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To the Graduate Council:

I am submitting herewith a thesis written by Renee Ashley Stancliffe entitled "Role of Beta-Hydroxy-Beta-Methylbutyrate (HMB) in Leucine Stimulation of Mitochondrial Biogenesis and Fatty Acid Oxidation." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael B. Zemel, Major Professor

We have read this thesis and recommend its acceptance:

Melissa Hansen-Petrik, Ling Zhao

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(Original signatures are on file with official student records.)

Role of Beta-Hydroxy-Beta-Methylbutyrate (HMB) in Leucine Stimulation of Mitochondrial Biogenesis and Fatty Acid Oxidation

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Renée Ashley Stancliffe

December 2012

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Dedication

To my husband

John Stancliffe

To my parents

John and Brenda Hanlin

To the memory of my grandmother

Imogene Drexl

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I would like to thank the distinguished faculty members who served on my committee: Professors Michael B. Zemel, Melissa Hansen-Petrik, and Ling Zhao. As my major professor Dr. Zemel provided guidance and encouragement throughout the course of this thesis. I am grateful for the opportunity to have worked with him on this project and to have been a member of his laboratory, which has shaped me personally and professionally in countless ways. Thank you to all of my committee members for their support, patience, encouragement, and suggestions. Additionally, I would like to express my gratitude to my lab-mates for their assistance and companionship throughout this thesis. Finally, I must thank my husband for his continual support and encouragement throughout this project, and for his pride in this accomplishment.

Abstract

Mitochondrial dysfunction and the resulting oxidative stress is widely recognized as a contributing factor to the development of numerous pathophysiologies including obesity, diabetes, cardiovascular disease, sarcopenia, liver disease, dementia, amongst others. Mitochondrial dysfunction results in a reduced mitochondrial number and oxidative capacity, causing an increase in free radical production and consequently oxidative stress. As such, the characterization of compounds that can upregulate mitochondrial biogenesis and function could provide the foundation for the development of therapeutic nutraceuticals that promote mitochondrial health, and consequently reduce oxidative stress. Leucine is well recognized to stimulate muscle protein synthesis, and we have recently demonstrated that leucine increases mitochondrial biogenesis and fatty acid oxidation (FAO) in muscle cells, although the mechanism of these effects is not clear. However, it is likely that that the leucine metabolites [alpha]-ketoisocaproic acid (KIC) and [beta]-hydroxy-[beta]-methylbutyrate (HMB) play a role. Once ingested, dietary leucine is transaminated by branched-chain aminotransferase (BCAT) to the [alpha]-keto analogue KIC. KIC is then metabolized into either isovaleryl-CoA via the branched chain [alpha]-ketoacid dehydrogenase (BCKD) enzyme, or HMB by the cytosolic enzyme KIC-dioxygenase (KICD). We investigated the roles of intact leucine versus KIC and HMB on markers of mitochondrial abundance and function in murine myotubes. All three compounds induced comparable increases in FAO. Both leucine and HMB increased myotube mitochondrial biogenesis, assessed fluorometrically via NAO binding. Consistent with this, HMB and leucine both stimulated expression of mitochondrial regulatory and component genes, which suggests that HMB mediates these effects of leucine. To confirm this, we transfected murine myoblasts with BCAT, BCKD, or KICD siRNA and investigated the role of intact

leucine versus HMB on myoblast mitochondrial abundance. Both HMB and leucine increased mitochondrial mass, while the knockdown of BCAT and KICD abated the leucine-stimulation of mitochondrial biogenesis. Consistent with this, BCAT siRNA transfected cells displayed reduced expression of key mitochondrial genes. This suggests that the leucine effects on muscle mitochondrial biogenesis are in fact mediated by the metabolite HMB.

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Part I

Introduction

β-hydroxy-β-methylbutyrate (HMB) is a metabolite of the branched chain amino acid (BCAA) leucine, and is produced endogenously by human skeletal muscle [1]. Of the BCAAs, leucine is the most widely investigated due to its varied physiological roles in mitochondrial function, protein synthesis, exercise recovery, glucose homeostasis and insulin action [2-8]. The anabolic effect of leucine on protein synthesis is largely mediated by the activation of mammalian target of rapamycin (mTOR) pathway in skeletal muscle, which results in the initiation of signal transduction pathways that enhance the activity and synthesis of proteins involved in translation [4,5,9]. It has been hypothesized that its metabolite HMB may contribute to these observations due to the myriad clinical trials involving HMB which demonstrate its efficacy as a supplement to support protein synthesis and muscle maintenance in a variety of populations and disease states [10-14]. Our lab has previously demonstrated leucine to increase mitochondrial biogenesis and fatty acid oxidation in murine muscle cells [2,3].Considering the similar results obtained following leucine and HMB supplementation on lean body mass and mTOR stimulation of protein synthesis, it is possible that HMB may also mediate the leucine stimulation of mitochondrial number and function. Accordingly, the primary objective of this project was to determine whether HMB mediates the effects of leucine on mitochondrial biogenesis and function. The following questions will be addressed:

1. What are the effects of the leucine metabolites KIC and HMB on mitochondrial biogenesis and fatty acid oxidation?
2. What is the role of HMB in mediating the leucine effects on mitochondrial biogenesis?

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Part II

Literature Review

2.1 Mitochondria: An Overview

Mitochondrial Dysfunction in Health and Disease

Mitochondria play an essential role in cellular metabolism and energy homeostasis, cell signaling, and apoptosis [1]. An increasing body of literature identifies mitochondrial dysfunction as a contributing factor to numerous pathophysiologies [1,2]. Given its central role in the cell, it is no surprise that damage to mitochondria ultimately leads to disease [2]. For example, mitochondrial dysfunction is recognized as a causative factor in a wide variety of disease states which include obesity, diabetes, cardiovascular disease, cancer, liver disease, and dementia [2-7]. Additionally, the accumulation of mitochondrial damage and the resultant oxidative stress has been identified as a potential mechanism for aging [8].

The common pathophysiology amongst these diseases stems from the production of reactive oxygen species that result in the accumulation of damage to mitochondrial DNA [1,3,8]. This ultimately leads to mitochondrial dysfunction, which consequently results in the generation of more reactive oxygen species, thus perpetuating a vicious cycle (Figure 2-1) [1,3,8]. This combination of oxidative stress and mitochondrial dysfunction is common to a number of conditions, making the identification of compounds that can attenuate oxidative stress and mitochondrial function of particular importance [1,9].

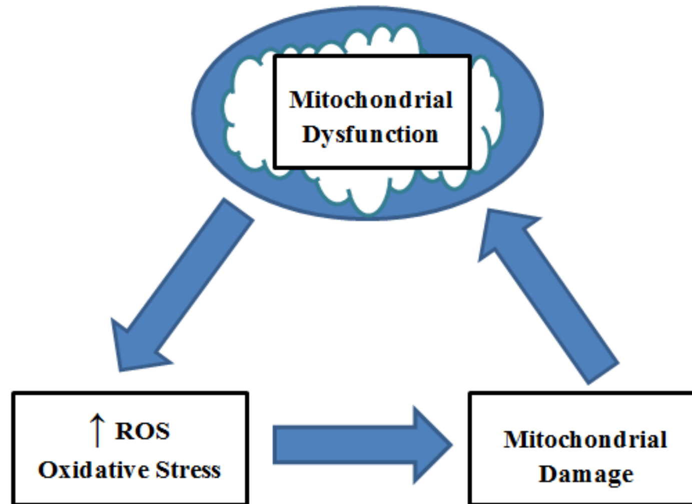

Figure 2-1. The Cycle of Mitochondrial Dysfunction. The production of ROS results in the accumulation of mitochondrial damage, leading to mitochondrial dysfunction. Mitochondrial dysfunction results in the production of more ROS and is accompanied by oxidative stress, thereby perpetuating a vicious cycle.

Mitochondrial Form and Function

Mitochondria are highly organized and dynamic organelles, which allows them to perform their myriad biological processes [1,10]. Mitochondria possess a double membrane organization, resulting in distinct compartments within the organelle [10]. The semi-permeable outer mitochondrial membrane encloses the organelle, separating it from the cytosol [10]. Between the outer and inner membrane is the intermembrane space, a location for protein transport [10]. The inner mitochondrial membrane serves as the site for oxidative phosphorylation and ATP synthases, and a location for protein transport [10]. The inner membrane is further compartmentalized to form cristae, which increases the surface area thereby augmenting the organelles ability to synthesize ATP [10]. Finally, the mitochondrial matrix is

the space within the mitochondrial membrane and is the location of the citric acid cycle as well as fatty acid oxidation [10].

While the primary function of mitochondria is often cited as ATP production, this organelle is essential for numerous cellular processes including cellular metabolism, cell signaling and apoptosis [1]. For example, in addition to being the site of the citric acid cycle, lipolysis, and oxidative phosphorylation, mitochondria contain enzymes essential for the synthesis of lipids, cholesterol, and heme and play a critical role in amino acid metabolism [1,10]. Additionally, the controlled generation of reactive oxygen species (ROS) by the mitochondria is critical for both cell signaling and apoptosis [1].

Mitochondrial Biogenesis

Due to the essential role the mitochondria have in bioenergetics, metabolism and cell signaling, there is tight regulation of mitochondrial mass and function [1]. This regulation allows for differentiations in mass, morphology and function dependent upon the organism or cell type with some cells containing a single mitochondrion and others possessing several thousand [1,10]. Strict control over mitochondrial dynamics also permits this organelle to have varying responses based upon alterations in the environment (i.e. physical activity, availability of nutrients, temperature, etc.) [1,10].

This control is achieved through mitochondrial biogenesis, which is an essential biological process required for growth and development as well as to meet the variable energy requirements of the cell [1,9]. Mitochondrial biogenesis requires the synthesis, import, and integration of macromolecules to the mitochondrial reticulum [1,11]. Mitochondrial biogenesis

is further complicated by the presence of dual genomes, and the necessary replication of the mitochondrial genome [1,11]. Roughly fifteen hundred proteins comprise the mitochondrial proteome, which are predominantly encoded by the nuclear genome [11]. The human mitochondrial genome is a circular segment of DNA that encodes a mere thirty-seven genes [1,11]. Of those thirty-seven genes encoded by mitochondrial DNA, thirteen encode proteins required for oxidative phosphorylation, twenty-two encode mitochondrial transfer RNA, and two encode mitochondrial ribosomal RNA [11]. Much of the mitochondrial proteome is committed to specialized functions, as evidenced by tissue-specific mitochondrial gene expression [12].

Comparison of mitochondria in multiple mouse tissues revealed significant conformity between protein levels and gene expression levels as measured by messenger RNA [12]. This suggests that the tight control of mitochondrial biogenesis is largely achieved through transcriptional regulation [1,12]. As such, the transcriptional regulation of mitochondrial biogenesis is achieved through the synchronized transcription of mitochondrial genes in both the nucleus and the mitochondria [13].

The Role of PGC-1α in Mitochondrial Biogenesis

PPARγ coactivator-1, or PGC-1α, is often referred to as the master regulator of mitochondrial biogenesis due to its ability to stimulate the expression of myriad mitochondrial genes [1]. PGC-1α possesses molecular features allowing it to interact with, and ultimately increase the transcription of, numerous mitochondrial genes [14]. The vital role of PGC-1α in mitochondrial biogenesis has been clearly demonstrated in transgenic studies [15-18]. The overexpression of PGC-1α in skeletal muscle results in increased mitochondrial abundance and

gene expression, and improved exercise performance [15,16]. In contrast, PGC-1α null mice display decreases in mitochondrial gene expression and impaired mitochondrial function [17,18].

While PGC-1α regulates the expression of numerous mitochondrial genes, its own expression is dictated by the energetic needs of the cell [14]. For example, PGC-1α is expressed in a tissue-specific manner exhibiting higher expression levels in tissues with elevated energy requirements [13,14]. Additionally, PGC-1α expression and activity is sensitive to external signals of increased energy needs [14]. For example, PGC-1α expression is increased following caloric restriction and physical activity [13,19]. Furthermore, the activity of PGC-1α is attenuated by posttranslational modifications by AMP-activated protein kinase (AMPK) and sirtuin1 (SIRT1) which are themselves activated by an increase in cellular energy needs [13,20,21]. This collectively implies that PGC-1α is a key influence in the long-term adaptation to the changing energy needs of the cell [1,13].

The Role of NRF-1 in Mitochondrial Biogenesis

Nuclear respiratory factor-1, or NRF-1, is a DNA binding transcription factor that regulates the expression of numerous mitochondrial genes [22]. NRF-1 stimulates the expression of a segment of the mitochondrial genome, specifically the genes that encode oxidative phosphorylation components and mitochondrial ribosomal proteins [22,23]. NRF-1 also controls the expression of transcription factor a, mitochondrial (TFAM) [23]. TFAM is a nuclear encoded gene that is partially responsible for the coordinated transcription of the nuclear and mitochondrial genomes during mitochondrial biogenesis [11,23]. Furthermore, NRF-1 activity is essential for the basal expression of mitochondrial genes as silencing of NRF-1 results

in significant reductions in the expression of said genes, and NRF-1 null embryos exhibit reduced mitochondrial DNA and mitochondrial membrane potential [24-26].

NRF-1 expression and activity is modulated by signals recognized to promote mitochondrial biogenesis [19,25,27-28]. For example, physical activity and/or increased levels of AMPK results in augmented expression of NRF-1 in muscle tissue [27,28]. Overexpression of PGC-1α in myotubes induces the expression of NRF-1 [19], while physical interaction between NRF-1 and PGC-1α enhances the activity of NRF-1 and results in greater expression of NRF-1-dependent genes [19,25]. Collectively this suggests that like PGC-1α, NRF-1 expression and activity is modulated by the energetic needs of the cell [19].

Measuring Mitochondrial Biogenesis

Mitochondria are highly polymorphic, exhibiting structural variants that are contingent upon cell type, metabolic needs of the cell, and the cell cycle stage [1,10]. Similarly, mitochondrial mass varies amongst different organisms and tissue types, and is specifically dependent upon the energy requirements of the cell [1,10]. In the literature, mitochondrial abundance is often utilized as an indicator of mitochondrial biogenesis [29,30]. This measurement is achieved through the use of a cell-permeable, mitochondrion-selective fluorescent dyes [30]. In order to fluorescently label the mitochondria, cells are incubated with nanomolar concentrations of fluorescent probe [31]. The probe is able to diffuse through the cell membrane and accumulate within the mitochondria, thus allowing researchers to measure mitochondrial mass via fluorescence [31]. 10-Nonyl acridine orange (NAO) and MitoTracker Green are two such fluorescent probes, often utilized for measuring mitochondrial mass

quantitatively as they accumulate within the mitochondria independent of membrane potential [29-31]. MitoTracker Green has the additional benefit of only fluorescing within the lipid environment of the mitochondria, thereby minimizing background fluorescence making it an ideal choice for qualitative imaging data [29,31].

While the aforementioned probes are useful for measuring mitochondrial mass, the data generated by said probes cannot distinguish changes in the size of mitochondria versus mitochondrial number [29-32]. Given that control of mitochondrial biogenesis is achieved through transcriptional regulation [1,12], gene expression levels of key mitochondrial regulatory and component genes should be used to confirm alterations in mitochondrial number rather than size [32]. For example, increased fluorescence measured via NAO and an upregulation in the expression of PGC-1α and/or NRF-1 are indicative of increased mitochondrial biogenesis [32]. The branched chain amino acid (BCAA) leucine significantly upregulates mitochondrial biogenesis, as measured by fluorescent probe and gene expression levels, in skeletal muscle cells [32].

2.2 Leucine

The Branched Chain Amino Acids

Leucine, isoleucine, and valine are categorized as BCAAs, a group of essential amino acids that play important roles in protein synthesis and energy production [33]. Of the BCAAs, leucine is the most widely investigated due to its varied physiological roles in mitochondrial function, protein synthesis, exercise recovery, glucose homeostasis and insulin action [32-38]. In

humans, between fifteen and twenty-five percent of total protein intake is in the form of BCAAs, and BCAAs comprise forty percent of the essential amino acids in body protein [39, 40]. As essential amino acid the BCAAs must be obtained through diet, and dairy products are one of the richest sources of BCAAs and of leucine in particular [39]. Estimated requirements for leucine in the diet vary greatly, ranging from one to twelve grams daily [41].

Chemical Profile of Leucine

Leucine is a branched-chain, hydrophobic amino acid composed of thirteen hydrogen atoms, six carbon atoms, two oxygen atoms, and a single nitrogen atom ($C_6H_{13}NO_2$) [10]. Due to its essentiality, leucine cannot be biosynthesized and must be consumed through diet [39]. Once ingested, dietary leucine is transaminated to α-ketoisocaproate (KIC) via the mitochondrial enzyme BCAA transferase (BCAT) [42]. The reversible BCAT reaction occurs predominantly extrahepatically, following this reaction KIC can enter one of two degradative pathways [42,43]. The principal route for leucine metabolism results in the generation of isovaleryl-CoA from KIC, through the enzymatic action of the mitochondrial branched-chain ketoacid dehydrogenase complex (BCKD) [42,44]. Following a series of reactions isovaleryl-CoA ultimately yields acetyl-CoA and acetoacetate, which can enter into the citric acid cycle [42,43].

The above pathway is common to isoleucine and valine, however, leucine has an alternative catabolic pathway available for the degradation of KIC [23]. β-hydroxy-β-methylbutyrate (HMB) can be produced from KIC via KIC-dioxygenase, a cytosolic enzyme found predominantly in the liver [42-44]. Under normal conditions, approximately five to ten percent of ingested leucine is converted into HMB [9,10]. Following this pathway, HMB can be metabolized to yield β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) which can be utilized for

cholesterol synthesis [9,22]. Leucine metabolism is summarized in Figure 2-2, adapted from Nissen and Abrumrad (1997) and Van Koverin and Nissen (1992) [42].

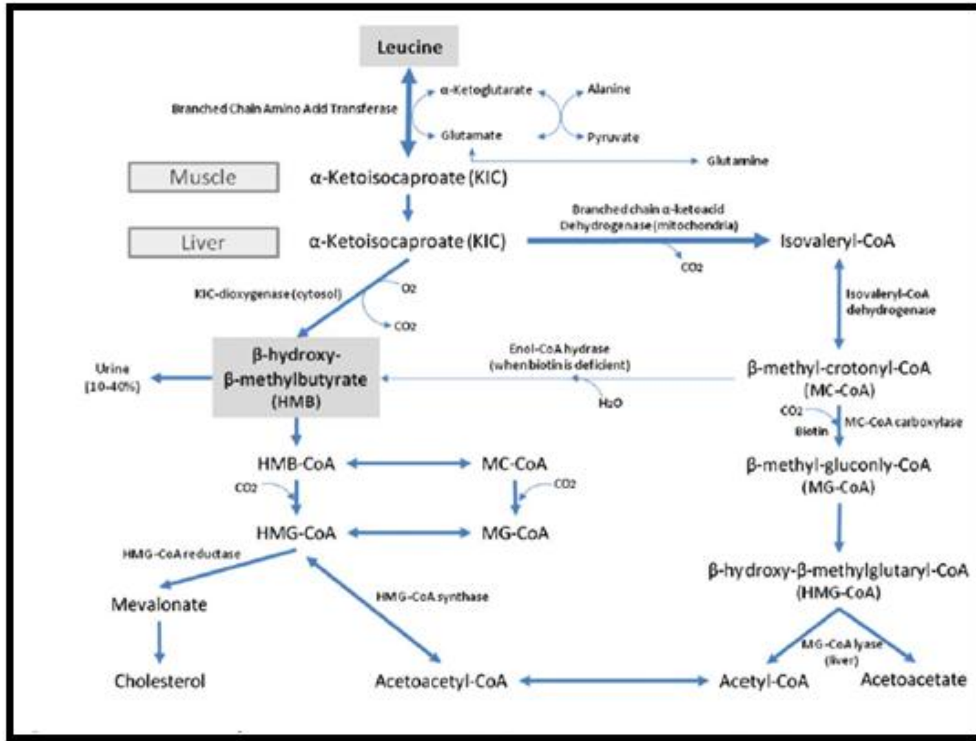

Figure 2-2. Leucine Catabolism.

Leucine and Protein Synthesis

While it is a function of all amino acids to serve as a substrate for protein synthesis, leucine possesses the unique ability to upregulate protein synthesis [45]. Leucine supplementation inhibits muscle catabolism during periods of caloric deprivation and prolonged endurance training, and stimulates muscle protein synthesis following exercise [46-48].

Furthermore, elimination of leucine during protein administration abates protein synthesis *in vitro*, while eliminating other amino acids has little to no effect on protein synthesis [45]. Additionally, a structural analogue of leucine (nor-leucine) enhances protein accretion despite the fact that it cannot act as a substrate for a growing polypeptide chain [45].

The anabolic effect of leucine on protein synthesis is largely mediated by the activation of mammalian target of rapamycin (mTOR) pathway in skeletal muscle, which results in the activation of S6 ribosomal protein kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) [49-52]. Leucine administration stimulates protein synthesis through the hyperphosphorylation of the translational repressor eIF4E-binding protein 1 (eIF4E-BP1), resulting in enhanced activity of the translation initiator eIF4E [45,52]. The activation of S6K1 results in increased synthesis of ribosomal proteins and elongation factors [52,53]. Through mTOR leucine is able to initiate signal transduction pathways that enhance the activity and synthesis of proteins involved in translation [52]. Although the majority of leucine's ability to promote protein accretion appears to be modulated by mTOR, leucine is also able to activate the eukaryotic initiation factor-4G (eIF4G) through an mTOR-independent mechanism [53]. While these effects are predominantly insulin-independent, baseline fasting levels of insulin are required in order to observe said effects [52,53].

Leucine & Mitochondrial Biogenesis

The benefits of BCAA supplementation extend beyond protein accretion, as a body of evidence has been accumulating that suggests that BCAAs, and leucine in particular, can promote mitochondrial health and potentially reduce oxidative stress [32,34,54-56].

Experiments in the model organism *Saccharomyces cerevisiae* have revealed that BCAAs may promote long-term survival in this species [54]. Furthermore, long-term administration of BCAA rich diet resulted in a significant increase in the lifespan of male mice, complemented by a concurrent increase in mitochondrial biogenesis and reduced oxidative damage in skeletal and cardiac muscle [55]. Our own laboratory has demonstrated that leucine significantly upregulates mitochondrial biogenesis and enhances FAO in murine myotubes [32,34].

2.3 HMB

HMB: The Leucine Metabolite

HMB, the leucine metabolite, is produced endogenously by skeletal muscle [57]. HMB has been the focus of considerable recent attention as a supplement to support protein synthesis and/or and muscle maintenance in athletes, the elderly, as well as in atrophic disease states such as advanced AIDS and cancer cachexia [43,58-61]. There is much overlap between the leucine and HMB literature as both compounds are known for their ability to promote protein synthesis and muscle health [43,57].

Lean Body Mass, Mitochondrial Function, and Health

Lean body mass (LBM) accounts for seventy-five percent of normal body weight, and includes all tissues except adipose tissue [62]. Skeletal muscle comprises the majority of LBM, the maintenance of which is critical in supporting whole-body protein metabolism, wound healing, physical strength, organ function, and immune function [62-64]. A progressive loss of

LBM, or sarcopenia, occurs naturally with age [58]. Injury and illness can cause or accelerate this loss of LBM [15]. For example, sarcopenia is exacerbated by several chronic conditions including AIDS, cancer, non-healing wounds, and congestive heart failure [62,65,66]. In addition to a loss of skeletal muscle, the sarcopenic phenotype is associated with a shift in muscle fiber type and a reduced ability to perform activities of daily living [65]. Sarcopenia is also associated with increased morbidity and mortality regardless of age, and documented consequences of LBM loss range from impaired immune function to physical disability to depression in the elderly [58,62,66].

Post-mortem histological examinations have demonstrated that sarcopenia results in an approximately fifty-percent reduction in total muscle fibers between the ages of twenty and eighty, with a disproportionate loss of fast-twitch muscle fibers [67,68]. The function of skeletal muscle is highly dependent upon the production of ATP by mitochondria [68]. Mitochondrial dysfunction results in a reduced mitochondrial number and oxidative capacity, causing an increase in free radical production and consequently oxidative stress [65,68]. Sarcopenic muscle is associated with mitochondrial dysfunction, demonstrating a clear relationship between muscle and mitochondrial health [65,67,68]. As such, the identification of compounds that can attenuate both the mitochondrial dysfunction and the loss of LBM associated with sarcopenia is an important focus in current medical research.

Chemical Profile of HMB

HMB has been documented to support muscle health in a variety of populations [43,58-61]. As a metabolite of leucine, HMB is produced endogenously following the consumption of dietary leucine [57].HMB is also found in a variety of food sources such a catfish, grapefruit,

alfalfa, and avocado and is available commercially as a supplement in the form of a calcium salt [43,69].

Following modest HMB consumption plasma levels peak within two hours, with an increased plasma response observed following larger doses of HMB [70]. Depending upon the dosage provided between ten and forty percent of HMB undergoes urinary excretion, with as much as eighty-six percent of HMB remaining in the body [43,70]. Animal toxicity studies have demonstrated no adverse effects following HMB exposure, and HMB supplementation in humans appears to be both safe and well tolerated in the diverse populations studied [42,43,70]. In clinical trials to date, the most frequently administered dose of HMB is three grams daily although optimal dosage and frequency have not been established [43]. The average person produces less than one gram of HMB endogenously daily, and would need to consume between thirty and sixty grams of leucine to achieve the results observed in standard supplementation studies of HMB [43,57].

Proposed Mechanism of Action

Within the current body of HMB literature, there is much to support the efficacy of supplementation on specific outcomes, and very few publications examining the mechanisms of action of the compound [42,43]. While these mechanisms remain unclear, they likely relate to the ability of HMB to modulate protein synthesis and degradation pathways and/or maintain the integrity of the sarcolemma [43].

HMB disrupts two pathways that promote proteolysis, caspase-8 and nuclear factor kappa B (NFκB) [71-73]. The activation of caspase-8 results in a cascade of events that ultimately downregulates protein synthesis, while NFκB stimulates proteosome activity [43,71]. HMB

inhibits the activation of caspase-8 and interferes with the protein degradation activities of NFκB, thus maintaining protein synthesis and preventing proteolysis [71-74]. HMB also appears to directly upregulate protein synthesis through the activation of mTOR (mammalian target of rapamycin), the intracellular protein that regulates protein synthesis [75].

Another possible mechanism by which HMB exerts its effect lies in its ability to provide readily available substrate for cholesterol synthesis in muscle cells, as this is critical for the integrity of myotube membranes [43,57]. There is evidence that damaged muscle cells are unable to produce adequate cholesterol, resulting in decreased sarcolemmal integrity and increased susceptibility to cell rupture [57]. The majority of HMB is metabolized into HMG-CoA, which is often termed the rate limiting step in cholesterol synthesis [43,57]. Thus, HMB is able to support the maintenance of sarcolemmal structure by providing an essential substrate for myotube cholesterol synthesis [43]. It is important to note that HMB supplementation does not result in an increase in circulating cholesterol [76,77]. HMB may play a role in the production of coenzyme Q, a downstream metabolite of HMG-CoA, which plays an important role in myocyte proliferation and mitochondrial function [78]. While the mechanisms of action of HMB may not be well understood, the efficacy of supplementation in a variety of populations on protein synthesis and muscle maintenance is well established [43].

Efficacy of HMB Supplementation

The use of HMB as an ergogenic aid has been extensively studied in healthy adults, alone and in combination with other amino acids [42,43]. Presently, more than twenty clinical trials support the efficacy of HMB supplementation in delaying muscle soreness, decreasing muscle

damage, improving strength and other performance markers, and increasing LBM [43]. These effects are more pronounced in untrained individuals, compared to experienced athletes [42].

The elderly have an increased risk for loss of LBM, and several studies have focused on HMB supplementation in this high-risk population [43,58]. For example, Vukovich et al. examined the effect of HMB supplementation in combination with resistance training in healthy, elderly men and women [59]. Thirty-one participants were randomized to either an HMB supplemented or a placebo group, and participated in an eight-week resistance training program [59]. The individuals receiving the HMB exhibited increased fat loss measured via computerized tomography scans, and a greater increase in upper and lower body strength compared to the placebo group [59]. A study conducted by Flakoll et al. studied the effects of supplementing HMB in combination with arginine and lysine versus a placebo in healthy elderly women [79]. The researchers hypothesized that the reductions in protein degradation observed with HMB, may attenuate the effects of sarcopenia [43,79]. After twelve weeks, the group receiving the HMB combination demonstrated increased leg and handgrip strength, and a twenty percent increase in protein synthesis relative to the placebo [79]. Based upon the available data, healthy elderly individuals exhibit increased strength and a more desirable body composition with HMB supplementation alone and in conjunction with an exercise program [43,59,79].

Much like the elderly, critically ill patients are at an increased risk for loss of LBM compared to the general population [62-65]. Due to its anti-catabolic properties HMB supplementation has been studied in numerous disease states associated with muscle atrophy [59-62]. Supplementation of HMB in conjunction with glutamine and arginine has been demonstrated to decrease muscle-wasting associated with advanced AIDS and cancer cachexia over a period of eight to twenty-four weeks [60,61]. In critical care patients, supplementation of

the aforementioned HMB combination resulted in increased protein synthesis while patients exhibited negative nitrogen balance during their stay in the intensive care unit [80]. Similarly, Cohen et al. demonstrated that supplementing exclusively HMB during negative nitrogen balance maintained LBM to a greater extent than placebo during a hospital stay [43].

While much of the research to date supports the efficacy of HMB supplementation in promoting protein synthesis and muscle maintenance, the literature is not without flaws. For example, the standard dosage of HMB supplemented is three grams per day provided in three equal dosages [43]. With few studies examining the topic, optimal dosage and frequency have yet to be established for HMB supplementation [42,43]. Additionally, studies relating to HMB supplementation are conducted by a core group of authors, many of whom profit from the sale of HMB, introducing the potential for bias in the research [43]. However, the HMB research has been sufficiently replicated in a variety of laboratory and clinical settings, suggesting the validity of the findings [42].

2.4 Research Objectives

Leucine versus HMB

The efficacy of HMB supplementation on protein synthesis and the maintenance of LBM in healthy and critically ill patients has been well documented, and similar effects have been observed with leucine supplementation. Amongst the BCAAs leucine has been most frequently used as an ergogenic aid in athletes, and has been demonstrated to increase protein synthesis in an mTOR dependent manner [43,57]. The mechanism by which these effects occur has not been

clearly established, and it has been postulated that these effects are in fact mediated by its metabolite HMB [43].

The association between muscle health and mitochondrial function has been well established, and our lab has recently demonstrated that leucine promotes mitochondrial biogenesis and stimulates fatty acid oxidation in murine muscle cells [32,34]. HMB has been recognized to promote muscle health in both exercise and clinical settings, but the effect of HMB on mitochondrial function has not been examined in the literature [43]. Based upon the similar results obtained from leucine and HMB supplementation on LBM and mTOR stimulation of protein synthesis, it is possible that HMB may also mediate the leucine stimulation of mitochondrial function. We hypothesize that the leucine attenuation of proteolysis, along with improved muscle health, and mitochondrial function are potentially mediated by its metabolite HMB. Accordingly, the primary objective of this project was to determine whether HMB mediates the effects of leucine.

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Part III

Role of Beta-Hydroxy-Beta-Methylbutyrate (HMB) in Leucine Stimulation of Mitochondrial Biogenesis and Fatty Acid Oxidation

3.1Abstract

Background: Leucine is well recognized to stimulate muscle protein synthesis, and we have recently demonstrated that leucine increases mitochondrial biogenesis and fatty acid oxidation (FAO) in muscle cells. However, the mechanism of these effects is not clear. It is hypothesized that the leucine metabolite HMB may play a role.

Methods: We tested the effect of leucine versus its metabolites (α-ketoisocaproic acid (KIC) and HMB)in the regulation of mitochondrial mass measured via fluorescence, and examined the expression levels of key mitochondrial regulatory and component genes. We also evaluated the effect of leucine versus the aforementioned metabolites on fat oxidation, one of the important functions of the mitochondria. Finally, we tested the effect of intact leucine on mitochondrial biogenesis while inhibiting leucine catabolism.

Results: KIC (0-0.5mM) and HMB (0-50μM) induced comparable increases in FAO (~ 60-70%, $p<0.001$) in C2C12 myocytes when compared to leucine (0-0.5mM). Both leucine and HMB increased myotube mitochondrial biogenesis (assessed fluorometrically via NAO binding) by ~ 50%, ($p<0.005$) in C2C12 cells. Consistent with this, HMB and leucine both stimulated the expression of mitochondrial regulatory (PGC-1α and NRF-1) and component (UCP3) genes ($p<0.01$) in murine myotubes. Transfection of C2C12 myocytes with branched-chain amino transferase (BCAT) or KIC dioxygenase (KICD) siRNA resulted in the suppression of the leucine-induced stimulation of mitochondrial biogenesis ($p<0.05$).

Conclusions: These data suggest that the leucine-induced stimulation of mitochondrial biogenesis and FAO is mediated by its metabolite HMB.

3.2 Introduction

The branched chain amino acids (BCAAs) leucine, isoleucine, and valine comprise more than one third of muscle protein, and have important physiological roles in protein synthesis and energy metabolism [1,2]. Amongst the BCAAs leucine is the most widely investigated due to its varied physiological roles in mitochondrial abundance and function, protein synthesis, exercise recovery, glucose homeostasis and insulin action [1,3-8]. β-hydroxy-β-methylbutyrate (HMB) is a metabolite of leucine, and is produced endogenously by human skeletal muscle [9].

Once consumed, dietary leucine is transaminated to α-ketoisocaproate (KIC) by the mitochondrial enzyme BCAA transferase (BCAT) [10]. The reversible BCAT reaction occurs predominantly extrahepatically, and following this reaction KIC can enter one of two potential degradative pathways [10,11]. The chief pathway for leucine catabolism results in the production of isovaleryl-CoA from KIC, through the enzymatic action of the mitochondrial branched-chain ketoacid dehydrogenase complex (BCKD) [10,12]. Following a series of reactions isovaleryl-CoA ultimately generates acetyl-CoA and acetoacetate, both of which can act as substrate for the citric acid cycle [10,11].

The aforementioned pathway is common to all of the BCAAs, however, leucine has an alternative degradative pathway available for the catabolism of KIC [12]. KIC is metabolized into HMB via KIC-dioxygenase, a cytosolic enzyme found predominantly in the liver [10-12]. Under normal conditions, approximately five to ten percent of ingested leucine is converted into HMB [9,11]. Following this pathway, HMB can be metabolized to yield β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) which can be utilized for cholesterol synthesis [9,10]. HMB is also found in a variety of food sources such a catfish, grapefruit, alfalfa, and avocado and is available commercially as a supplement in the form of a calcium salt [9,10].

HMB has been the focus of considerable recent attention as a supplement to support protein synthesis and muscle maintenance in athletes, the elderly, as well as in atrophic disease states such as advanced AIDS and cancer cachexia [11,13-16]. Due to the overlap in the literature with regard to leucine and HMB supplementation and protein accretion, it has been suggested that HMB may mediate the effects of leucine [11].Our lab has previously demonstrated leucine to increase mitochondrial biogenesis and fatty acid oxidation in murine muscle cells [3,4]. Accordingly, the primary objective of this project is to determine whether HMB mediates the effects of leucine on mitochondrial biogenesis and FAO.

To accomplish this, C2C12 murine myotubes were treated with physiologically relevant concentrations of leucine, KIC, or HMB to explore the direct effect of leucine versus its metabolites on mitochondrial biogenesis and fatty acid oxidation. Mitochondrial abundance was measured by fluorescence as an indicator of mitochondrial biogenesis, and the expression of key mitochondrial regulatory and component genes will be measured to confirm a change in mitochondrial number. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), often termed the master regulator of mitochondrial biogenesis and transcription factor nuclear respiratory factor 1 (NRF1) was utilized as additional markers of mitochondrial biogenesis [3,4]. Mitochondrial uncoupling protein 3 (UCP3), a mitochondrial component gene, was also utilized to indicate a change in mitochondrial number [3,4]. The effects of leucine, KIC, and HMB on fatty acid oxidation was also assessed, and myotube fat oxidation was measured using tritiated-palmitate as previously described [3,17]. Key experiments were repeated following the knockdown of BCAT, BCKD, and KICD via siRNA transfection, to evaluate the roles of KIC and HMB in the leucine-induced stimulation of mitochondrial biogenesis and FAO.

3.3 Materials and Methods

Cell Culture

C2C12 myoblasts were incubated at a density of 8,000 cells/cm^2 (T75 flask) and grown in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO_2. Cultures were re-fed every 1 to 2 days until 80 to 90% confluency had been achieved. Confluent C2C12 myoblasts were induced to differentiate with a standard differentiation medium (DMEM with 2% horse serum and 1% penicillin-streptomycin), and fed with fresh differentiation medium daily until myotubes were fully formed.

Treatment of Cells

Leucine, KIC, and HMB were freshly diluted in medium before treatment of cells. Cells were incubated in serum free medium overnight and then washed with fresh medium, re-fed with medium containing the different treatments (0-0.5mM leucine, 0-0.5mM KIC, and 0-50µM HMB), and incubated at 37°C in 5% CO_2 for 48 hours before analysis.

Fat Oxidation

Fat oxidation of muscle cells was measured as previously described [17], with minor modifications. C2C12 myotubes were rinsed twice with phosphate-buffering saline (PBS) and incubated in substrate mixture containing 22µM unlabeled palmitate plus 5µCi [^{3}H]palmitate in Hank's basic salt solution containing 0.5mg/ml BSA for two hours. Negative controls were created by treating cells with methanol for 30s to eliminate cellular metabolism. The reaction medium was then collected from cells and treated with 0.2ml 10% trichloroacetic acid. The protein precipitate was then removed via centrifugation, and supernatants were treated with 6N

NaOH and then applied to a poly-prep chromatography column with 1ml Dowex-1. The ^3H_2O was passed through the column and the following 1ml of wash was collected and radioactivity was measured with a liquid scintillation counter.

Total RNA Isolation

RNA extraction was performed per the manufacturer's instructions using total cellular RNA isolation kit (Ambion, Austin, TX). The concentration and purity of the isolated RNA was then measured spectrophotometrically (A280/A260 between 1.9 and 2.1).

Quantitative Real-Time PCR

18s, PGC-1α, NRF1, and UCP3 were quantitatively measured using an ABI 7300 Real-Time PCR System (Applied Biosystems, Branchburg, NJ) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). Primer and probe sets were purchased from Applied BiosystemsTaqMan Assays-on-Demand and utilized according to manufacturer's instructions. A standard curve was created using serial diluted pooled total RNA (1.5625-25ng). Reactions of quantitative RT-PCR for standards and unknown samples were performed per ABI 7300 Real-Time PCR System and TaqMan Real-Time PCR Core Kit instructions. The mRNA quantitation for each sample was normalized using the corresponding 18s quantitation.

Measurement of Mitochondrial Mass

Nonylacridine orange (NAO) (Invitrogen, Carlsbad, CA), a mitochondrial probe, was used to analyze mitochondrial mass by fluorescence (excitation 485nm and emission 520nm). A fluorescence microscope (Leica, Lasertechnik GmbH, Heidelberg, Germany) linked to a Hamamatsu color chilled 3CCD camera (Hamamatsu, Japan) was used to generate qualitative

imaging data. Quantitative data were obtained via fluorescence microplate reader (Promega, Fitchburg, WI), with the intensity of fluorescence expressed as arbitrary units per μg of protein.

Gene Silencing

siRNA-annealed oligonucleotide duplexes for BCAT, BCKD, and KICD and negative control were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA) and C2C12 cells were transfected using the siRNA Reagent System (Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer's instructions. The percent knockdown was assessed and optimized via Real-Time PCR as described previously.

Statistical Analysis

All data are presented as mean ± SEM. Data were evaluated by one-way or ANOVA, and significantly different group means ($p<0.05$) were then separated by the least significant difference test using SPSS (SPSS Inc, Chicago, IL).

3.4 Results

We have previously shown leucine to upregulate FAO in murine myotubes [4]. Consistent with this observation, we found the leucine metabolites KIC and HMB to induce comparable increases in FAO in C2C12 cells compared to leucine (Figure 3-1). Prior data generated by our laboratory has demonstrated leucine to induce significant increases in mitochondrial mass and to augment the expression of key mitochondrial regulatory and component genes in murine myotubes [3]. Similar effects were observed in C2C12 cells following treatment with KIC and HMB. All three compounds significantly increased

mitochondrial abundance in the muscle cells compared to the control group (Figure 3-2). Likewise, treatment with leucine and its metabolites increased the expression of mitochondrial biogenesis regulatory genes PGC-1α and NRF-1, and the mitochondrial component gene UCP3 (Figure 3-3).

To further investigate the role of HMB in mediating the leucine-induced effects on mitochondrial biogenesis, we knocked down BCAT, BCKD, and KICD using siRNA in C2C12 myocytes. BCAT and BCKD siRNA transfection successfully decreased the corresponding mRNA by approximately thirty to forty percent (Figure 3-4). Due to low expression levels, KICD knockdown could not be assessed. BCAT knockdown showed a significant reduction in the leucine-induced expression of mitochondrial regulatory genes PGC-1α and NRF-1, and the mitochondrial component gene UCP3 (Figure 3-5). This effect was not observed in leucine treated murine myocytes following BCKD or KICD knockdown. However, knockdown of BCAT and KICD abated the leucine-induced stimulation of mitochondrial mass while BCKD knockdown had no effect (Figure 3-6).

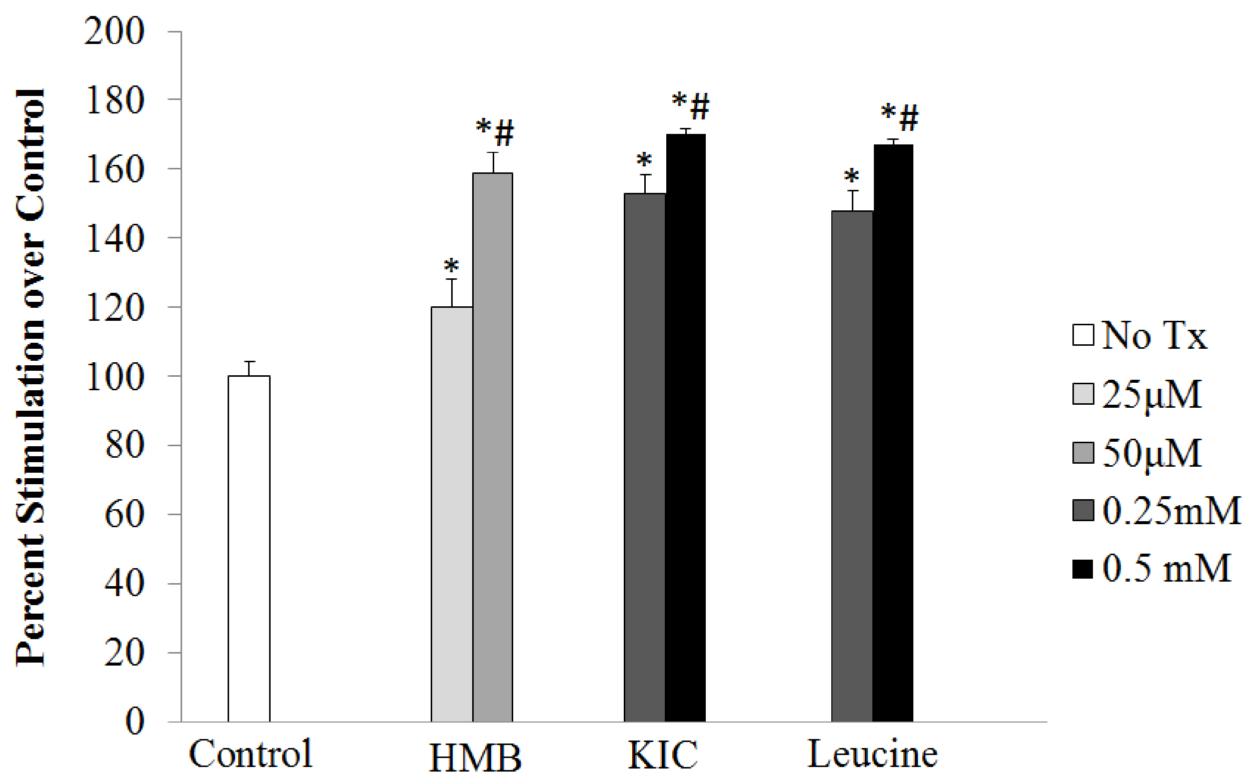
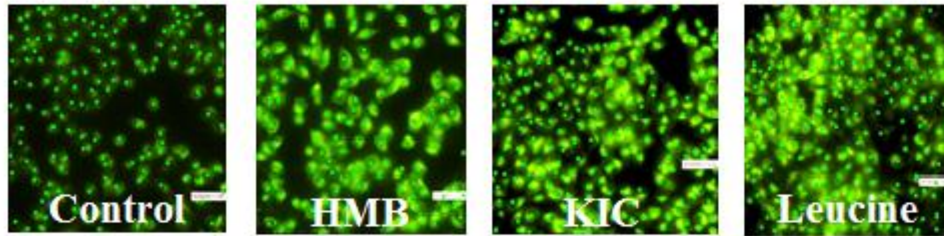


Figure 3-1.The effect of HMB, KIC, and leucine on fatty acid oxidation in C2C12 muscle cells. FAO was determined by palmitate oxidation. C2C12 myotubes were treated with one of two concentrations of HMB, KIC, or leucine for 48 hours. Values are presented as mean ± SEM, $n = 6$. Means with * differ compared with control group $p<0.001$, and means with # differ by concentration $p<0.05$.

A



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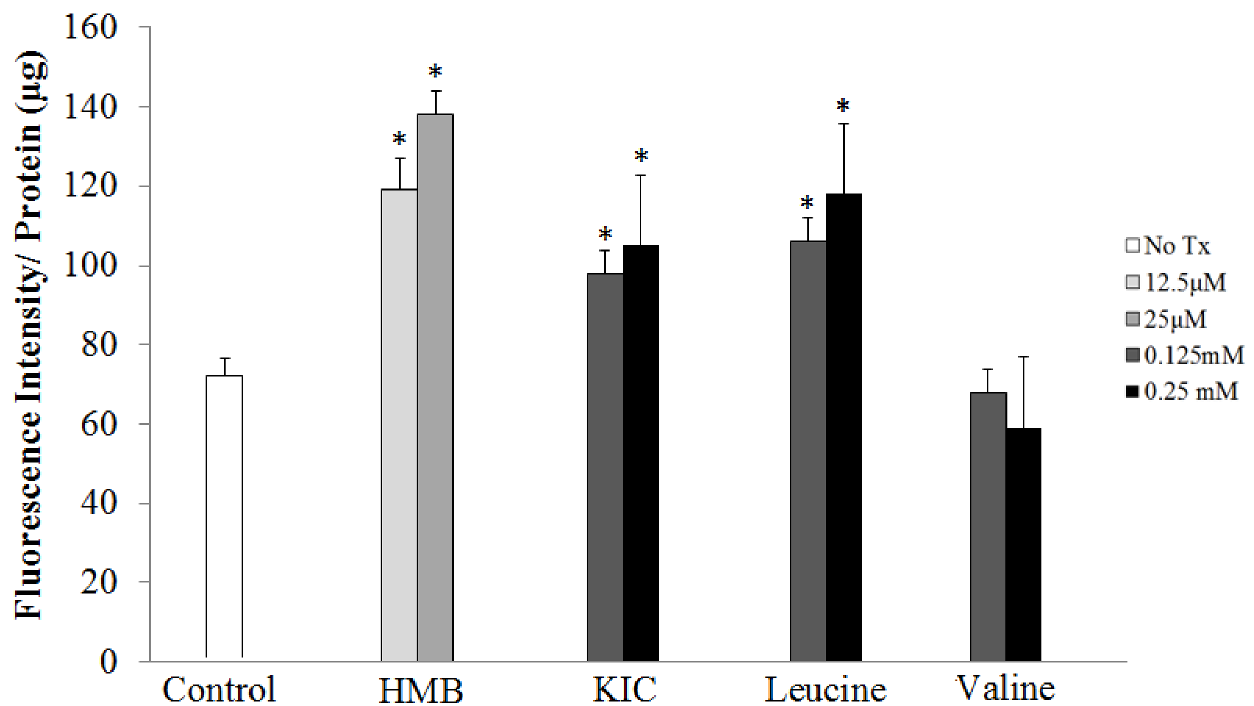


Figure 3-2.The effect of HMB, KIC, and leucine on mitochondrial mass by NAO binding in C2C12 myotubes. (A) The images show fluorescent photographs of mitochondrial density and (B) the bars show the quantitative data in response to treatment. Cells were treated with one of two concentrations of HMB, KIC, or leucine for 48 hours. Values are presented as mean ± SEM, $n = 12$. Means with * differ compared with control group $p<0.005$.

A

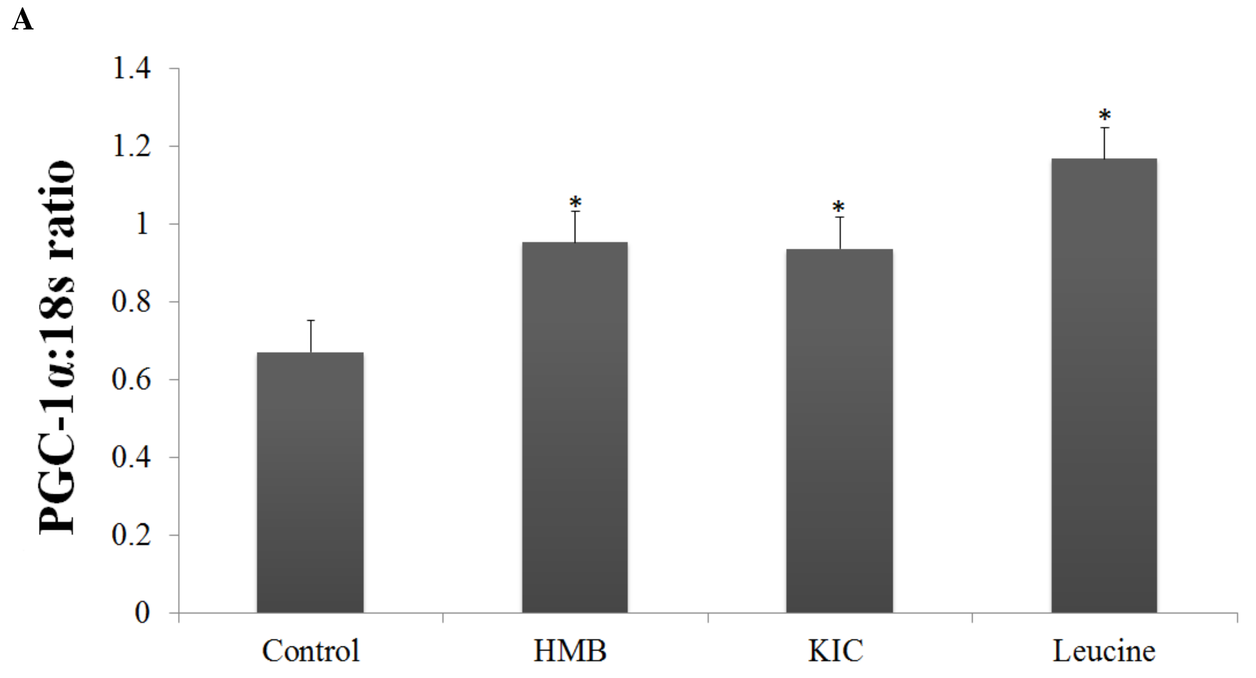
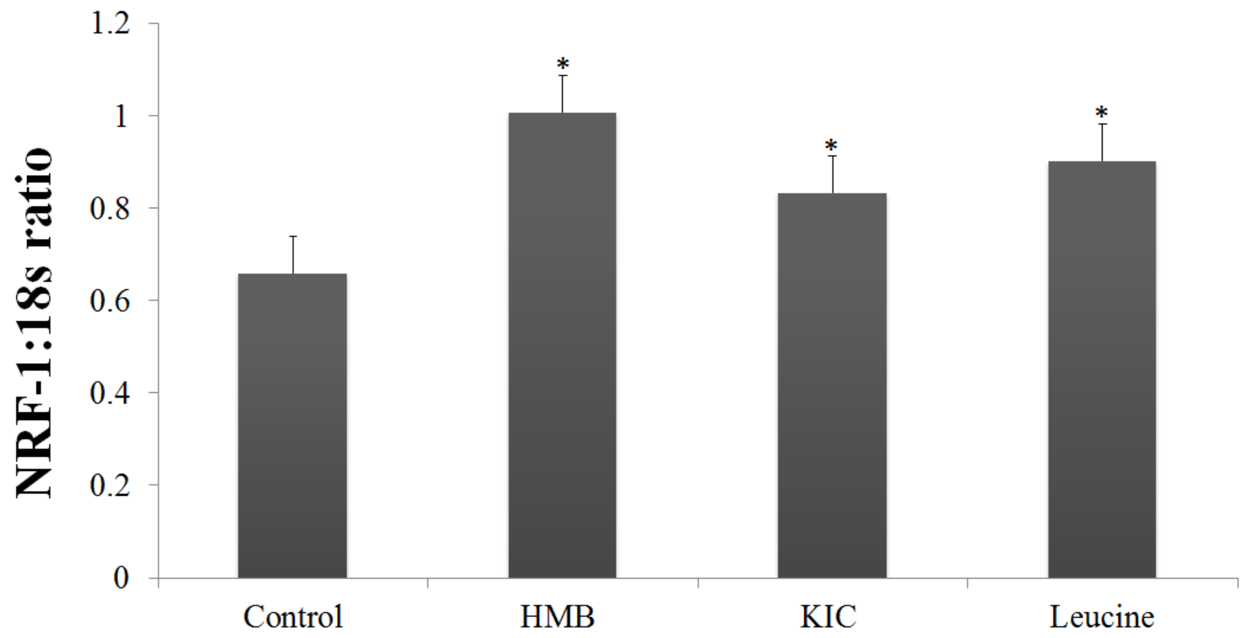


Figure 3-3. The effect of HMB, KIC, or leucine on (A) PGC-1α, (B) NRF-1, and (C) UCP3 gene expression in C2C12 myotubes; expression of each gene is normalized to 18s expression. Cells were treated with HMB (12.5μM), KIC (0.125mM), or leucine (0.125mM) for 48 hours. Values are presented as mean ± SEM, n = 6. Means with * differ compared with control group $p<0.01$.

Figure 3-3 Continued

B



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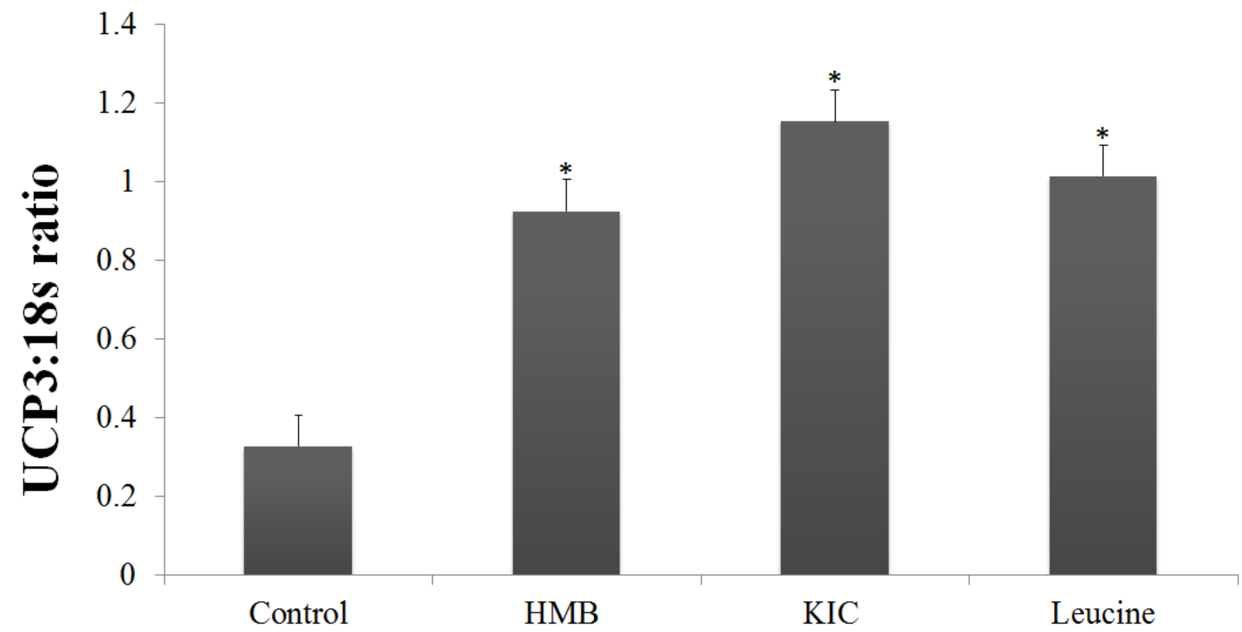

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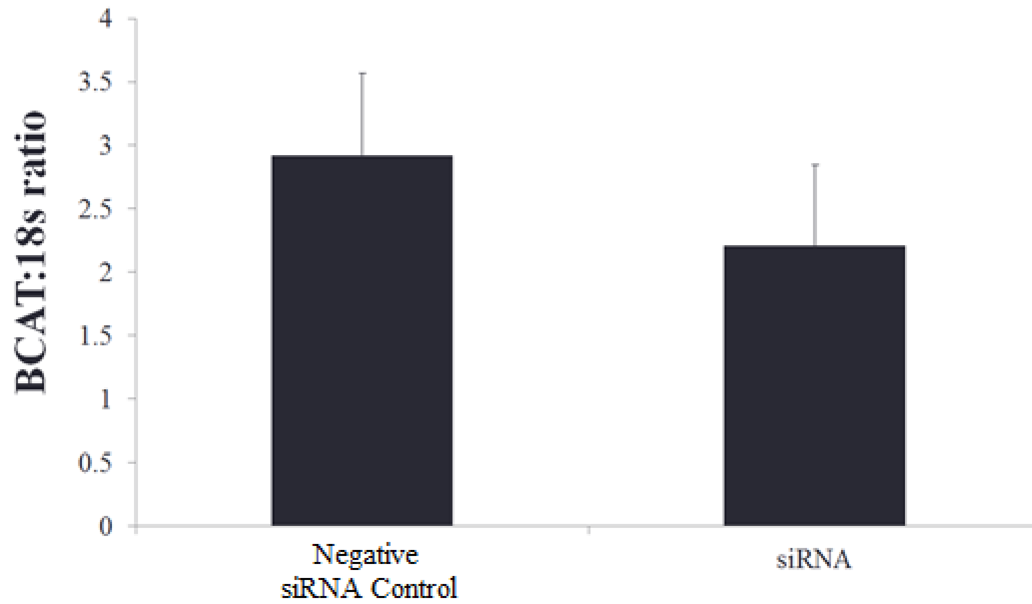


B

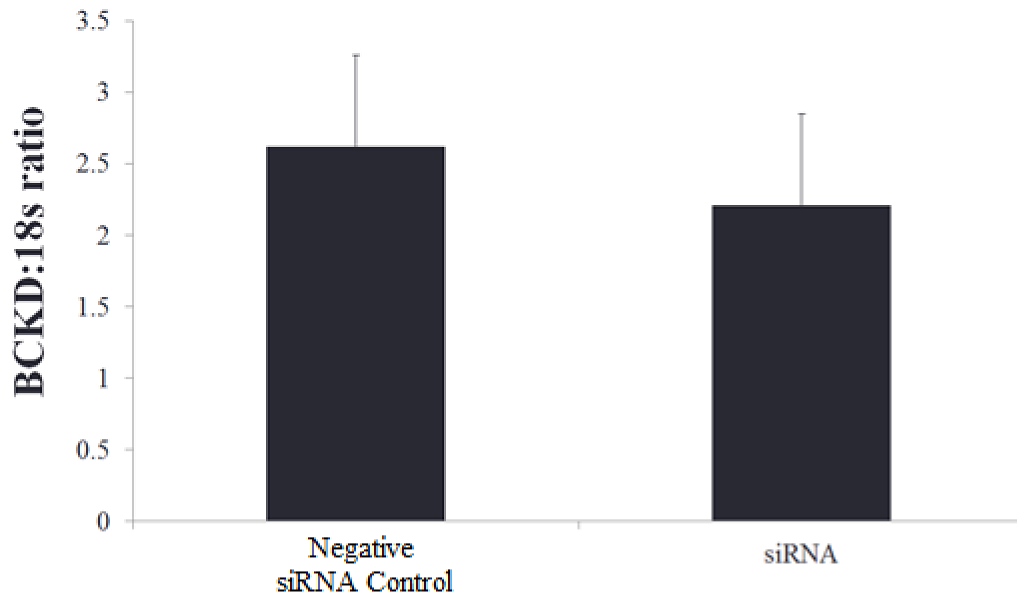


Figure 3-4. The expression of (A) BCAT and (B) BCKD in C2C12 myocytes with or without (A) BCAT and (B) BCKD siRNA transfection; expression of each gene is normalized to 18s expression. Values are presented as mean ± SEM, $n = 6$.

A

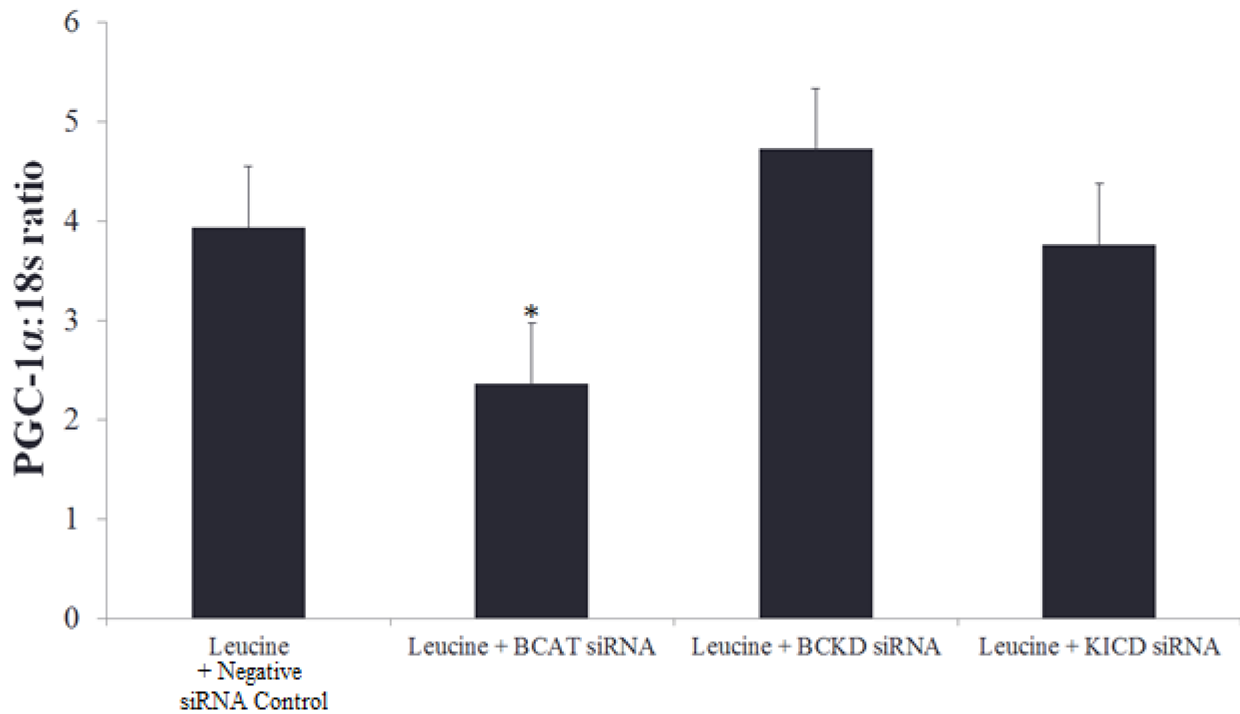
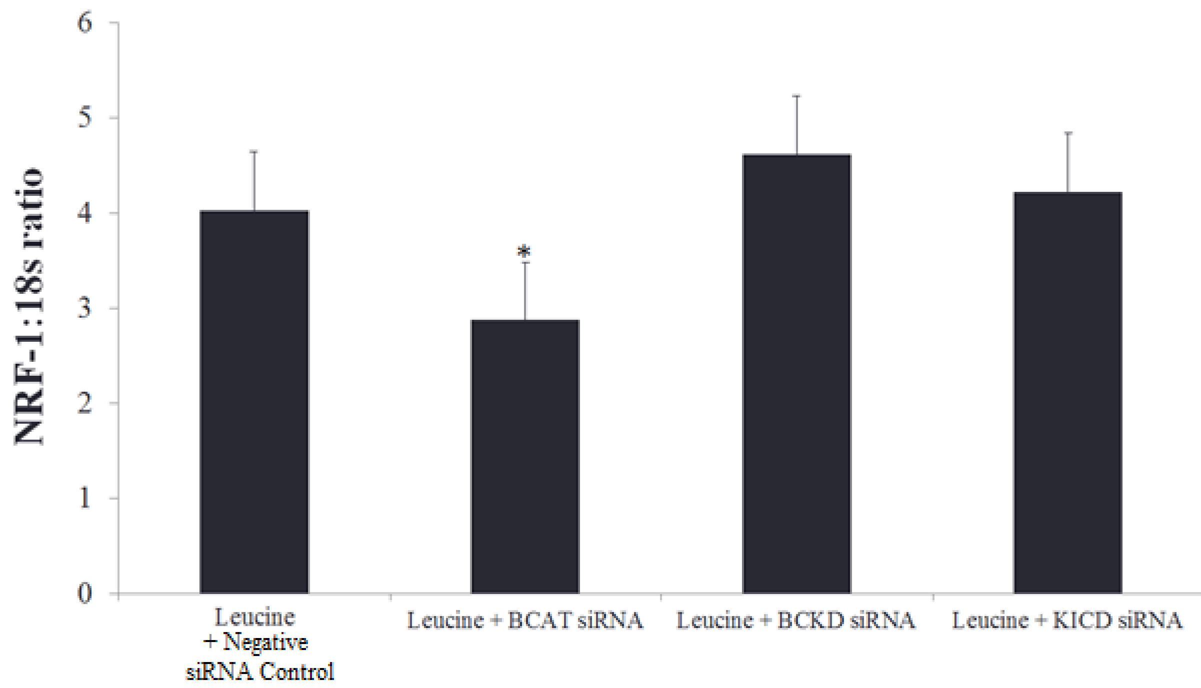


Figure 3-5. The effect of leucine on (A) PGC-1α, (B) NRF-1, and (C) UCP3 gene expression in C2C12 myocytes with or without BCAT, BCKD, and KICD siRNA transfection; expression of each gene is normalized to 18s expression. Cells were transfected with Control siRNA, BCAT, BCKD, or KICD siRNA, and then treated with leucine (0.5mM) for 48 hours. Values are presented as mean ± SEM, n = 6. Means with * differ compared with control group $p<0.05$, and means with # differed from other siRNA groups $p<0.01$.

Figure 3-5 Continued

B



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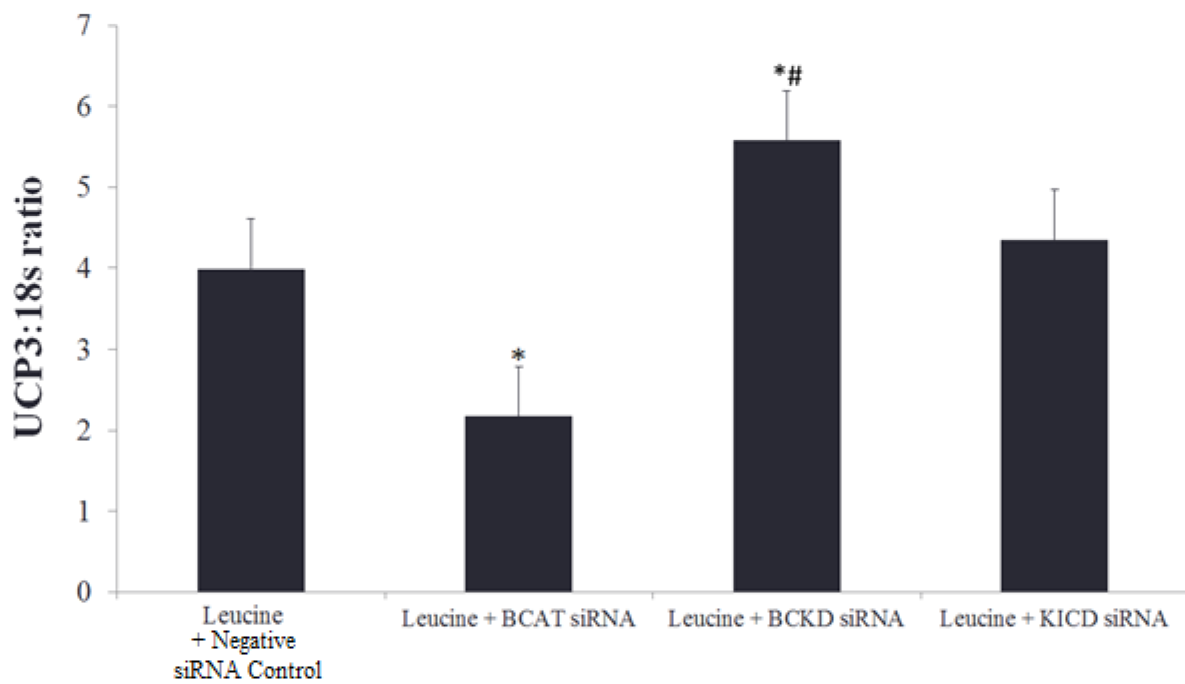

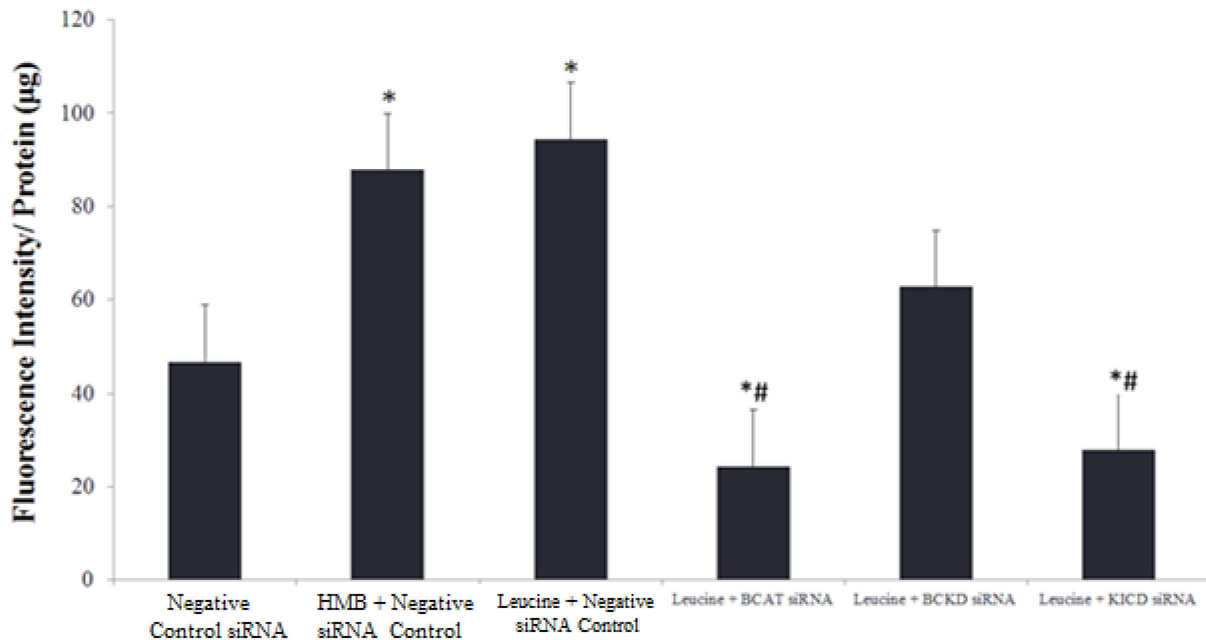

Figure 3-6. The effect of HMB or leucine on mitochondrial mass by NAO binding in C2C12 myocytes with or without BCAT, BCKD, and KICD siRNA transfection. Cells were transfected with Control siRNA, BCAT, BCKD, or KICD siRNA, and then treated with HMB (50µM) or leucine (0.5mM) for 48 hours. Values are presented as mean ± SEM, n = 12. Means with * differ compared with control group $p<0.05$, and means with # differed from other siRNA groups $p<0.01$.

3.5 Discussion

The efficacy of HMB administration on protein accretion and the maintenance of lean body mass (LBM) in a variety of populations is well established, and leucine supplementation exerts similar effects [1]. Leucine has been identified to be a useful ergogenic aid in athletes, and the resultant increase in protein synthesis has been demonstrated to be mTOR dependent [9,11]. The upregulation of protein synthesis following HMB supplementation also appears to be the result of the direct activation of mTOR [18]. Considering the vast areas of overlap in the literature between the two compounds, it is not surprising that HMB has been suggested mediate the effects of leucine [11].

This study was designed to evaluate the roles of the leucine metabolites, KIC and HMB, in the leucine-induced stimulation of mitochondrial biogenesis and fat oxidation. The results demonstrate that both KIC and HMB stimulate mitochondrial biogenesis and fat oxidation at levels comparable with leucine, and leucine catabolism is required to elicit the leucine-induced stimulation of mitochondrial biogenesis. Collectively this suggests that a down-stream metabolite, possibly HMB, mediates the effects of leucine on mitochondrial biogenesis.

This experiment is novel in its aim to determine the potential role of HMB in the mediation of leucine-modulated effects on mitochondrial number and function. The concentration of leucine selected for this study is based upon typical plasma levels achieved following a high protein meal or after leucine administration in mice [3,4]. The concentration of HMB utilized is ten percent of that of leucine to emulate the *in vivo* catabolism that would occur under normal conditions [11]. To address the possibility that the effects of leucine were non-specific effects of branched chain amino acid availability, key experiments incorporated the

same concentration of valine as an additional control [3,4]. Fatty acid oxidation was utilized as a marker of mitochondrial function, and leucine and both metabolites induced significant increases in muscle fat oxidation (Fig. 3-1). Consistent with improved mitochondrial function, all three compounds induced similar increases mitochondrial mass (Fig. 3-2) and amplified the expression of key genes associated with mitochondrial biogenesis (Fig. 3-3). This data is consistent with previous work demonstrating leucine's effects on mitochondrial biogenesis and function [3,4].

Although the catabolism of leucine to HMB is a one-way reaction, data from the previous experiments cannot distinguish whether HMB mediates the effects of leucine or merely mimics them. As such, the use of knockdown experiments was utilized to differentiate the roles of HMB, KIC, and leucine on mitochondrial biogenesis. The leucine stimulation of mitochondrial mass and expression of mitochondrial genes was abated with the knockdown of BCAT and KICD (Fig. 3-5 and 3-6). Preventing the catabolism of KIC to isovaleryl-CoA through the knockdown of BCKD resulted in increased expression of UCP3 and did not abate the leucine-induced stimulation of mitochondrial abundance (Fig. 3-5 and 3-6). This suggests that catabolism of leucine is essential to the stimulation of mitochondrial biogenesis, although the active downstream metabolite is uncertain, while the catabolism of KIC to isovaleryl-CoA is not. Despite the limited success of siRNA transfection, the knockdown of all three enzymes resulted in clear phenotypic shifts in mitochondrial abundance. However, the inefficient knockdown of BCAT, BCKD, and KICD is clearly a study limitation (Fig. 3-4).

In summary, the present data demonstrate that leucine, KIC, and HMB attenuate mitochondrial biogenesis and function in murine myotubes. Additionally, the catabolism of leucine appears to be required for the observed mitochondrial effects. Collectively, this indicates

that HMB or another downstream target is the active metabolite of leucine, and mediates its effects on mitochondrial biogenesis.

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Part IV:

Conclusions

The use of HMB as a nutritional supplement to promote protein accretion and the maintenance of lean body mass has been demonstrated to be efficacious in a variety of populations and clinical settings. Additionally, there is significant overlap in the literature between HMB and its amino acid precursor leucine with regard to anabolic effects on total body protein. Previous data generated by our laboratory has shown leucine to promote mitochondrial biogenesis and to stimulate mitochondrial function. It was our hypothesis that HMB mediated these effects of leucine, and the present study supports this concept. We have demonstrated the leucine metabolites KIC and HMB to have a comparable effect on mitochondrial biogenesis, as measured by mitochondrial mass and the expression of key regulatory and component genes, as leucine in murine myotubes. Likewise, we have shown these metabolites to enhance fat oxidation at levels similar to leucine. Furthermore, we have shown that the effects of leucine on mitochondrial biogenesis are abated when leucine metabolism is inhibited, which indicates that the catabolism to HMB is required to produce this effect. These data imply that a downstream metabolite of leucine, possibly HMB, is the active metabolite of leucine and mediates the leucine-induced effects on mitochondrial biogenesis and fat oxidation in skeletal muscle.

Vita

Born and raised in Los Angeles, California, Renée Stancliffe (née Hanlin) graduated from Occidental College in 2005 with a B.A. in biology. After teaching middle school and high school math and science, she returned to school as a student at the University of Tennessee. In 2009 she earned a B.S. in nutrition, and remained at the University of Tennessee as a graduate student in the nutrition department. She completed the dietetic internship in 2011, and defended her thesis in August 2012. Renée currently lives in Dallas with her husband, and their three pets.