice without supplemental leucine. STAT3 and P4EBP1 were both decreased in the cancer groups and were unaffected by leucine. AMPK was increased when cancer was present. When both cancer and leucine were present, we found PMTOR to be decreased; however, there was no effect with cancer or leucine alone. Additionally, there were no changes in puromycin incorporation suggesting that there was no change in protein synthesis in the liver.

Even though the mice had tumors, the actual cachexia had not progressed beyond the early stages. There are 3 stages of cachexia: pre-cachectic, cachectic, and refractory cachectic (Penet & Bhujwalla, 2015). If we had allowed the cachexia to progress further and supplemented leucine longer, we would have expected to see changes in inflammation and protein synthesis. For example, one study found that STAT3 phosphorylation in the liver increased with the progression of cachexia (Narsale et al., 2015). Further, they also found that AKT and S6 phosphorylation were both decreased as cachexia progressed (Narsale et al., 2015). Overall, there tends to be a decrease in protein synthesis as the cachexia progresses (White et al., 2011). This suggests that if our mice had progressed further, we may have seen more pronounced effects of leucine.

Leucine upregulates MTOR signaling in many healthy and diseased conditions (Du, Shen, Zhu, & Ford, 2007). Further, chronic leucine supplementation in rats has been found to increase protein synthesis rates in adipose tissue, skeletal muscle and the liver (Lynch, Hutson, Patson, Vaval, & Vary, 2002). Cachexia suppresses MTOR activity and downstream signaling (White et. al, 2011). It has been found that during the initial stages of cachexia, phosphorylation of 4EBP1 is reduced in skeletal muscle (White et al., 2011). Leucine supplementation has been found to increase 4EBP1 phosphorylation through MTOR signaling (Millward, 2012). In our study, we found decreased 4EBP1, which further supports that our leucine dose was not high enough to elicit a response. In skeletal

muscle, AMPK has been shown to increase as cachexia progresses (White et al., 2011). Further, any stress that depletes ATP will lead to the activation of AMPK (Viollet et al., 2006). In cancer, increased glycolysis and lactate production leads to less available ATP (Solaini, Sgarbi, & Baracca, 2011). Therefore, we can expect to find increased AMPK in our cancer groups. In our study, we found increased AMPK phosphorylation. AMPK negatively impacts protein synthesis; therefore, we expected total protein synthesis to be decreased as a result of increased AMPK. However, by measuring puromycin, we found no changes to overall protein synthesis even though we expected decreased protein synthesis because of increased AMPK activation. This suggests that although AMPK is activated in the liver, liver protein synthesis is not suppressed through AMPK. One potential explanation for this is that PGC1 is activated by AMPK to increase mitochondrial biogenesis, therefore, we would expect increased AMPK to impact our levels of PGC1. However, we found no changes in PGC1alpha levels. Previous studies in muscle have shown a decrease in PGC1alpha with the progression of cachexia. It is possible that the increase in AMPK acts to preserve PGC1alpha levels and maintain mitochondrial function. Further research needs to be done to confirm this.

Our dose of leucine for this study had no impact on liver protein synthesis, regardless of the presence of cancer. These findings suggest that the liver may possess an alternate pathway it can activate to maintain protein synthesis and liver function. Another possibility is that liver protein synthesis is maintained as a side effect of maintaining other liver functions. The liver, amongst other roles, metabolizes fats, proteins, and carbohydrates. It also serves as a storage location for glycogen. In cancer, glycogen stores are of importance because of the increased energy demands on the body. Therefore, the body may attempt to preserve the liver to preserve this energy supply. Thus, as a coincidence, the protein synthesis pathway may be preserved.

For this study, we can go back and look at other proteins such as AKT or P70S6K. AKT is known to signal to mTOR to increase protein synthesis. P70S6K is known to be downstream from mTOR and regulate ribosomal biogenesis. By looking at these two proteins we can get more of a view on what is happening on both sides of mTOR. Knowledge of how other proteins are affected by our conditions may help us discern other possible pathways. Further, because we have saved tissues and muscles

from this study, we can look at a systemic view of the interaction. In future studies, we can supplement more leucine than we did in this study. Our results show that our level of leucine supplementation was not enough to elicit the expected response, therefore, altering the dose may yield different results. Another possible modification would be to allow the cachexia to progress further as our actual cachectic progression was low. By allowing cachectic progression, STAT3 should increase, allowing us to match the results found in other studies. Either one of these modifications could be made and follow similar methods and procedures to the ones we followed.

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