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The effectiveness of protein, leucine and β-hydroxy-β-methylbutyrate on cell-signaling pathways controlling protein turnover in red and white gastrocnemius muscles of rats

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The effectiveness of protein, leucine and β-hydroxy-β-methylbutyrate on cell-signaling pathways controlling protein turnover in red and white gastrocnemius muscles of rats

by

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There are so many people I'd like to thank since my thesis would never have been finished without their help. First of all, I would like to thank my advisor Dr. John Ivy for his excellent teaching and guidance. I would also like to thank the members in exercise physiology and metabolism laboratory. There is Zhenping Ding, teaching me a lot of techniques and helping me deal with countless occasions. Then there is Yi-Hung Liao (Henry), with his patience and instruction for my work process. Jeff Bernard also taught some other techniques in my first year. And then there are Ming and Joowon, helping with my study as good assistants. It is my pleasure in Dr. Ivy's lab in these two years and everyone made my experience enjoyable.

Wanyi Wang

Abstract

The effectiveness of protein, leucine and β-hydroxy-β-methylbutyrate on cell-signaling pathways controlling protein turnover in red and white gastrocnemius muscles of rats

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The University of Texas at Austin, 2011

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Whey protein supplementation, containing large amount of leucine, has been a traditional intervention to maintain net protein balance in the past decades. It has been recognized that leucine alone is able to stimulate protein synthesis by activating mTOR and its related downstream pathway without affecting protein degradation, whereas its metabolite β-hydroxy-β-methylbutyrate (HMB) is known to attenuate protein degradation when provided chronically. However, the mechanism of HMB's benefit remains unclear. To address how HMB regulates protein synthesis and degradation signaling pathways, we compared one dose of whey protein (187.5mg/kg), HMB (400mg/kg) or leucine (1.4g/kg) by oral gavage. Blood was collected at 0, 45 and 90 min for blood glucose and plasma insulin analysis. Red and white gastrocnemius muscle was taken separately 90 min after

gavage. Blood glucose was reduced by leucine at 45 and 90 min post gavage. Plasma insulin was enhanced by leucine at 45 min and then decreased at 90 min post gavage, whereas HMB decreased plasma insulin through 90 min post gavege. Western blot analysis showed that HMB phosphorylated Akt in red gastronemius, and enhanced phosphorylation of mTOR in both types of muscles. Leucine phosphorylated mTOR, p70s6k and 4E-BP1 in both red and white gastronemius. Regarding protein degradation signals, phosphorylation of FOXO3A was enhanced by HMB, but not in the other treatment groups. Whey protein had no effect on those cellular signaling. Our results indicate that both HMB and leucine may stimulate protein synthesis through the mTOR pathway in red and white gastrocnemius muscles by different degrees with leucine more effective than HMB. HMB may have a greater effect than leucine on limiting protein degradation by phosphorylating Akt and FOXO3A in red and white gastrocnemius muscles. A combination of HMB and leucine, as a new interventional strategy, is predicted to maximize protein accretion by increasing protein synthesis as well as inhibiting protein degradation.

Table of Contents

INTRODUCTION

Greater attention to muscle mass loss has occurred in the past decades, due to related decreases in muscle power, strength, and quality of life. In humans, muscle loss starts to occur at 25-30 years old and there is ~10% muscle loss by 50 years of age. With aging, muscle loss can reach up to ~30% after 50 years of age (Hurley 1995). This atrophy occurs mainly in Type II fibers, especially in Type IIb muscle fiber.

Whey protein supplementation is a common strategy to increase protein synthesis, because it provides a good balance of essential amino acids as substrate (Houston et al. 2008). Also, whey protein can stimulate insulin secretion, which activates the mTOR pathway to increase protein synthesis (Tremblay and Marette 2001). Even though amino acids stimulate protein synthesis, not all amino acids can activate protein synthesis signaling pathways to the same degree. Leucine, as a significant branch-chain amino acid, has been shown to attenuate skeletal muscle atrophy through stimulating protein synthesis (Zanchi, Nicastro and Lancha 2008), via phosphorylation of mTOR and its downstream factors, p70s6k and 4E-BP1 (Anthony et al. 2000). β-hydroxy-β-methylbutyrate (HMB), the metabolite of leucine, has also been reported to prevent skeletal muscle atrophy, but this appears to be done by decreasing protein degradation (Holecek et al. 2009).

However, it is not clear how an acute supplement of HMB affects protein synthesis or degradation related signaling pathways, and whether HMB is more effective than leucine. Since HMB is the metabolite of leucine, we hypothesized that both HMB and leucine would affect protein synthesis and degradation signaling pathways, and HMB would have a better effect on blocking protein degradation compared to leucine or a whey protein supplement alone.

METHODOLOGY

ANIMAL CARE

All of the animal research and procedures were approved by the Institutional Animal Care and Use Committee, at The University of Texas at Austin. Female Sprague-Dawley rats (n=29) age 9-10 months were obtained from Charles River Laboratories (Wilmington, MA). They were housed 2 per cage in an animal room maintained at 21℃ with a 12:12-h light-dark cycle. This reverse light cycle ensures that the rats are tested during their "waking" hours and fasted during their sleeping cycle. They were fed a standard laboratory diet (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and provided water ad libitum. The rats were allowed to acclimate for one week after arriving on campus. During the week of familiarization, each rat was periodically handled and gavaged using an empty syringe. After one week of acclimation and when rats weighed between 350-420g, they were randomly assigned into 4 treatment groups.

EXPERIMENTAL DESIGN

Following a 12-14h fast, rats were taken to the experimental room. Rats were wrapped in a towel and the end of their tails cut with a razor blade. The first drop of blood was discarded. The second drop of blood was used to measure blood glucose using a portable glucose analyzer (One Touch Ultra 2; LifeScan Inc., Milpitas, CA) at 0, 45, and 90 min. We collected another 500µl blood into a 1.5ml test tube containing 0.1ml EDTA (24mg/ml, pH 7.4) at each time point and put the tube on ice. Right after taking blood at 0 min, rats were intubated with one of four solutions.

The treatment solutions consist of 1) control: Krebs-Henseleit Buffer (KHB); 2) whey protein (WP): 187.5mg/kg body weight; 3) HMB: 400mg/kg body weight; 4) Leucine: 1.4g/kg body weight. KHB was mixed by 10 x KHB stock I and II at pH 7.4. All rats were fed 10 ml/kg. Based on previous recommendations, whey protein was given as 187.5 mg/kg prepared as 18g/L in 50 ml KHB; HMB was given as 400 mg/kg prepared as 3g CaHMB·H_2O in 50 ml KHB (Pimentel et al. 2011); leucine was given 1.4

g/kg prepared as 7g in 50 ml KHB (Anthony et al. 2000). All the supplementations were supplied by Abbott Laboratory (Abbott Nutrition, Columbus, OH)

Immediately after taking blood at 90min, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg of body weight) at which time red and white gastrocnemius were excised, frozen clamped in liquid nitrogen, and stored at -80℃ for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight).

PLASMA INSULIN ANALYSES

Blood samples maintained on ice were centrifuged at 3000g for 10 min at 4℃ and then the supernatant aliquoted to several test tubes and stored at -80℃ for insulin analysis. All samples were tested in duplicate. Plasma insulin was measured using a radioimmunoassay kit (Linco Research, St. Charles, MO) with CV<10%. The ^{125}I-labeled insulin was added into glass tubes containing standards, controls, and plasma samples. Rat insulin antibody was then added into the tubes. All tubes were incubated at 4℃ overnight (20-24 hours). During that time, ^{125}I-labeled and unlabeled insulin compete each other to bind to the antibody. The next day, the antigen-antibody complex was precipitated by adding precipitating solution, incubation, and spinning down. Then all tubes were counted in a gamma counter for 2mins. Standards were used to build up a standard curve.

MUSCLE HOMOGENIZATION

We weighed ~60μg red and white gastronemius separately and homogenized in ice-cold homogenization buffer (containing 20mM Hepes, 2mM EGTA50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM Benzamidine, 0.5 mM Na Vanadate) with a glass tissue grindle pestle (Corning Life Science, Acton, MA). Homogenates were centrifuged at 13,000g for 15 minutes at 4℃. Supernatants were aliquoted into several test tubes and stored at -80℃ for later analysis.

PROTEIN QUANTIFICATION

A modified method of the Lowry Assay (Lowry et al. 1951) was used to determine muscle sample protein concentration. Copper-protein complex reaction occurs when copper is reduced. The copper-protein complex reduces Folin and Ciocalteu's Phenol reagent, which produces a blue color that was measured by spectrophotometer at 750nm. A standard curve (protein concentration versus absorption) was then produced and a linear regression formed. The protein concentration of each sample was determined from the linear regression equation.

WESTERN BLOT

Signaling proteins, including Akt/PKB, mTOR, p70s6k, 4E-BP1, FOXO3A and α-tubulin, were determined by Western Blotting. Sample proteins (100 µg) was combined with an equal amount (1:1) of sample buffer (1.25M tris, 20% glycerol, 20% SDS, 0.25% bromophenol, β-mercaptoethanol, pH 6.8) and boiled at 95°C for 10 min. Then, equal amounts of muscle proteins (60-100 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels for 80 min at 130V. The resolved proteins were transferred onto a nitrocellulose membrane (NC) using a wet transfer unit for 2 h at 90V. The NC was then blocked in freshly prepared 7% non-fat dry milk in Tris-buffered saline (TBS) with 0.06% Tween20 (TTBS-MLK) for 20 minutes at room temperature with shaking. The blocked NC was then incubated with either affinity purified anti-phospho-Akt/PKB (ser473), anti-phospho-mTOR (ser2448), anti-phospho-p70s6k (thr389), anti-phospho-4E-BP1 (thr37/46), anti-phospho-FOXO3A (ser318/321), or anti-α-tubulin (Cell Signaling Technology, Inc., Danvers, MA) overnight at 4 ℃. The primary antibodies were diluted to either 1:800 (phospho-Akt/PKB, phospho-mTOR, phospho-p70s6k, α-tubulin) or 1:650 (phospho-4E-BP1, phospho-FOXO3A) with freshly prepared TTBS-MLK. The next day, the NC membrane was washed 5min x 3 times with TTBS-MLK which is followed by incubation for 1h at room temperature with species-specific (anti-rabbit) immunoglobulin G (IgG) secondary antibodies (Cell Signaling Technology,

Inc., Danvers, MA). The secondary antibodies were diluted to 1:800 in TTBS containing 2% NFDM. After 3 additional 5 min washes, the NC membrane was visualized by Western Lightning Plus-enhanced chemi-luminescense (ECL) in accordance to the manufacturer's instructions (Perkin Elmer Inc., Waltham, MA). Signaling Protein blots were quantified by a ChemDoc ECL detection system and Quantity One software (both Bio-Rad, Hercules, CA). The protein phosphorylation was normalized to the internal control (α-tubulin).

STATISTICAL ANALYSIS

A one-way ANOVA was performed on all muscle sample data. Blood glucose and plasma insulin data was performed using a two-way repeated ANOVA. Significance was considered at $p<0.05$. All statistical analyses were completed using PASW v19.0 software (PASW Inc., Chicago, IL) and all values expressed as means ± standard error (SE).

RESULTS

Animal Characteristics

There were no differences in average body weight among the different treatment groups Control (CTRL) 381.0 ± 4.8g; Whey Protein (WP) 388.3 ± 7.3g; HMB 387.1 ± 10.9g; Leucine (LEU) 379.6 ± 11.6g.

Blood glucose level

Blood glucose concentration was determined following a 14 h fast and at 45 and 90 min after one treatment dose. As shown in Fig. 1, there were no differences in fasting glucose among treatment groups. There were small changes in the blood glucose response for CTRL, WP, and HMB through 90 min; however, blood glucose was significantly decreased at 45 and 90 min for LEU supplementation compared to CTRL, WP, and HMB (Fig. 1A). The glucose area under the curve (AUC) was also lower for LEU relative to the other treatment groups (Fig. 1B). There were no differences in glucose AUC among the other treatment groups.

Plasma insulin level

Plasma insulin was increased at 45 min post leucine supplementation, but had returned to baseline by 90 min. HMB reduced plasma insulin at 45 min post treatment compared to the fasting insulin level. There were no significant differences among treatments at each time point (Fig. 2A). Insulin AUC was significantly increased with the leucine treatment, but decreased with HMB treatment compared to CTRL (Fig. 2B).

Akt/PKB^{ser473} was phosphorylated by HMB in red gastrocnemius muscle

Akt/PKB protein phosphorylation at site ser473 was significantly increased 90 min post HMB supplementation compared to WP and CTRL in red gastrocnemius muscle, but not in white gastrocnemius. There were no significant differences among the other treatment groups in either muscle fiber type (Figure 3A and B).

mTOR^{ser2448} was phosphorylated by both HMB and Leucine in red and white gastrocnemius muscle

mTOR protein phosphorylation at site ser2448 was significantly increased 90 min post HMB supplementation compared to CTRL and WP in red gastrocnemius (Figure 4A). mTOR ^{ser2448} phosphorylation was further enhanced with the LEU treatment in red gastrocnemius compared to HMB (Figure 4A). In white gastrocnumius, both HMB and LEU phosphorylated mTOR compared to CTRL and WP, but there was no significant difference between HMB and LEU treatments (Figure 4B).

p70s6k^{thr389} was phosphorylated by Leucine in red and white gastrocnemius muscle

p70s6k phosphorylation at site thr389 was significantly increased for LEU supplementation compared to all other treatments in both red and white gastrocnemius (Figure 5A and B). p70s6k phosphorylation had a tendency to be increased by HMB in both types of muscle, but there were no significant differences.

4E-BP1$^{thr37/46}$ was phosphorylated by Leucine in red and white gastrocnemius muscle

4E-BP1 phosphorylation at site thr37/46 was significantly increased 90 min post LEU compared with HMB, WP and CTRL in red and white gastrocnemius muscle. There were no significant differences among the other treatment groups (Figure 6A and B).

FOXO3A$^{ser318/321}$ was phosphorylated by HMB in red and white gastrocnemius muscle

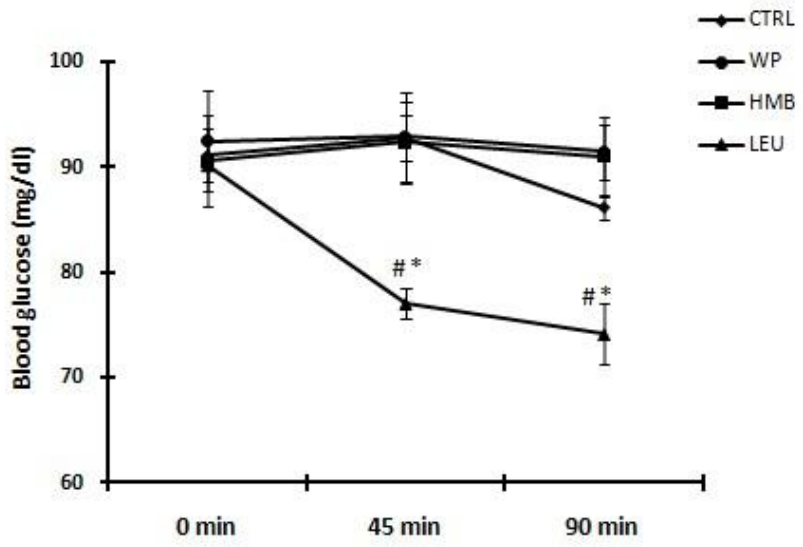
FOXO3A protein phosphorylation at site ser318/321 was significantly increased for HMB compared with WP and CTRL both in red and white gastrocnemius muscle (Figure 7A and B). In addition, phosphorylation of FOXO3A was also higher with HMB treatment compared to LEU in white gastrocnemius muscle (Figure 7B). There was no difference in phosphorylation of FOXO3A between WP and CTRL for both muscle fiber types.

Abundance of alpha tubulin was the same for all treatments.

We probed our membranes with an antibody that detects endogenous levels of total alpha-tubulin protein. No statistical differences were detected between the treatment groups (Fig. 8A and B). This suggests that equal amounts of protein were loaded for all the treatments.

FIGURES

A



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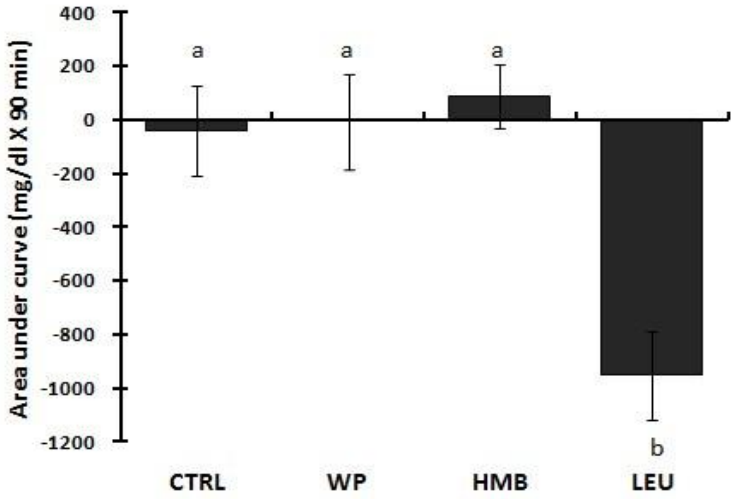
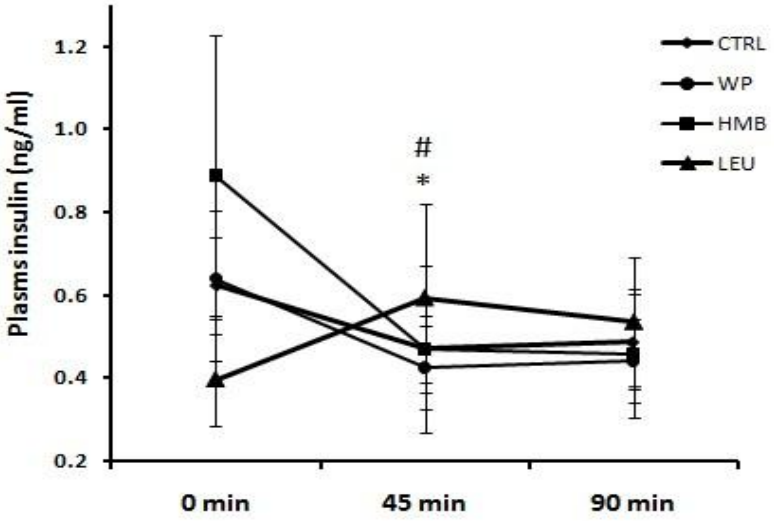


Figure 1. (A) Blood glucose after 14 h fast, and 45 and 90 min post supplementation. Values are means ± SE. *, $p<0.05$ LEU vs. all other treatments at the same time point. #, $p<0.05$ LEU at 45 and 90min vs. 0min. (B) Blood glucose AUC. Values are means ± SE. Means with different letters are significantly different from each other ($P<0.05$).

A



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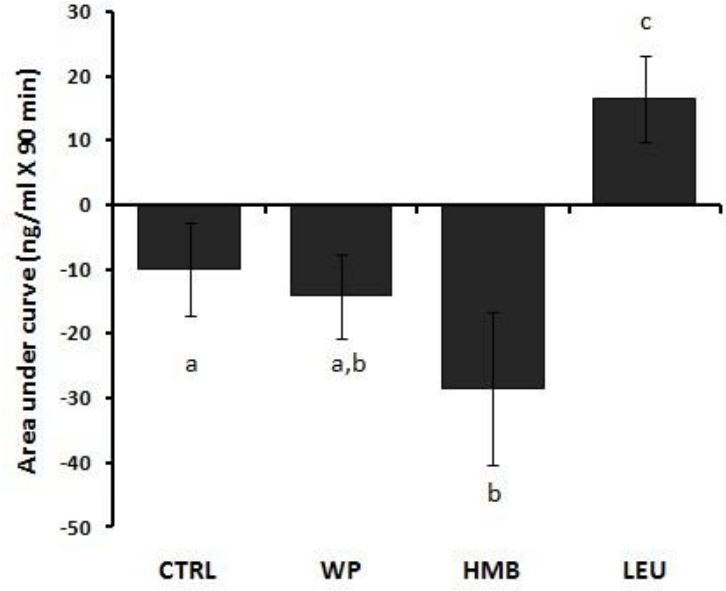
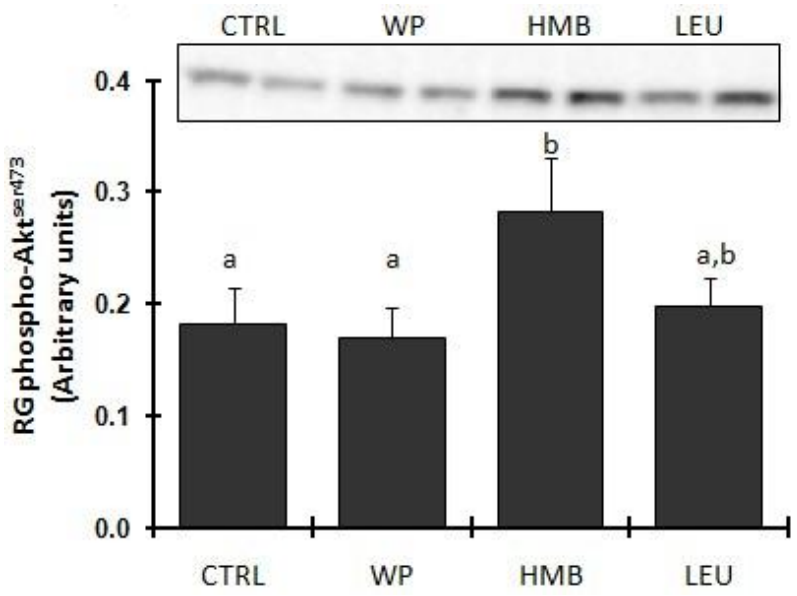


Figure 2. (A) Plasma insulin after 14 h fast, and 45 and 90 min post supplementations. *, $p<0.05$ HMB at 45 vs. 0min. # $p<0.05$ LEU at 45 min vs. 0 and 90 min. (B) Plasma insulin AUC. Values are means ± SE. Means with different letters are significantly different from each other ($P<0.05$).

A



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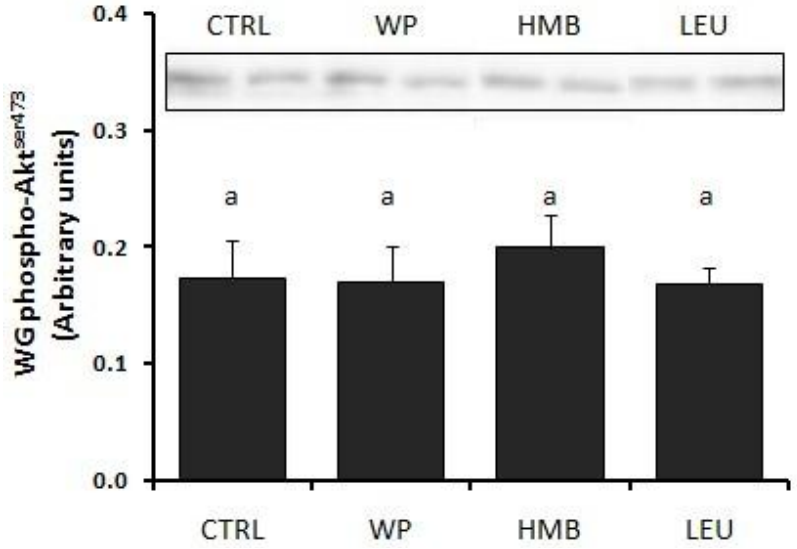
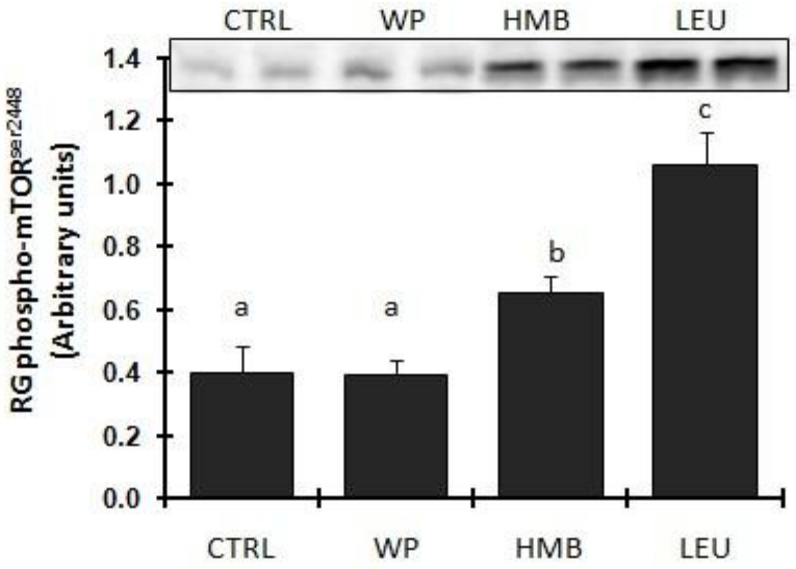


Figure 3. (A) Red gastrocnemius and (B) white gastrocnemius Akt Ser-473 phosphorylation 90 min after each treatment. Values are means ±SE. Means with different letters are significantly different from each other ($P < 0.05$).

A



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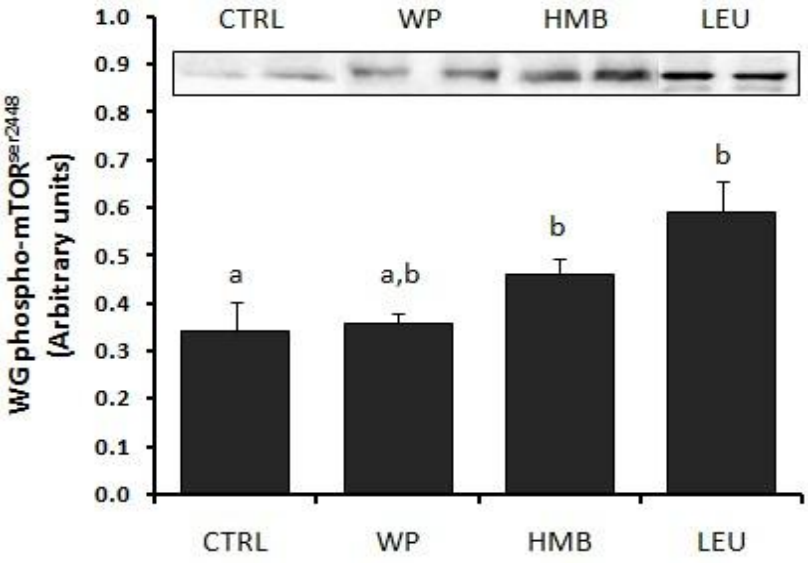
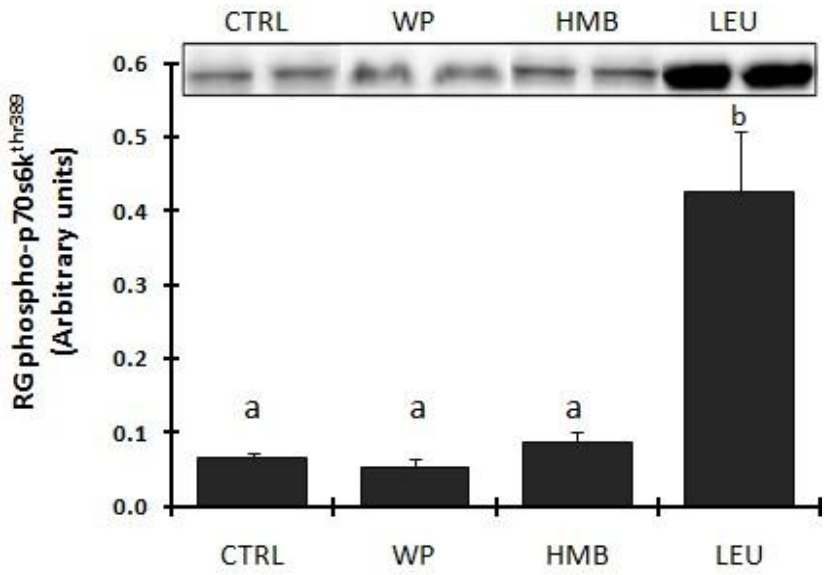


Figure 4. (A) Red gastrocnemius and (B) white gastrocnemius mTOR ser2448 phosphorylation 90 min after each treatment. Values are means ±SE. Means with different letters are significantly different from each other ($P < 0.05$).

A



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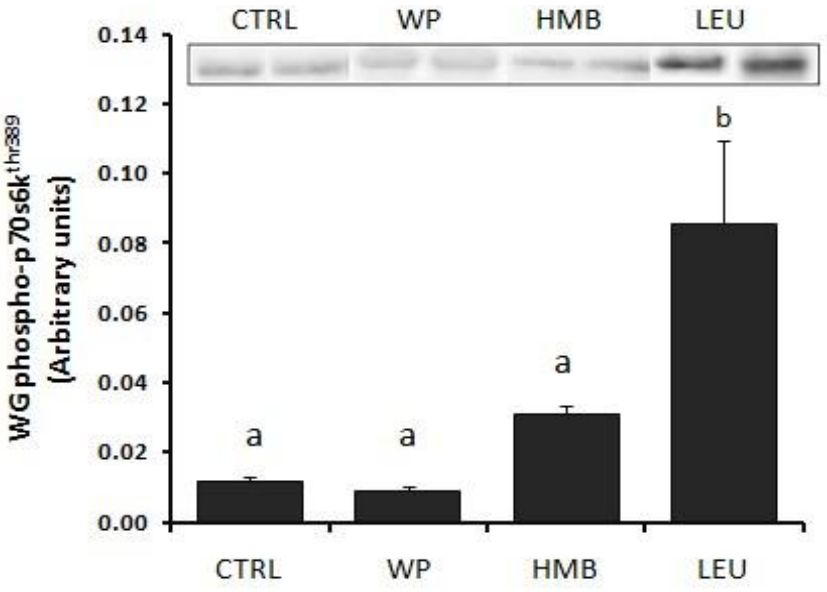


Figure 5. (A) Red gastrocnemius and (B) white gastrocnemius p70s6k-thr389 phosphorylation 90 min after each treatment. Values are means ±SE. Means with different letters are significantly different from each other ($P < 0.05$).

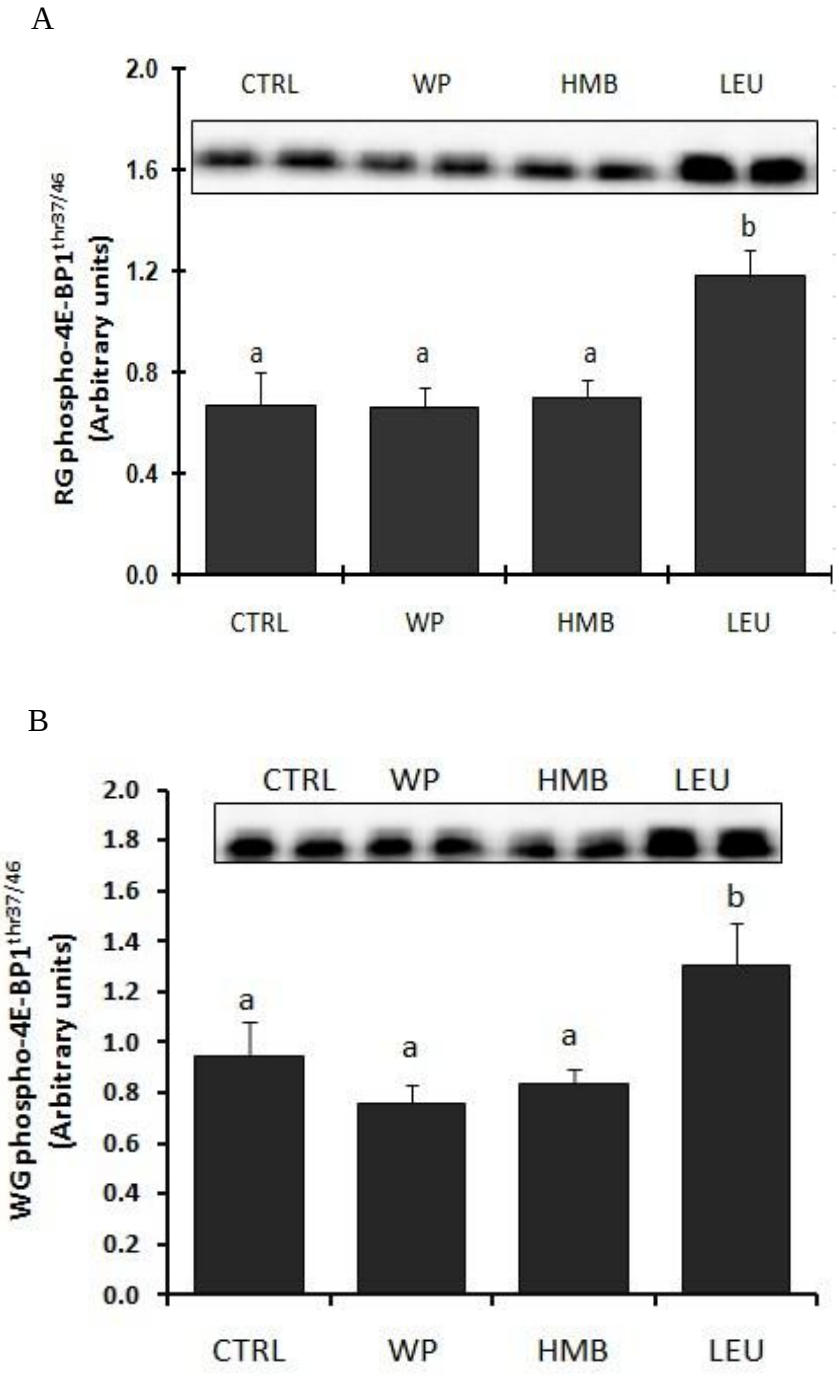
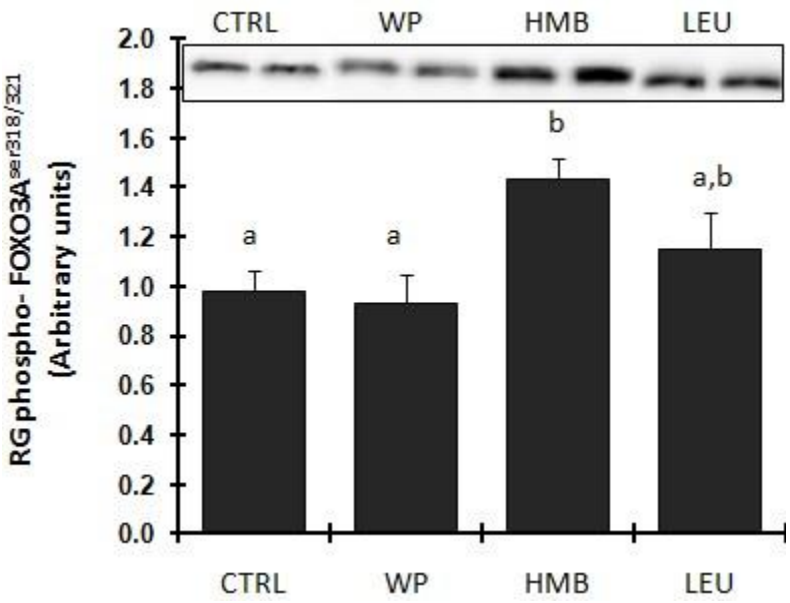


Figure 6. (A) Red gastrocnemius and (B) white gastrocnemius 4E-BP1-thr37/46 phosphorylation 90 min after each treatment. Values are means ±SE. Means with different letters are significantly different from each other ($P < 0.05$)

A



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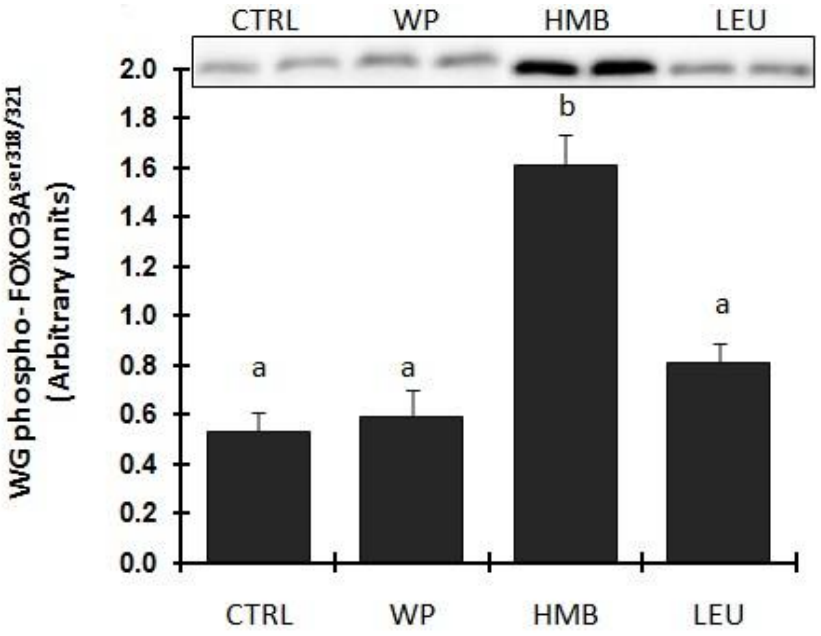
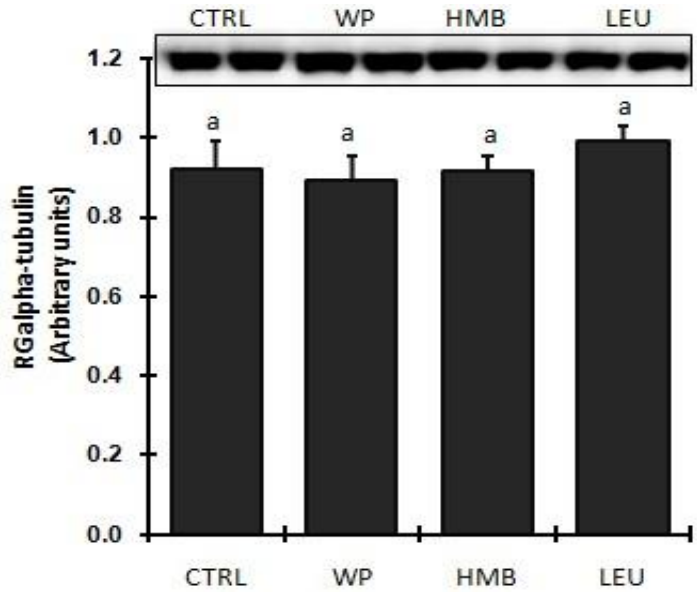


Figure 7. (A) Red gastrocnemius and (B) white gastrocnemius FOXO3A-ser318/321 phosphorylation 90 min after each treatment. Values are means ±SE. Means with different letters are significantly different from each other (P <0.05).

A



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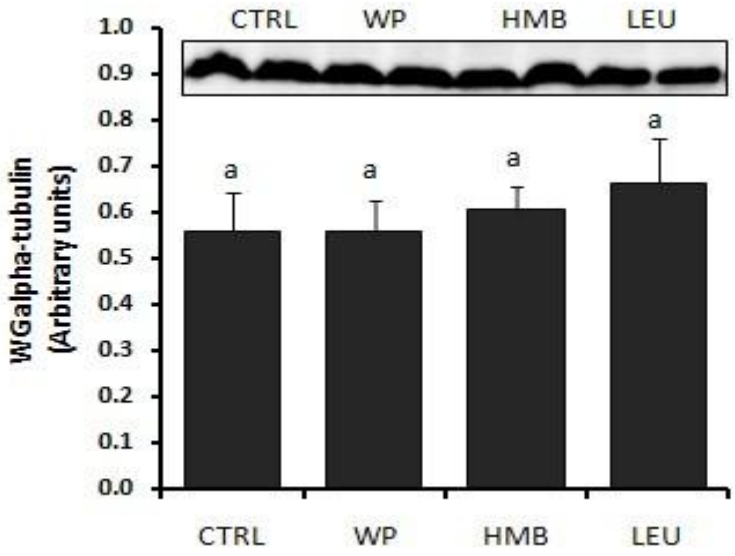

Figure 8. Total protein abundance of alpha-tubulin in red and white gastrocnemius muscles was used to evaluate the amount of protein equally loaded. Values are expressed as means ±SE. No significant difference among the means was detected.

Discussion

The primary finding of the present study is that acute treatment of both HMB and leucine were able to partly increase cell signaling responsible for mRNA translation initiation to different degrees. Leucine appeared to be more effective in controlling protein synthetic signals; whereas HMB appeared to have a greater effectiveness limiting protein degradation as it caused a greater phosphorylation of FOXO3A. These findings implies that administered together they may have the capacity of stimulating protein synthesis and inhibiting protein degradation thus maximizing protein accretion.

Greater attention has been paid to loss of muscle mass in recent decades due to related decreases in muscle function and quality of life. Different intervention strategies are recommended to prevent protein degradation or stimulate protein synthesis to enhance protein accretion. As described by previous studies, the insulin-dependent signaling pathway predominates protein synthesis both in animals and humans (Baar and Esser 1999, Terzis et al. 2008). Insulin is able to phosphorylate Akt/PKB through activation of PI3K. Once phosphorylated, Akt can phosphorylate and thus activate mTOR. Activated mTOR is then able to enhance phosphorylation of p70s6k and 4E-BP1 (Dennis, Fumagalli and Thomas 1999). Multi-site phosphorylation of 4E-BP1 will dissociate from eIF-4E which allows it to form eIF4F by binding to eIF4G (Wang and Proud 2006). eIF4F is a central factor in the active ribosome. Therefore, phosphorylation of 4E-BP1 plays an important role in ribosome activation. In addition, phosphorylation of p70s6k by mTOR results in activation of rpS6, which is related to translation of mRNA and elongation factors (Roux et al. 2007). Also, p70s6k can phosphorylate glycogen synthesis kinase-3 which then inhibits eIF2B to increase mRNA translation. Both of these sub-branched pathways from mTOR ultimately increase the capacity of protein synthesis.

Akt/PKB also plays an important role in regulation of protein degradation. Its downstream protein, forkhead box O (FOXO) 3A has been shown to promote atrophy in skeletal muscle (Sandri et al. 2004). FOXO3A is a transcription factor in the nucleus,

which can enhance E3 ligases such as atrogin-1 protein expression (Zanchi et al. 2010). Such enzymes are involved in the activity of the proteasome pathway, which then results in proteolysis. FOXO3A can be inhibited by phosphorylation at sites thr318/321 by Akt.

With regard to protein synthesis, our current study observed that leucine treatment resulted in the phosphorylation of mTOR and its downstream proteins p70s6k and 4E-BP1 in both red and white gastrocnemius muscle. These results suggest that leucine is able to stimulate protein synthesis through activating the mTOR pathway. The evidence for leucine activating mTOR directly in the absence of insulin has been previously reported (Byfield, Murray and Backer 2005, Nobukuni et al. 2005). Moreover, these studies also reported phosphorylation of mTOR by leucine results in the activation of p70s6k and inhibition of 4E-BP1 (Anthony et al. 2000, Byfield et al. 2005), which is consistent with our findings. However, we cannot eliminate the possibility that leucine activated the mTOR pathway in part through an insulin-dependent pathway. Therefore, we investigated the phosphorylation status of Akt/PKB. We found that phosphorylation of Akt/PKB was not stimulated by leucine in either muscle fiber types, which agrees with the findings of Liu et al. (Liu et al. 2002). Since Akt phosphorylation was not increased and the increase in plasma insulin was marginal following the leucine treatment, we conclude that leucine stimulates mTOR through an insulin-independent process.

HMB, as the metabolite of leucine, has been shown to slow the biological decrease in muscle mass and strength in elderly individuals (Vukovich, Stubbs and Bohlken 2001). It is worth noting that there are no adverse reactions reported by subjects consuming HMB (Vukovich et al. 2001), whereas high amounts of leucine intake have been reported to have some side effects such as hypoglycemia (Digeorge, Auerbach and Mabry 1963). Moreover, it has been suggested that leucine's activation of protein synthesis may be due to its conversion to HMB (Wilson, Wilson and Manninen 2008). We therefore investigated the effect of HMB on the intracellular signaling pathways controlling protein synthesis and degradation. It was demonstrated that HMB increased Akt/PKB phosphorylation in red gastrocnemius, but not in white gastrocnemius. Similar results were reported by Kornasio et al. (Kornasio et al. 2009). In contrast, Pimentel et al.

(Pimentel et al. 2011) found that the chronic treatment of HMB did not affect phosphorylation of Akt in the extensor digitorum longus (EDL) muscle of rats. These different findings are most likely due to differences in sampling of muscle. In their research, Pimentel et al. tested Akt signaling 15-18 hours after HMB oral gavage. It is highly unlikely that Akt would not remain phosphorylated for this length of time. In addition, Norton et al. has shown that most insulin-related signaling responses occur shortly after treatment gavage, usually within 1-3h (Norton et al. 2009). The mechanism by which HMB phosphorylates Akt is unknown. It is unlikely insulin was responsible for the increased phosphorylation of Akt, as insulin levels declined following HMB treatment. It is important to note, however, that other potent hormones are secreted in response to HMB that could influence the insulin signaling pathway. Insulin-like growth factor (IGF)-1α is another important hormone involved in muscle protein synthesis and it is capable of activating Akt/PKB (Skurk et al. 2005). One recent study reported that HMB increased serum IGF-1 concentration (Gerlinger-Romero et al. 2011). Therefore, it is suggested that HMB activated Akt/PKB through the IGF-1 pathway in the present study.

Akt/PKB downstream proteins were also measured in the current study. We found that, relative to the control group, HMB increased phosphorylation of mTOR in both red and white gastrocnemius, but not to the same degree as leucine treatment. Furthermore, HMB did not increase the phosphorylation of 4E-BP1 or p70s6k relative to control in either red or white gastrocnemius. Eley et al. (Eley et al. 2007) found that HMB was able to promote phosphorylation of p70s6k and 4E-BP1. They also reported that hyperphosphorylation of 4E-BP1 increased association of eIF3E and eIF4G either with HMB alone or in combination with proteolysis-inducing factor (PIF), a catabolic signal (Eley et al. 2007). This discrepancy is likely related to differences in experimental models used. Eley et al (Eley et al. 2007) used murine myotubes *in vitro* with PIF to test the effect of HMB on the mTOR/p70s6k pathway. However, our *in vivo* model is more complicated, containing many factors that could affect the degree of phosphorylation such as secretion of hormone and the distribution of blood flow. Even though HMB did

not enhance phosphorylation of p70s6k and 4E-BP1 in our study, we still cannot rule out the possibility that HMB activates these proteins in *in vivo*, because these proteins were tested only at 90 min post gavage. However, it is unlikely HMB would have the same effect as leucine on protein synthesis, because mTOR was not activated by HMB to the same degree as by leucine.

Based on our results, it appears that leucine has a strong capability to increase protein synthesis through activating the mTOR pathway, as previously noted (Pimentel et al. 2011, Anthony et al. 2000). HMB, however, did not appear to activate proteins critical for activation of protein synthesis. In addition, as mentioned above, leucine has been predicted to stimulate protein synthesis via HMB, because 60g leucine intake is believed to be metabolized into 3g HMB (Wilson et al. 2008). However, based on our results, it is unlikely that leucine's stimulation of protein synthesis is due to its conversion to HMB.

There is, however, strong evidence that HMB can block proteolysis in skeletal muscle due to aging, stress of exercise, AIDS, renal failure, and cancer (Smith, Mukerji and Tisdale 2005, Wilson et al. 2009, Wilson et al. 2008). Often these conditions involve progressive wasting of muscle, and a number of studies demonstrate the ability of HMB to prevent such muscle wasting. For example, Vulovich et al. (Vukovich et al. 2001) was able to increase fat-free mass in 70-year-old adults by providing HMB for 8 weeks. Smith et al. (Smith et al. 2005) was able to decrease proteasome-proteolysis activity in mice with tumor with a 9-day supplement of HMB and preserve the lean body mass of the mice. There are, however, few studies showing HMB's effect on inhibition of protein degradation in normal sedentary models. Additionally, the underlying mechanism(s) by which HMB attenuates proteolysis still requires further investigation *in vivo*.

In our current study, phosphorylation of FOXO3A was enhanced following HMB administration compared to control in red and white gastrocnemius. Our finding that HMB treatment resulted in phosphorylation of Akt in red gastronecmius and there was a trend for phosphorylation of white gastrocnemius suggests that Akt/PKB was responsible for phosphorylation of FOXO3A. Inhibited FOXO3A via its phosphorylation will block the enzymes involved in the proteasome and the ubiquitin pathways, and inhibit protein

degradation. Leucine, however, did not result in phosphorylation of FOXO3A, suggesting that leucine does not regulate protein degradation through the Akt/FOXO3A pathway similar to HMB.

Our results also demonstrated that whey protein (187.5mg/kg) in the amount provided had no effect on plasma or muscle measurements. This finding is probably the result of an insufficient amount of protein provided to see an acute effect like demonstrated in other studies (Anthony et al. 2007, Morrison et al. 2008).

In summary, our findings suggest that leucine was able to stimulate protein synthesis through the mTOR pathway. Although leucine was effective in stimulating protein synthesis, HMB appeared more able to inhibit protein degradation as indicated by its ability to phosphorylate FOXO3A. Therefore, the combination of leucine and HMB may be an optimal nutritional intervention to maintain net protein balance under conditions that increase proteolysis by increasing protein synthesis and reducing protein degradation without adverse health effects. Future research should evaluate the combined effects of leucine and HMB on protein synthesis and degradation and determine the optimal dosage for use under different conditions.

BACKGROUND LITERATURE

INTRODUCTION

Sarcopenia, characterized as loss of muscle mass, starts to occur after 25 years old. During aging, sarcopenia becomes more and more severe, especially in the type IIb muscle fiber type. It has been reported that loss of skeletal muscle mass is due to multiple reasons, including degenerated enzymatic activity, reduced capacity of hormone regulation, etc., which then cause increased protein degradation and decreased protein synthesis (Orgel 1963, Lecker et al. 1999, Squier 2001). Current intervention is focusing on reversing decreased protein synthesis as well as preventing gradual protein degradation.

One common strategy to maintain muscle mass in aged individuals is to consume high amounts of protein. Protein, as a source of amino acids for muscle protein synthesis, is a strong stimulator of pancreatic insulin release. Both protein and insulin play critical roles in muscle protein synthesis by activating the mTOR pathway (Tremblay and Marette 2001). Whey, casein, and soy are three popular protein sources in dairy products. Whey and casein have been recognized as high quality proteins due to their high branched-chain amino acids components, especially leucine. Compared to casein, whey protein is digested faster and has been found to produce a better muscle protein synthetic response in older people (Phillips, Tang and Moore 2009, Dangin et al. 2003). However, high amounts of protein consumption may have side-effects in elderly, such as an increase in kidney burden.

Another strategy is giving leucine alone. Leucine has been found to activate mTOR and its downstream proteins. This benefit has been attributed to the conversion of leucine to its metabolite β-hydroxy-β-methylbutyrate (HMB) (Hider, Fern and London 1969, Caperuto et al. 2007). HMB has been previously demonstrated to attenuate sarcopenia and decelerate the biological decrease in muscle strength in elderly individuals (Vukovich et al. 2001). Nevertheless, the mechanisms involved in prevention

of sarcopenia and how HMB stimulates protein accretion have not been described clearly. Moreover, most studies with HMB supplementation are chronic and focus on conditions resulting in rapid muscle loss, such as patients with cancer, excess stress, and ADIS. There are few reports about HMB treatment in normal (healthy and sedentary) situation or the age that protein starts to degrade.

THE COMPOSITION OF MUSCLE FIBER TYPES

Skeletal muscle is composed of distinct types of fibers that differ in their capillary density, enzymes, and metabolism. The major forms of muscle fiber types are classified as Type I fiber fibers (slow-twitch motor units) and Type II fiber (fast-twitch motor units) based on myosin heavy chain expression. Type II fibers are further subclassified into Type lla and Type llb fibers. Type I fibers contain more mitochondria, blood vessels and high myoglobin levels, etc. Therefore, Type I fibers are responsible for posture and oxidative exercise (Pette and Spamer 1986). Conversely, Type IIb fibers have stronger contraction capability and are responsible for power and strength. Type IIa is the intermediate between Type I and Type IIb fibers. Individual muscles are a mixture of 3 types of muscle fibers, but their proportions vary based on different muscle actions. For example, the soleus is composed of Type I muscle fiber. Gastrocnemius and quadriceps are the mixture of Type IIa and Type IIb. Extensor digitorum longus (EDL) has much more Type llb. There are many intrinsic and extrinsic factors affecting muscle fiber changes such as gender, age, hormones, physical activities, etc. Aging is associated with the reduction of enzymes involved in energy metabolism, especially in the levels of the enzymes of the glycolytic pathway (Bass, Gutmann and Hanzlikova 1975). Some studies have shown decreased capacity to oxidize substrate through reductions in mitochondrial enzyme (Beyer et al. 1984, Cartee and Farrar 1987), but not in others (Young, Chen and Holloszy 1983, Bass et al. 1975). Therefore, the degree of muscle atrophy differs in various muscles dependent on fiber types, and aging has a negative impact on muscle function, especially power and strength. Type II fibers have been reported to lose more size (atrophy) than Type I fibers with aging (Lexell 1995). Such a selective denervation

of Type II fibers within aging indicates the different functions in different fiber types and may also suggest different degree of regenerations between fiber types with treatments. In addition, it is not clear whether both Type IIa and Type IIb undergo protein degradation and muscle atrophy at the same level.

PROTEIN METABOLISM AND ITS RELATED CELL-SIGNALING PATHWAY IN CONTROLLING mRNA TRANSLATION

Protein content is dependent upon the rate of protein synthesis and the rate of protein degradation. A positive net protein balance occurs when protein is synthesized in excess of degradation, whereas a negative net protein balance occurs when the breakdown of proteins exceeds synthesis. Insulin has the capacity to increase protein synthesis by activating the mammalian target of rapamycin (mTOR) pathway, which is a key regulator in controlling protein synthesis both in human and animal studies (Baar and Esser 1999, Terzis et al. 2008). Rapamycin, the specific inhibitor of mTOR, was used to inhibit muscle hypertrophy in rodents, which further demonstrates the important role of mTOR in protein synthesis control (Bodine et al. 2001). Insulin first binds to the α-subunit of the insulin receptor (IR) on the extracellular side of plasma membrane, which causes autophosphorylation of the β-subunit of the receptor. Then, pleckstrin homology (PH) domain on insulin receptor substrate (IRS)-1 binds to the activated IR to trigger Src homology 2 (SH2) domain on another downstream targets bound to IRS, such as phosphoinositide 3-kinase (PI3K) . PI3K has two subunits and with conformational change p85 subunit activates the catalytic subunit p110 domain. This in turn converts phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) on the intracellular side of plasma membrane. PIP3 recruits the PH domain containing protein, such as PDK1, Akt, and aPKC. Akt phosphorylates mTOR, which then phosphorylates its downstream factors: eukaryotic initiation factor 4E binding protein-1 (4E-BP1) and ribosomal protein S6 kinase (p70S6K) (Dennis et al. 1999). Eukaryotic initiation factor 4E (eIF-4E) is necessary for activating mRNA translation initiation. Hyperphosphorylation of 4E-BP1 will cause it to unbind to eIF-4E and allow

eIF-4E to bind to eIF4G to start formation of eIF4F (Wang and Proud 2006). The latter is the central factor in the active ribosome. On the other hand, phosphorylation of p70s6K by mTOR leads to activation of ribosomal protein S6 (rpS6), which results in translation of specific mRNA for ribosomal proteins, elongation factors, and binding proteins (Roux et al. 2007). Both sub-branched pathways from mTOR ultimately increase the capability for protein synthesis.

Akt is not only playing an important role in protein synthesis via the mTOR pathway but is also important in inhibiting protein degradation through phosphorylation of akt-forkhead box O-class (FOXO-3A) (Brunet et al. 1999). It has been shown that FOXO3A mRNA expression is increased in muscle in the fasted state and some other muscle atrophy states, such as aging and cachexia (Giresi et al. 2005, Lecker et al. 2004). Furthermore, FOXO3A dephosphorylation is sufficient to induce atrophy in whole muscle (Sandri et al. 2004).

AGE-RELATED LOSS OF MUSCLE MASS

Elderly individuals have a marked loss of muscle mass, known as sarcopenia, resulting in decreasing muscle strength and functionality. In aging humans, there is a 10% loss of muscle mass from 25 to 50 years of age, and muscle loss can reach up to ~30% after 50 years old (Hurley 1995). Selective atrophy occurs more in Type II muscle fibers than Type I, and occurs more in old women than men (Petrella et al. 2005).

Sarcopenia is a multifactor problem. One of the important mechanisms causing muscle loss in aging is the loss of effective neuro-innervations over time. It is known that contraction of skeletal muscle depends on a signal from a motor neuron. Each axon terminal of a motor neuron is attached to the synapse at a neuromuscular junction (NMJ). Age-related neuromuscular changes are associated with degeneration of NMJ, fragmentation of nerve terminals, and loss of motor neurons. This results in the denervation of myofibers and subsequent loss of muscle mass (Luff 1998).

Hormonal changes during aging are another crucial factors causing loss of muscle

mass. Testosterone is one of the primary hormones for enhancing muscle protein synthesis, muscle mass and strength. The gradual reduction of testosterone in men over 60 years old causes loss of muscle protein, thereby leading to loss of muscle mass. However, unlike testosterone, the reduction of estradiol level in women during menopause does not affect muscle mass and muscle strength. Conversely, insulin-like growth factor (IGF)-1α is another important hormone involved in muscle protein synthesis. Nevertheless, the circulating and intramuscular IGF-1α levels are markedly reduced with aging (Petrella et al. 2006), suggesting that age-related muscle wasting is in part due to the deficiency of IGF-1α.

Post absorptive protein balance between young and old people is not different (Volpi et al. 2001), but protein turnover after a meal decreases with age, resulting in net protein loss (Katsanos et al. 2006). Also, it has been demonstrated that the concentration of mTOR and p70S6K in skeletal muscle was lower in elderly individuals than in the young (Cuthbertson et al. 2005). Furthermore, phosphorylation of these kinases is blunted in the elderly relative to the young when given a certain amount of protein or amino acids (Cuthbertson et al. 2005). Because mTOR is the key signaling protein to mediate protein synthesis through the phosphorylation of 4E-BP1 (Gingras et al. 1999, Wang et al. 2005) and p70S6K (Dennis et al. 1999), these findings suggest that the efficiency of cellular machinery for protein synthesis is impaired in aged skeletal muscle. Collectively, it can be concluded that, during aging, these cellular and physiological impairments lead to gradual muscle wasting through increased protein degradation and/or attenuated protein synthesis. Therefore, the current theory of developing therapeutic intervention is focusing on decelerating or reversing age-related muscle wasting by re-activation of the muscle protein synthesis system and/or interruption of signaling pathways involved in muscle protein degradation.

Numerous interventional strategies have been used to maintain protein net balance in elderly, such as exercise, nutritional supplementation, medical therapy, and so forth. Nevertheless, in some circumstances, older individuals are unable to move freely and exercise, so nutritional supplementation would be a better option to attenuate protein

degradation or stimulate protein synthesis. Dietary protein is a common supplement. It is acceptable to large populations, including the elderly, because of its palatability and lower cost compared to other nutritional supplementation. As mentioned above, protein degradation occurs since 25 years old. Interventional strategies to prevent muscle loss via increasing protein synthesis or/and decreasing protein degradation are also important beginning with young adults.

EFFECT OF PROTEIN SUPPLEMENTATION ON MUSCLE PROTEIN SYNTHESIS

Protein synthesis requires 20 amino acids for peptide bond formation. Recent studies suggest that high-protein dietary supplements are sufficient interventions for increasing muscle mass and strength in aging individuals, because protein provides a rich amount of amino acids as substrates for protein synthesis in the body (Houston et al. 2008). In addition, protein intake can stimulate insulin secretion, a primary anabolic hormone in the body, which also can stimulate muscle protein synthesis through the mTOR pathway as mentioned above (Tremblay and Marette 2001). Whey, casein, and soy proteins are all natural proteins found in daily food. Both whey and casein proteins contain high portion of branched-chain amino acids (BCAAs), which are main resources responsible for protein synthesis. Casein protein appears more responsive to inhibition of protein degradation, while whey protein has a greater effect on promoting protein synthesis. Compared to casein, whey protein is digested faster, thus whey protein is considered to be the best protein source for older individuals to rapidly elevate circulatory amino acid levels (Dangin et al. 2002, Dangin et al. 2003). It also contains a high concentration of leucine, an important BCAA in the regulation of protein synthesis through mTOR pathway (Boirie et al. 1997).

Muscle atrophy in the elderly, however, may not be attenuated by protein supplementation alone. The elderly have a decreased sensitivity and responsiveness of protein synthesis associated with lower phosphorylation of anabolic signaling pathways and decrements in mRNA expression (Cuthbertson et al. 2005). Moreover, a

recommendation in that amount of protein may lead to some side-effect such as impairing renal function. However, it has been formed that a moderate serving of protein with high doses of essential amino acids are capable of stimulating muscle protein synthesis to a similar extent in the elderly compared to the young (Paddon-Jones and Rasmussen 2009). Therefore, supplementation of small amounts of essential amino acids has been recommended to maintain protein net balance.

EFFECT OF LEUCINE SUPPLEMENTATION ON MUSCLE PROTEIN SYNTHESIS

Amino acids are able to stimulate protein synthesis, but not all AAs have the same effectiveness. Leucine has been proven as a therapeutic strategy in attenuation of skeletal muscle atrophy in both *in vitro* and *in vivo* studies. Leucine promotes protein synthesis without affecting protein degradation (Zanchi et al. 2008). It exerts an anabolic effect which is similar to the effect of insulin. Compared to protein supplementation, leucine costs more and is less palatable. It has been known that protein synthesis in the elderly is much lower than in the young. This anabolic resistance can be fully countered by increasing the portion of leucine in the diet (Katsanos et al. 2006). The importance of leucine in regulating protein metabolism has been demonstrated in both human and rats models.

The mechanism for regulating protein synthesis between leucine and protein are partly different. Leucine alone does not stimulate the mTOR pathway via activating PI3K or its downstream target Akt, which are seen in the insulin and IGF-1α signaling pathway (Nobukuni et al. 2005). Thereby, leucine may not mainly stimulate insulin secretion. Instead, leucine stimulates protein synthesis in skeletal muscle via enhancing 4E-BP1 phosphorylation and p70S6K activity, which are downstream factors in the mTOR pathway (Anthony et al. 2000). In vitro, essential amino acids can phosphorylate mTOR directly, then promote p70S6K to stimulate protein synthesis independent of insulin (Byfield et al. 2005). However, old individuals have a lower protein synthesis sensitivity compared to young adults when given the same amount of AA, especially leucine.

(Dardevet et al. 2000). Some studies show that this reduced anabolic response to AA with aging may be related to dysregulated mTOR and its downstream signaling. (Cuthbertson et al. 2005, Guillet et al. 2004). It also appears that given a sufficient amount of leucine may trigger protein synthesis to a similar rate in elderly compared to youth, which suggests that the elderly should take protein supplements with sufficient leucine. In chronic studies, leucine supplementation was able to promote protein synthesis both in skeletal muscle and adipose tissue in rats without affecting insulin and IGF-1 level in plasma. (Lynch et al. 2002) Nevertheless, large amounts of leucine intake have been reported to cause diarrhea and may also impact renal function over time in the elderly. Thus, high-quantities of leucine intake may not be optimal to maintain a high protein synthesis in old adults.

Some studies also reported that leucine has little effect on anticatabolism. In 22-month old rats, a single leucine (5%) supplement meal fully restored the defective postprandial inhibition of proteasome-dependent proteolysis (Combaret et al. 2005). In addition, giving leucine alone or with other amino acids also inhibits whole-body protein degradation in dogs (Frexes-Steed et al. 1992b). However, the concentration of leucine required to prevent proteolysis is 10-20 times more than the requirement for protein synthesis. As we know, β-hydroxy-β-methylbutyrate (HMB) is the metabolite of leucine and α-ketoisocaproate (KIC). In human metabolism, every 100g leucine intake could be metabolized into 5g HMB; hence, the evidence above suggests that leucine has its effect on proteolysis via its metabolite HMB.

B-HYDROXY-B-METHYLBUTYRATE (HMB)

β-hydroxy-β-methylbutyrate (HMB) is reported to increase lean body mass, especially in older people, patients with AIDS, renal failure and cancer. It is worth noting that there are no adverse reactions reported by subjects consuming HMB (Vukovich et al. 2001).

1) Effect of HMB on protein degradation

Elderly people show more proteolysis than the young in a fasted state (Volpi et al. 2001). Several lines of evidence reveal that HMB is capable of blocking proteolysis in skeletal muscle. For example, a 9-day supplementation of HMB attenuates protein degradation and subsequently preserves lean body mass in mice with tumor via lowering proteasome-proteolytic activity (Smith et al. 2005). Additionally, HMB is also found to attenuate tumor necrosis factor-α-(TNF-α) and angiotensin II-induced muscle protein degradation through inactivation of ubiquitin-proteasome pathway (Eley, Russell and Tisdale 2008). Moreover, another study shows that HMB positively impacts protein metabolism by primarily inhibiting proteolysis rather than promoting protein synthesis (Holecek et al. 2009). Although the inhibitory effects of HMB on ubiquitin-proteasome pathway and protein degradation were only found in *in vitro* models and mice with tumors, these findings still raise the possibility for use of HMB to prevent muscle protein loss in older adults and even young adults. Based on the ability of HMB to inhibit proteolysis, it is likely that leucine's anticatabolic effects on protein degradation in skeletal muscle may be attributed to its conversion to HMB.

2) Effect of HMB on protein synthesis

In addition to the protective effect of HMB on protein degradation, both human and animal studies have shown that leucine and α-ketoisocaproate (KIC) supplementation significantly enhances protein synthesis only in catabolic states with increased proteolysis (e.g. starvation or burn trauma) (Sapir et al. 1983, Sax, Talamini and Fischer 1986, Bruzzone et al. 1991, Frexes-Steed et al. 1992a), suggesting that these anti-catabolic properties of leucine and its metabolites appear to be effective in decelerating the loss of muscle mass during aging. As the metabolite of leucine and KIC, HMB can also activate muscle protein synthesis through the insulin-related signaling pathway (Eley et al. 2007). HMB treatment for one month is able to phosphorylate both mTOR and p70s6k to induce muscle hypertrophy in EDL (Pimentel et al. 2011), but the same results do not appear in the soleus muscle. In addition, HMB appears to enhance protein synthesis by increasing

intramuscular IGF-1 gene expression. Additionally, a current *in vitro* study also showed that HMB is capable of stimulating IGF-1 mRNA expression in human myoblasts (Kornasio et al. 2009). Overexpression of IGF-1 has been proved to prevent age-related myofiber atrophy through activation of the mTOR pathway (Musaro et al. 2001). Nevertheless, it has not been clear whether this enhancing effect of HMB on IGF-1 gene expression also exists in *in vivo*. Furthermore, there have been no investigations focusing on whether acute treatment of HMB could activate protein synthetic related cell signaling pathways in aged or young adult mammalian models. Thus, the underlying mechanism(s) by which HMB increases protein synthesis and attenuates proteolysis requires further investigation.

Based on the positive effects of whey protein, leucine and its metabolite HMB on maintaining muscle protein balance when protein starts to degrade due to aging, we propose this project to detect the impact and the underlying mechanism(s) of these three treatments on protein synthesis and partial protein degradation pathways in young adult rats' skeletal muscle.

SUMMARY

A number of reports have demonstrated that intake of whey protein or essential amino acids, especially leucine, can stimulate protein synthesis in skeletal muscle by either increasing insulin secretion from the pancreas or activating the mTOR pathway directly, which further phosphorylates p70s6k and 4E-BP1 to increase ribosomal assembly, translation initiation, and elongation. Older adults have a gradual increase in protein degradation and less protein synthesis, due to dysregulated protein synthetic signaling in skeletal muscle. Larger intakes of whey protein or leucine are recommended to keep positive net protein balance, but too much whey protein or leucine intake can lead to adverse effects in the elderly. HMB, a metabolite of leucine, has been reported to prevent protein degradation as well as stimulate protein synthesis in long-term treatment, and it does not appear to cause any side effect. However, acute treatment of HMB and its

mechanisms on protein synthesis and degradation *in vivo* have not been examined. Therefore, if our research is able to demonstrate that acute HMB treatment can have a greater effect on the protein synthesis signaling pathway than protein or leucine treatment, HMB would be an optimal nutritional intervention for prevention of muscle loss and maintaining positive net protein balance.

APPENDIX A- MUSCLE HOMOGENIZATION

Muscle homogenization buffer (100ml, pH7.4)

Reagent	Concentration (mM)	Amount (g)
HEPES	20	0.477
EGTA	2	0.076
NaF	50	0.209
KCl	100	0.746
EDTA	0.2	0.0074
β-Glycerophosphate	50	1.08
DTT	1	0.015
PMSF	0.1	0.00174
Benzamidine	1	0.0157
Soduim vanadate	0.5	0.0092

Add reagents in order to 80ml ddH_2O, adjust pH to 7.4, and bring the volume to 100ml with stirring on a magnetic plate.

Homogenization procedure;

1. Weigh approximately 70mg of muscle sample;
2. Dilute muscle to 1:8 (wt/vol) in homogenization buffer;
3. Homogenize muscle on ice (3 x 10sec strokes) with a glass tissue grinder pestle at 3000rpm by an electrically-powered stirrer;
4. Centrifuge muscle samples at 14,000g for 10 minutes at 4 ℃;
5. Aliquot the supernatant into 1.5ml test tubes and store them at -80 ℃

APPENDIX B- WESTERN BLOT

30% Acrylamide and 1% Bisacrylamide Mixture (200ml)

Add 58g Acrylamide and 2g Bisacrylamide in order to 150ml ddH_2O stirring on a magnetic plate and bring to 200ml volume with ddH_2O. Filter this mixed solution through Whatman #1 filter paper and stored in a dark bottle due to the high light sensitivity at 4 ℃.

1.5M Tris, pH8.8 (500ml)

Add 90.82g Trisbase to 400ml ddH_2O stirring on a magnetic plate and bring to 500ml volume with ddH_2O. Adjust pH to 8.8 with 12N and 1N HCl. Store it at 4 ℃.

1.0M Tris, pH 6.8 (500ml)

Add 60.57g Trisbase to 400ml ddH_2O stirring on a magnetic plate and bring to 500ml volume with ddH_2O. Adjust pH to 6.8 with 12N and 1N HCl. Store it at 4 ℃.

10% SDS (100ml)

Add 10g SDS to 80ml ddH_2O stirring on a magnetic plate and bring to 100ml volume with ddH_2O. Filter this solution through Whatman #1 filter paper.

20% SDS (100ml)

Add 20g SDS to 80ml ddH_2O stirring on a magnetic plate and bring to 100ml volume with ddH_2O. Filter this solution through Whatman #1 filter paper.

10% APS (1ml)

Add 0.1g APS to 1.5ml test tube with 1ml ddH_2O and vortex until dissolve. Make fresh daily.

10 x Running Buffer (2L)

Reagent	Amount (g)
Trisbase	60.56
Glycine	288.4
SDS	20g

Add the reagents into 1.5L ddH_2O stirring on a magnetic plate and bring to the volume with ddH_2O

10x TTBS (2L, pH7.4)

Reagent	Amount
TrisBase	24.2 g
NaCl	175.36 g
Tween 20	12 ml

Add the reagents into 1.5L ddH_2O stirring on magnetic plate and bring to the volume

Ponceau S.

Reagent	Amount
Dye-Ponceau S	0.05 g
Acetic Acid	2.5ml

Add the reagents into 50ml ddH_2O and vortex until dissolve. Recycled to be used later.

Wet-transfer Buffer (2L for 2 tanks)

Reagent	Amount
Glycine	28.8g
Trisbase	6.04g
Methanol	300ml
SDS	0.75g

Add reagents in order into 1400ml ddH_2O stirring on a magnetic plate. Bring the volume into 2L with ddH_2O.

0.25% Bromophenol Bule

Add 0.0125g Bromophenol Bule into 5ml ddH_2O

Sample buffer (50ml)

Reagent	Amount
1.25M Tris-HCl (Ph 6.8)	5ml
Glycerol	10ml
20% SDS	5ml
B-Mercaptoethanol	2.25ml
0.25% Bromophenol Blue	1.6ml

Add reagents in the order listed to 20ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

Western blot procedure

1. Preparing resolving gel solution (10% resolving gel for 2 gels):

Reagent	Amount
H_2O	4 ml
Acrylanide mix	3.3 ml
1.5M Tris (pH 8.8)	2.5 ml
20% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.004 ml

Add all reagents above into 50ml test tube. Then mix them and fill ¾ full caster with a glass pipette. Overlay with 300ul butanol. Allow 1h for gel to polymerize.

2. After gel polymerization, pour off butanol from the resolving gel and rinse between casting plates with ddH_2O and dry it with KimWipes.
3. Preparing stacking gel solution (for 2 gels)

Reagent	Amount
H_2O	3.4 ml
Acrylanide mix	0.83 ml
1.0M Tris (pH 6.8)	0.63 ml
20% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

Add al reagents above into 15ml test tube. Then mix them and fill the caster with stacking gel solution then put combs into place. Allow 45 min for stacking gel to polymerize.

4. Preparing samples

a. Take the samples our of -80 ℃ and thaw them on ice

b. Dilute samples 1:2 with sample buffer in labeled microcentrifuge tubes.

c. Vortex and place in boiling water (~90-95 ℃) for 8min

5. Preparing 1 x Running buffer

   Add 100ml 10x Running buffer into 900ml ddH_2O stirring on a magnetic plate.

6. After stacking gel has polymerized, carefully remove the combs and assemble gel apparatus. Fill inner chamber and out chamber with 1 x running buffer.

7. Load 10 ul of standard, 12-20 ul of sample, or 8 ul of molecular weight marker to corresponded gel lane.

8. Electrophresis at 170V for 1h.

9. After the electorphoresis, prepare for wet-transfer buffer showed as above.

10. Cut 9.5 x 6cm rectangle Nitric Cellular Membrane, label it, and place membrane in wet-transfer buffer for 15min on a shaker.

11. Carefully separate casing plates, cut off stacking gel and place resolving gel in wet-transfer buffer for 15min on a shaker.

12. Soak four of the spongy pads and filter paper (2 for each gel) in the wet-transfer buffer for a few minutes.

13. Making the gel “sandwich”

a. Place cassette in a container filled with transfer buffer. Black side down.

   From bottom to top, add a spongy pad, a filter paper, a gel, a NC membrane, another filter paper, another spongy pad. Roll out bubbles with wetted glass tube.

b. Close cassette. Place in transfer apparatus.

c. Add ice to the small container and place in transfer apparatus as well.

d. Fill the transfer apparatus with transfer buffer and add the top piece with electrodes to the apparatus.

e. Place transfer apparatus in a larger plastic container fill the large plastic container with ice.

f. Electrophresis at 90V for 2h.

14. During the transfer, prepare 1 x TTBS.
    Add 100ml 10 x TTBS into 900ml ddH_2O stirring on a magnetic plate.
15. Prepare 7% non-fat dry milk (NFDM). 30ml per membrane.
    Add 4.2g NFDM into a 100ml beaker containing 60ml 1 x TTBS stirring on a magnetic plate.
16. After the transfer, take out the "sandwich". Stain each NC membrane with Ponceau S. for 5 seconds and put membrane into 1 x TTBS on a shaker to wash out Ponceau S. on Membrane.
17. Place NC membrane a container with 7% NFDM and block for 20min with gently agitation.
18. During blocking, prepare antibody with 2% NFDM. 10ml per membrane
    Add 0.4g NFDM into 20ml 1 x TTBS stirring on a magnetic plate. Then add appreciate ratio of antibody into 2% NFDM
19. After blocking the membrane, wash 3 x 5min in 25ml TTBS.
20. Incubate membrane with the primary antibody overnight at 4 ℃ with gently agitation.
21. After overnight incubation with the primary antibody, wash membrane 3 x 5min in 25 ml TTBS.
22. Incubate membrane with species specific secondary antibody for 1h at room temperature with gently agitation.
23. After incubation with the secondary antibody, wash membrane 3 x 5min in 25 ml TTBS.
24. Visualize protein bands using an ECL detection kit and Bio-Rad ChemiDoc detection system according to the manufacturer's instructions.
25. Quantify the density of bands using Quantity One Analysis software.

APPENDIX C- LOWERY PROTEIN ASSAY

1. Thaw one aliquot of 5mg/ml bovine serum albumin (BSA) stock (-20℃) and serially dilute it with deionized water to generate a standard curve as follows:

	Water (ml)	Protein (ml)	Protein Conc.(mg/ml)
Blank	1.0	0	0
A	1.8	0.2 of stock	0.5
B	0.2	0.8 of A	0.4
C	0.5	0.5 of B	0.2
D	0.5	0.5 of C	0.1
E	0.5	0.5 of D	0.05
F	0.5	0.5 of E	0.025

Set up duplicate 12x75mm glass assay tubes of each standard

2. Dilute samples with deionized water in 1:30 ratio. Keep on ice. Set up duplicate glass assay tubes for each sample

3. Make Solution A: add 48 ml of sodium carbonate (20 g/l of 0.1 NaOH) to a 125 ml Erlenmeyer flask. Gently layer 1 ml of 2% sodium potassium tartate over the surface of the sodium carbonate. Then gently layer 1 ml of 1% cupric sulfate over the sodium potassium tartate. Mix Solution A by swirling, then by vigorous vortexing

4. To the duplicate 12x75mm glass assay tubes, add 0.1 ml of standards or samples. Add 1.0 ml of Solution A to each tube. Next vortex each individual tube. Incubate tubes at room temperature for 10 minutes

5. Dilute phenol reagent 1:2 with deionized water. After the tubes have incubated for the 10 minutes, add 0.1 ml of the diluted phenol reagent into each glass tube while vortexing the glass tube. Incubate at room temperature for 30 minutes

6. Turn on the spectrophotometer and let the bulb warm up at a wavelength of 750 nm. Once the 30 minutes incubation are over, blank the spectrophotometer with the

"blank" tube. Then read every sample. Use the standard curve to determine protein concentration of the samples.

APPENDIX D- RAT INSULIN ASSAY

Day one:

1. Dilute all samples in half with assay buffer.
2. Thaw all the solutions. Hydrate ^{125}I-insulin with entire contents of Label Hydrating Buffer. Allow to sit at room temperature for 30mins, with occasional gentle mixing.
3. Pipette 200ul of Assay Buffer to the Non-specific binding (NSB) tubes (3-4) and 100ul to reference tubes (5-6). No buffer is added to tubes 7 through the end of the assay.
4. Pipette 100ul of standards and quality controls in duplilcate.
5. Pipette 100ul of each sample in duplicate.
6. Pipette 100ul of hydrated ^{125}I-insulin to all tubes.
7. Pipette 100ul of rat insulin antibody to all tubes except total count tubes (1-2) and NSB tubes (3-4).
8. Vortex, cover, and incubate at 4 ℃ overnight (20-24 hous)

Day two:

1. Add 1ml of cold precipitating reagent to all tubes except total count tubes (1-2) on ice.
2. Vortex and incubate 20mins at 4 ℃
3. Spin down all tubes except total count tubes (1-2) for 20mins at 3,000xg at 4 ℃.
4. Immediately decant the supernatant of all tubes except total count tubes (1-2), drain tubes for 60 seconds, and then blot excess liquid from lip of tubes.
5. Count all tubes in a gamma counter for 2mins and get the concentration based on the standard curve.

APPENDIX E- TREATMENTS SOLUTION PREPARATION

1. Make 10 x Krebs-Henseleit Buffer (KHB) Stock 1 and 2

   KHB Stock 1 10x (500 ml)

Chemical	Concentration	Amount (g)	FW	Catalog #
NaCl	1.6 M	33.895	58.44	SX0420-3
KCl	46 mM	1.715	74.56	P-217
KH_2PO_4	11.6 mM	0.789	136.1	P-0662
$NaHCO_3$	253.0 mM	10.625	84.01	S-6014

   KHB Stock 2 10x (500 ml)

Chemical	Concentration	Amount (g)	FW	Catalog #
$CaCl_2$ $2H_2O$	25 mM	1.8375	147	C-3881
$MgSO_4$ $.7H_2O$	4.46 mM	1.4295	246.48	M-1880

2. Make 200 ml 1x KHB combining 20 ml of Stock 1 and 2 with 150 ml ddH_2O, pH solution to 7.4 and then bring volume to 200 ml by adding more ddH_2O.
3. Making whey protein (WP) solution:

   Amino acids components per 100 g whey protein

Amino acids	Amount (g)
arginine	2.4
Glutamine	7.4
Histidine	1.7
Isoleucine	6.7
Leucine	11.9
Lysine	9.7
Methionine	2.0
Phenylalanine	3.3

Threonine	7.2
Tryptophan	2.1
Valine	6.2

Add 937.5 mg WP in 25 ml KHB stirring on a magnetic plate and bring to 50ml volume with KHB.

4. Making HMB solution:

   Add 3g CaHMB $\cdot H_2O$ in 40 ml KHB stirring on a magnetic plate and bring to 50ml volume with KHB

5. Making leucine solution:

   Add 7g leucine in 35 ml KHB stirring on a magnetic plate for 15 min and bring to 50ml volume with KHB, and then keep stirring on the magnetic plate at 50 ℃ at least overnight until rats are gavaged the next day.

APPENDIX F- RAW DATA

Blood glucose data

# of rats	treatment	0 min (mg/dl)	45 min (mg/dl)	90 min (mg/dl)	AUC (mg/dl x90 min)
1	CTRL	89.5	81.0	82.5	-540.0
6	CTRL	87.0	92.5	80.5	101.3
11	CTRL	82.0	90.5	90.5	573.8
13	CTRL	87.0	91.5	84.5	146.3
18	CTRL	83.5	95.5	89.0	663.8
23	CTRL	96.5	87.5	85.5	-652.5
44	CTRL	106.0	102.5	88.5	-551.3
2	WP	93.0	99.0	105.5	551.3
7	WP	87.5	100.5	86.0	551.3
12	WP	84.5	81.0	81.5	-225.0
17	WP	96.5	91.0	91.0	-371.3
22	WP	102.5	98.0	98.0	-303.8
27	WP	91.0	88.0	87.0	-225.0
32	HMB	90.0	89.5	90.5	-11.3
33	HMB	107.0	106.5	100.0	-180.0
34	HMB	82.0	89.0	90.0	495.0
35	HMB	90.5	85.5	86.5	-315.0
36	HMB	93.0	107.5	95.5	708.8
37	HMB	76.5	67.5	78.0	-371.3
39	HMB	95.0	101.0	96.5	303.8
16	LEU	98.0	83.0	80.5	-1068.8
21	LEU	91.5	81.5	89.0	-506.3
26	LEU	82.5	76.5	67.5	-607.5
40	LEU	106.0	80.0	71.0	-1957.5
41	LEU	85.0	77.0	77.0	-540.0
42	LEU	85.5	64.0	63.5	-1462.5
43	LEU	83.0	77.5	70.5	-528.8

Plasma insulin data

# of rats	treatment	0 min (ng/ml)	45 min (ng/ml)	90 min (ng/ml)	AUC (ng/ml x90 min)
1	CTRL	1.622	0.908	1.020	-45.675
6	CTRL	0.274	0.248	0.132	-4.365
11	CTRL	0.322	0.498	0.352	8.595
13	CTRL	0.338	0.342	0.384	1.215
18	CTRL	0.388	0.470	0.512	6.480
23	CTRL	0.636	0.344	0.276	-21.240
44	CTRL	0.788	0.484	0.736	-14.850
2	WP	0.296	0.384	0.352	5.220
7	WP	0.688	0.510	0.418	-14.085
12	WP	1.000	0.364	0.438	-41.265
17	WP	0.522	0.404	0.514	-5.490
22	WP	0.890	0.578	0.548	-21.735
27	WP	0.444	0.314	0.370	-7.515
32	HMB	0.880	0.290	0.356	-38.340
33	HMB	2.760	1.506	1.210	-91.305
34	HMB	0.164	0.022	0.038	-9.225
35	HMB	0.298	0.270	0.242	-2.520
36	HMB	0.922	0.346	0.558	-34.110
37	HMB	0.234	0.026	0.100	-12.375
39	HMB	0.956	0.822	0.708	-11.610
16	LEU	0.380	0.670	0.292	11.070
21	LEU	0.238	0.228	0.176	-1.845
26	LEU	0.856	1.438	1.006	29.565
40	LEU	0.260	0.398	0.404	9.450
41	LEU	0.848	1.631	1.112	41.175
42	LEU	0.428	0.976	0.688	30.510
43	LEU	0.128	0.060	0.060	-4.590

Western blotting data

# of RG	treatment	p-akt/STD	p-mTOR/STD	p-p70s6k/STD	p-4E-BP1/STD	p-FOXO3A/STD	a-tubulin/STD
1	CTRL	0.337	0.160	0.083	0.409	0.720	0.900
6	CTRL	0.270	0.600	0.056	0.257	0.670	1.026
11	CTRL	0.112	0.320	0.060	0.940	0.996	0.869
13	CTRL	0.142	0.603	0.088	0.908	1.142	0.936
18	CTRL	0.135	0.564	0.034	0.836	1.255	1.094
23	CTRL	0.148	0.459	0.090	1.047	1.029	0.908
44	CTRL	0.129	0.100	0.046	0.305	1.050	0.720
2	WP	0.148	0.296	0.026	0.320	0.890	0.949
7	WP	0.150	0.265	0.074	0.753	0.640	0.932
12	WP	0.114	0.337	0.089	0.565	1.132	0.939
17	WP	0.301	0.493	0.027	0.833	1.364	0.996
22	WP	0.168	0.452	0.067	0.823	0.891	0.831
27	WP	0.136	0.516	0.030	0.659	0.685	0.722
32	HMB	0.236	0.770	0.066	0.909	1.790	0.966
33	HMB	0.539	0.436	0.151	0.883	1.202	0.726
34	HMB	0.248	0.874	0.103	0.494	1.251	0.948
35	HMB	0.231	0.590	0.040	0.887	1.494	0.923
36	HMB	0.206	0.705	0.046	0.615	1.604	1.032
37	HMB	0.152	0.592	0.080	0.655	1.224	0.953
39	HMB	0.357	0.586	0.123	0.471	1.473	0.852
16	LEU	0.131	1.178	0.230	1.470	1.167	0.915
21	LEU	0.111	0.792	0.244	1.183	0.962	0.991
26	LEU	0.232	1.004	0.377	0.951	0.697	0.992
40	LEU	0.144	1.590	0.869	1.575	1.887	1.158
41	LEU	0.287	1.080	0.485	0.933	1.070	1.071
42	LEU	0.254	0.871	0.434	1.234	0.867	0.954
43	LEU	0.226	0.908	0.335	0.902	1.390	0.857

# of WG	treatment	p-akt/STD	p-mTOR/STD	p-p70s6k/STD	p-4E-BP1/STD	p-FOXO3A/STD	a-tubulin/STD
1	CTRL	0.199	0.553	0.009	0.702	0.284	0.331
6	CTRL	0.277	0.337	0.009	1.097	0.420	0.407
11	CTRL	0.295	0.167	0.008	0.684	0.210	0.570
13	CTRL	0.130	0.490	0.012	1.475	0.467	0.581
18	CTRL	0.108	0.469	0.007	1.375	0.766	0.809
23	CTRL	0.096	0.133	0.013	0.662	0.845	0.538
44	CTRL	0.105	0.381	0.021	0.608	0.723	0.680
2	WP	0.207	0.335	0.008	0.744	0.560	0.555
7	WP	0.199	0.286	0.008	1.101	0.915	0.402
12	WP	0.280	0.387	0.012	0.586	0.839	0.686
17	WP	0.088	0.573	0.013	0.712	0.392	0.415
22	WP	0.150	0.362	0.006	0.694	0.447	0.735
27	WP	0.097	0.719	0.006	0.709	0.392	0.547
32	HMB	0.197	0.742	0.030	0.780	1.852	0.457
33	HMB	0.361	0.673	0.034	1.100	2.169	0.500
34	HMB	0.144	0.433	0.035	0.970	0.825	0.636
35	HMB	0.190	0.598	0.041	0.710	1.066	0.663
36	HMB	0.170	0.647	0.021	0.650	2.595	0.672
37	HMB	0.162	0.558	0.025	0.890	1.259	0.680
39	HMB	0.171	0.393	0.031	0.770	1.467	0.630
16	LEU	0.132	0.828	0.028	2.188	0.598	0.575
21	LEU	0.149	0.603	0.059	0.820	1.034	0.417
26	LEU	0.147	0.556	0.017	1.362	0.454	0.699
40	LEU	0.160	0.626	0.190	1.190	0.983	0.621
41	LEU	0.223	0.479	0.074	1.064	0.982	0.817
42	LEU	0.222	0.737	0.151	1.205	0.769	0.914
43	LEU	0.145	0.310	0.081	1.328	0.829	0.611

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