8-2016

Long-Term Supplementation with Leucine Does Not Prevent Development of Obesity in Rats Fed a High-Fat Diet

Dameon Smith
University of Arkansas, Fayetteville

Long-Term Supplementation with Leucine Does Not Prevent Development of Obesity in Rats Fed a High-Fat Diet

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

Dameon Smith
University of Arkansas
Bachelor of Science in Biology, 2012
Bachelor of Science in Education, 2012

August 2016
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

_____
Dr. Jamie I. Baum
Thesis Director

_____
Dr. Tyrone Washington
Thesis Committee Member

_____
Dr. Sun-Ok Lee
Thesis Committee Member

_____
Dr. Walter Bottje
Thesis Committee Member

_____
Dr. Jeffery Wolchok
Thesis Committee Member

ABSTRACT

Excess dietary fat consumption has been implicated in the development of obesity and diabetes. Obesity can be characterized by a disproportionate increase in fat mass compared to lean body mass. However, if muscle mass can be increased or maintained in obesity, this may facilitate weight loss by increasing the body's overall metabolic capacity. Historically, supplementation with the branched-chain amino acid leucine has been shown to increase muscle protein synthesis via the protein kinase mTORC1. Recent studies suggest that supplementation with leucine also has the potential to reduce weight gain and fat deposition in high-fat fed, obese mice. The objective of this study was to determine if long-term dietary leucine supplementation prevents development of obesity in rats meal-fed a high fat (60%) diet. Male, Sprague-Dawley rats (n=30/dietary treatment) were meal-fed (3 meals/day) either a control diet (C), control+leucine (CL), high-fat (HF), or high-fat+leucine (HFL) for 42 days. HF/HFL-fed rats gained, 28 g more than rats fed C/CL diets ($p<0.05$). Plasma insulin levels in the fasted condition were not significant between groups; however, thirty minutes postprandial the rats supplemented with leucine, both on a high-fat and control diet, had significantly higher insulin levels than the control group ($p<0.05$). Free fatty levels in the fasted condition did not differ between the control diet and the control diet with leucine supplementation. However, plasma free fatty acid levels in the fasted condition were significantly higher in the high-fat with leucine group when compared to the high-fat group without leucine supplementation ($p<0.05$). Rats supplemented with leucine had significantly higher fat mass ($p<0.05$) and significantly lower muscle mass ($p<0.05$) compared to their respective controls. No significant difference was detected in 4E-BP1 and AKT phosphorylation between diets or between time points. PGC-1α expression in fasted conditions did not differ between leucine supplemented groups and their respective

controls. At 30 minutes postprandial, only the control diet with leucine supplementation was observed to have significantly greater PGC-1α expression than the high-fat diet with leucine supplementation ($p<0.05$). Taken together, supplementation with leucine does not prevent development of obesity in high-fat fed rats.

TABLE OF CONTENTS

List of figures

Chapter 1: Introduction

More than one-third of the U.S. adult population is obese. Obesity has become an epidemic and major public health concern. Obesity contributes to several health risks including: stroke, heart disease, type-2 diabetes, and cancer [1,2]. These rank among the leading causes of death in the United States and may be reduced or prevented by preventing development of obesity.

Obesity can be characterized by a disproportionate increase in fat mass compared to lean body mass. Obesity is defined as having a body mass index of 30 or higher [1]. This increase in fat mass occurs when the energy balance (energy intake = energy expenditure) is altered [3]. When more energy is being consumed than expended weight gain, specifically fat, occurs. To address the disease of obesity and the health risks it poses, the issue of energy imbalance must be addressed. Adjusting the macronutrient composition (carbohydrates, protein, and fat) of an individual's diet can aid in the treatment of obesity [3,4]. Specifically, protein content of a weight-loss diet is important for maintaining muscle mass and to maximize fat loss [3,5]. The branched-chain amino acids in protein are attributed with a muscle sparing effect in a hypo-caloric diet [3]. Among these branched-chain amino acids, leucine is the primary activator of the cellular machinery associated with muscle protein synthesis [6].

The mammalian target of rapamycin complex 1 (mTORC1) is activated by leucine concentration in the cell. This complex triggers muscle protein synthesis, as well as activates genes responsible for fatty acid synthesis and fatty acid oxidation [3,7,8]. It may therefore be possible to supplement leucine to a diet in order to activate the cellular mechanisms necessary oxidize fat and maintain muscle in order to shift body composition to a leaner state. Additionally, supplementing leucine to a diet that induces obesity could delay or prevent the onset of obesity.

Chapter 2: Objectives

The objectives of this study were to: 1) determine if the supplementation of leucine to a high-fat diet, designed to induce obesity, would prevent or delay the onset of obesity in male, Sprague- Dawley rats and 2) to determine the role of dietary leucine supplementation in fat metabolism of male, Sprague-Dawley rats fed a high-fat diet.

Chapter 3: Literature Review

Background

According to the Center for Disease Control (CDC) it is currently estimated that over one-third of the United States adult population is obese [1]. Obesity is defined as having a body mass index (BMI) of 30 or higher and individuals with a BMI of 25 to 29.9 are classified as overweight. Obesity is not just a problem in the United States, the World Health Organization (WHO) estimates that worldwide obesity has double since 1980 [2]. Additionally, in 2008 it was estimated that 35% of adults 20 years of age and older worldwide were overweight, while 11% were obese [2]. Obese individuals are at an increased risk for type-II diabetes, stroke, and even cancer [1]. In 2008, it was estimated that the annual cost of obesity in the United States was 147 billion dollars [1].

Obesity is caused by energy imbalance and correcting this energy imbalance is essential for treating obesity [9]. Energy balance is achieved when the amount of energy, or kilocalories, that is taken in equals the amount of energy being used by an organism. When more energy is being taken in than is being expended, weight gain occurs. Conversely, when more energy is being utilized than taken in, weight-loss occurs [10].

In a study by Layman et al. [3], twenty-four women were placed on two reduced energy diets (1700 kcal/day) one group consumed twice the recommended daily allowance (RDA) of protein (1.6g/kg of body weight/day) about a 1.4 carbohydrate to protein ratio, while the other group was given the standard RDA of protein(0.8g/kg of body weight/day) about a 3.5 carbohydrate to protein ratio. It is important to also note that the fat and fiber content of both diets were kept the same (approximately 50g/d total fat intake and 20g/d total fiber intake,

respectively). These diets were calorically balanced by removing carbohydrates from the group that received more protein, while keeping the fat content uniform in both diets. After 10 weeks on the dietary interventions the group that received more protein lost more fat mass relative to lean mass loss than the group consuming the lower protein [3,11]. This highlights that the macronutrient composition (carbohydrates, protein, and fat) can alter body composition and subsequently health.

High protein diets are attributed to greater weight-loss and improved body composition when compared to carbohydrate diets [3,11]. It is further theorized that the branched-chain amino acids (BCAAs), in addition to decreased caloric intake, are the major components of high protein diets that generate this metabolic effect [6, 10]. There are three BCAAs: valine, isoleucine, and leucine. These amino acids are unique because they are the only amino acids not metabolized in the liver [10]. Instead, they bypass first pass metabolism and are transported to muscle tissue where they are metabolized [12]. This is done through the transamination of BCAAs which results in fuel substrates, first substrates, for energy production in the mitochondria and the production of alanine [10,12]. The alanine that is produced from BCAAs is in turn converted to glucose in the liver via the glucose-alanine cycle, which helps maintain blood glucose levels [10]. Stabilizing blood glucose levels can aid in controlling appetite [10].

The branched-chain amino acid leucine is unique among the BCAAs because it is the primary amino acid that triggers protein synthesis [6]. In a study conducted by Anthony et al. [6], rats were fasted for 18 hours then given either saline (control), valine, isoleucine, or leucine, and after one hour the rats were sacrificed. Only rats treated with leucine had increased muscle protein synthesis [6]. Similar results were demonstrated in a study conducted by Norton et al. [5] where whey protein, a protein high in leucine, was shown to have the highest fractional protein

synthetic rate in muscle tissue, while wheat protein, a protein low in leucine, had a far lower fractional synthetic rate [5]. When the leucine content of the wheat protein was supplemented to the level of whey protein the fractional synthetic rate of the wheat protein increased to the same level as whey protein [5]. This demonstrates that leucine is the key amino acid in stimulating muscle protein synthesis.

mTORC1 signaling and protein synthesis

The muscle protein synthetic response is triggered when the leucine concentration inside the cell increases and causes activation of mTORC1 (mammalian target of rapamycin complex 1; **Figure 1**) [5,6,13]. The mammalian target of rapamycin complex 1 (mTORC1), found in every animal cell, incorporates both intracellular (inside the cell) and extracellular (outside the cell) signals to regulate cellular metabolism, growth, and the survival of the cell [13]. The activation of the mTORC1 complex subsequently phosphorylates a binding protein, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which binds eukaryotic translation initiation factor 4E (elF4E), an initiation factor for the transcription of muscle protein synthesis; this binding of elF4E inactivates muscle protein synthesis [13]. When 4E-BP1 is phosphorylated it releases elF4, which migrates to the nucleus of the cell to form the initiation complex on the DNA molecule then initiates transcription of the genes related to muscle protein synthesis [4,14,15]. Similarly, ribosomal protein S6 kinase beta-1 (p70S6K) is activated by mTORC1 and phosphorylates ribosomal protein S6 when migrates the nucleus to initiate transcription of protein synthetic genes. This protein synthetic response is essential in maintaining muscle mass, which, in turn, helps to maintain energy metabolism. As muscle mass decreases metabolic activity in the body will decrease [4,14,15,16]. Alternatively, some studies have suggested that a reduction in proteolysis, the breakdown of protein, could be a contributing cause of muscle mass

regulation in long-term leucine supplementation studies. In a study by Sugawara et al.[17], rats were fed a low-protein diet and supplemented with 1.5% leucine in the drinking water. A suppression effect on protein degradation was observed in the leucine supplemented group [17]. However, this suppression of protein degradation is less documented than the protein synthetic response to leucine supplementation. Taken together leucine supplementation may play a role in both the reduction of protein degradation and the stimulation of protein synthesis [4,5,14-19].

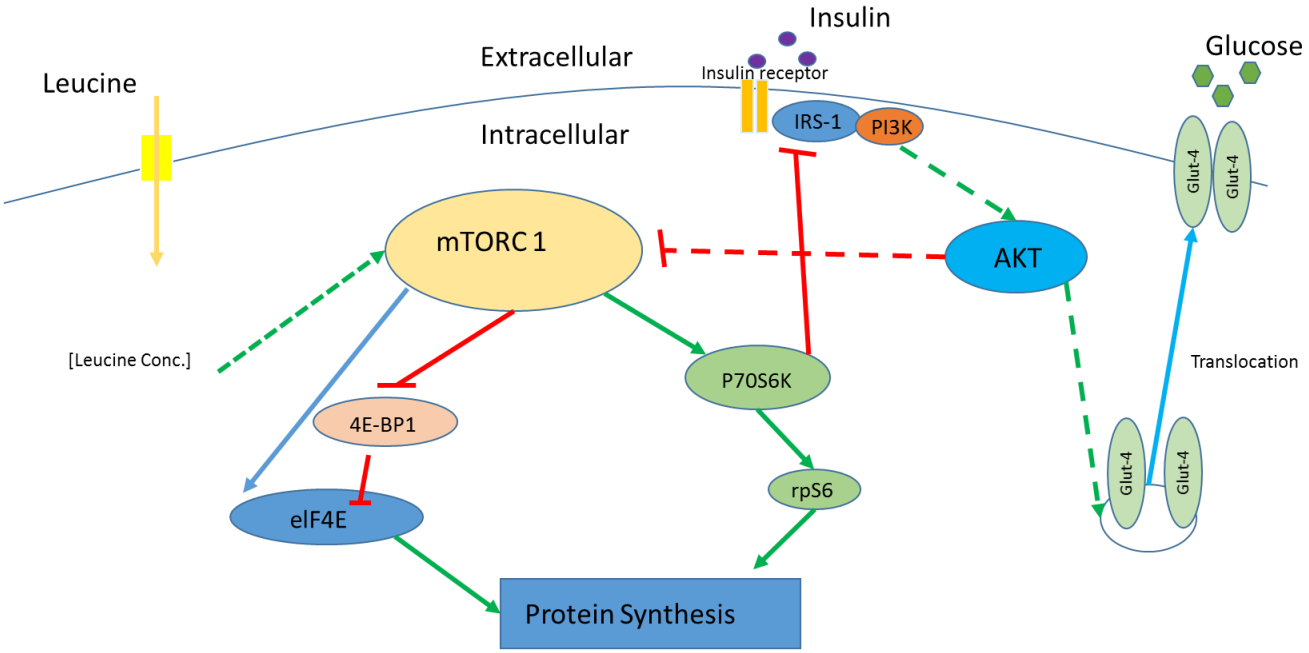


Figure 1. *Leucine acts through the mTORC1 signaling pathway to stimulate protein synthesis. Adapted from Laplante M. and D. M. Sabatini [13]. As the concentration of leucine inside the cell increases mTORC1 is phosphorylated leading to the phosphorylation of 4E-BP1 and P70S6K. The phosphorylation of these triggers protein synthesis inside the cell. Additionally, [13]. Insulin Receptor Substrate-1 (IRS-1) is activated by insulin triggering a signaling cascade that phosphorylates Akt this phosphorylation stimulates inhibition of mTORC1 and also triggers Glut-4 to translocate to the cell membrane to facilitate glucose transport. Leucine can stimulate the phosphorylation of mTORC1 and subsequently P70S6K, this can inhibit IRS-1 and disrupt the inhibition of Akt on mTORC1 [13].*

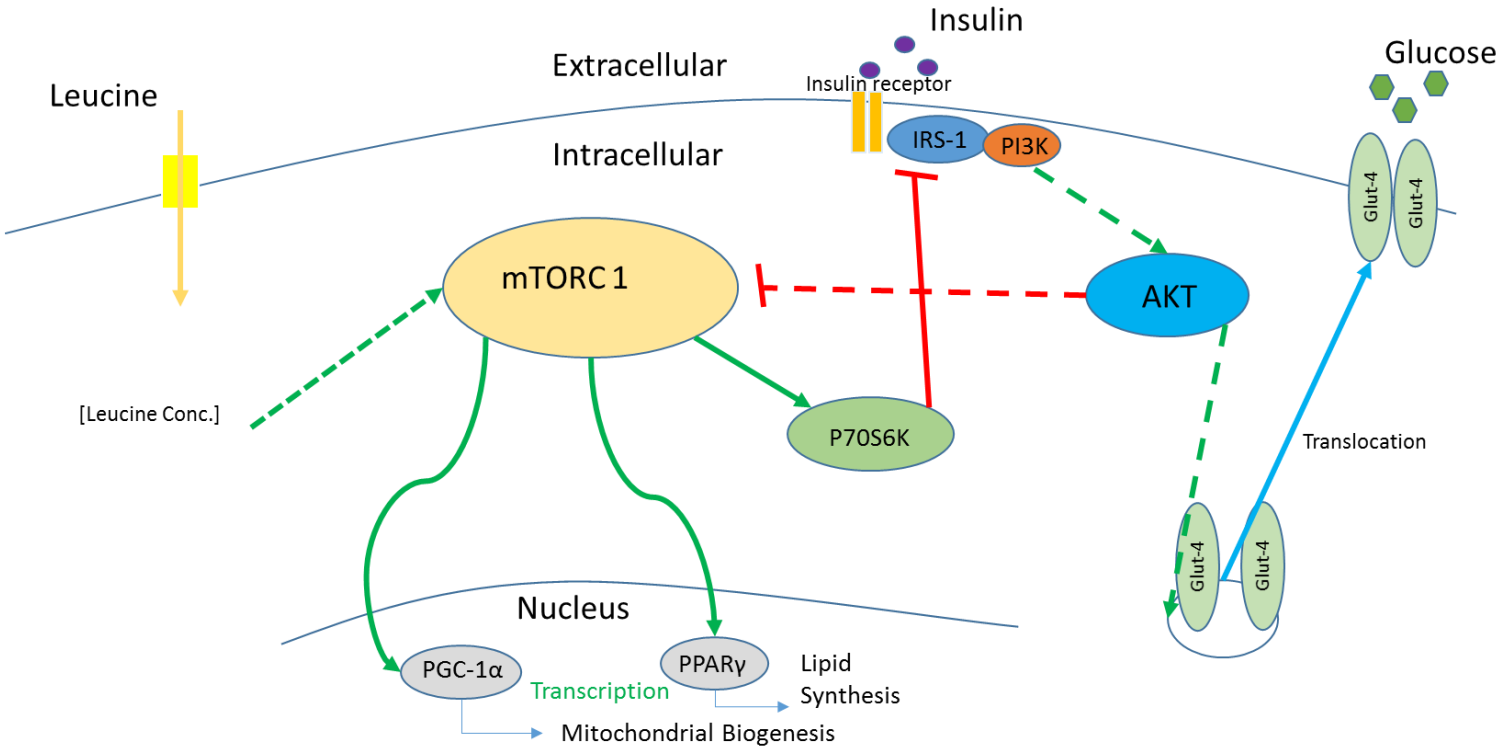


Figure 2. The potential role of leucine *in regulation of gene transcription via the mTOR pathway. Adapted from Laplante M. and D. M. Sabatini continued [13]. As the concentration of leucine inside the cell increases mTORC1 is phosphorylated and then translocates to the nucleus where it signals the transcription of genes related to mitochondrial biogenesis (PGC-1α) and lipid synthesis (PPARγ). Leucine can stimulate the phosphorylation of mTORC1 and subsequently P70S6K, this can inhibit IRS-1 and disrupt the inhibition of Akt on mTORC1 [13].*

The role of leucine in glycemic response

Leucine stimulates the release of insulin from the pancreas and may also modulate the uptake of insulin into both skeletal muscle tissue and adipose tissue [20]. Insulin can stimulate muscle protein synthesis through the insulin-PI3K pathway, this pathway causes downstream activation of the mTORC1 pathway. However, phosphorylation targets downstream of mTORC1 can affect insulin sensitivity by a feedback loop which phosphorylates insulin receptor substrate 1 (IRS1) [20]. If the cell becomes insensitive to insulin, glucose uptake is restricted (**Figure 1**), however, if insulin sensitivity increases then more glucose is taken up by the cell and used as a metabolic fuel source [20,21]. Glucose levels, as well as insulin, may be effected by leucine supplementation. In a study conducted by Binder et al. [8], mice were fed a high-fat diet (60% fat) or standard chow for 8 weeks. At 8 weeks, they were returned to a standard chow diet with standard water or supplemented with 1.5% leucine (wt./vol.) in the drinking water for 21 weeks. This study found that leucine supplementation improved glucose tolerance in both previously high-fat fed and standard fed groups. However, it is important to note that leucine supplementation in combination with the conversion to standard chow from a high-fat diet had a greater improvement in glucose levels. Additionally, insulin sensitivity improved with the exposure to standard chow but leucine did not stimulate any further effect [8]. A similar study adding leucine to the drinking water was conducted, however this study supplemented leucine in the drinking water while mice were being fed a high-fat diet for 17 weeks [7]. In standard chow conditions leucine supplementation had no effect on glucose or insulin tolerance. However, fasting plasma insulin levels were lower in mice fed high-fat with leucine supplemented in the drinking water compared to mice fed high-fat with standard water. Also, the high-fat fed group

with leucine in the drinking water showed a greater sensitivity to insulin than the high-fat fed group with standard water [7].

In contrast, leucine supplementation has also been shown to have no effect on glucose tolerance, plasma glucose level, and insulin glucose levels in non-obese mice supplemented with leucine to a diet containing adequate protein levels [22]. A study by Li et al. [14], was conducted by supplementing leucine to the drinking water high-fat fed and standard chow fed rats. This study supplemented leucine to the drinking water in 4 different amounts 0%, 1.5%, 3.0%, and 4.5% leucine for 24 weeks. Leucine supplementation had no effect on fasting glucose levels for any of the groups. The high-fat diet induced an increase in fasting insulin levels, this was attenuated by leucine supplementation, regardless of concentration supplemented. Additionally, the decrease in insulin sensitivity associated with a high-fat diet was rescued by leucine supplementation regardless of leucine concentration. In this study, no additional insulin sensitivity was observed in rats fed a standard chow diet [14].

Mechanistically, in high-fat fed rats Akt and mTOR phosphorylation increased at the basal level compared to controls. As a result, when insulin is present in animals fed a normal diet an increase in the phosphorylation of Akt occurs, however, when insulin is present in a high-fat diet this increase phosphorylation does not occur, the result is insulin resistance [18]. Long-term leucine supplementation enhances insulin stimulated phosphorylation of Akt and mTOR in muscle, liver, and adipose tissue. Also, insulin receptor substrate 1 (IRS1) phosphorylation has been associated with insulin resistance, but the phosphorylation of IRS1 may be associated with insulin sensitivity [18].

Some studies suggest that chronic leucine supplementation initiates a feedback loop from mTOR through S6k1 to and trigger insulin resistance; other studies suggest that leucine triggers

the phosphorylation of IRS1, subsequently improving insulin sensitivity [18,23]. A possible explanation for this difference in observations could be related to the dose of leucine. Li et al. [18] identify that acute high levels of leucine could trigger insulin resistance, while small dose such as the 1.5% leucine in water could promote insulin sensitivity. Therefore, insulin sensitivity through the IRS1/AKT/mTOR/S6k1pathway may be dose dependent. Taken together supplementation of leucine to animals fed a high-fat diet tends may improve insulin sensitivity. [18,23]. However, in another study by Newgard et al. [21] high-fat fed rats were supplemented with BCAAs, in the chow, and showed the same insulin resistance as high-fat fed rats. A chronic phosphorylation of the mTORC1 pathway and IRS-1 was also observed suggesting a possible resistance to insulin [21]. Considering all arguments, it is not completely clear why insulin sensitivity is influenced one way or another with leucine supplemented in high-fat diets [7,8,14,18,22].

Leucine supplementation and body composition

Body composition is key in ensuring health [1]. Muscle is a metabolic tissue, the more muscle tissue an organism has the more energy can be expended. Supplementing leucine to facilitate a change in body composition has shown different effects. In a study by Li et al.[14], leucine was supplemented to the drinking water of rats fed either a high-fat diet or normal chow diet in 4 different amounts 0%, 1.5%, 3.0%, and 4.5% leucine for 24 weeks. The authors observed that rats fed a high-fat diet supplemented with leucine had a significantly higher body weight than all other groups, including the high-fat control. This was also accompanied by an increase in fat mass in leucine supplemented high-fat groups [14]. In contrast, a similar study supplementing 1.5% leucine in the drinking water of mice observed a significant reduction in the body weight of high-fat fed mice supplemented with leucine compared to the high-fat control

mice. Additionally, significant reduction of both visceral and subcutaneous body fat was observed in the high-fat fed mice supplemented with leucine when compared to the high-fat control mice [18].

The difference could be due to the different species or diet interventions used in the experiments. Leucine supplemented to a normal diet does not seem to elicit a change in body composition [22]. Noatsch et al. [22], observed this when mice were fed an adequate protein diet supplemented with leucine (4.5g/100g of diet). The authors observed no significant changes in body weight, energy expenditure, or body composition as a result of leucine supplementation [22]. Interestingly, a study conducted by Binder et al. [7] supplementing 1.5% leucine to the drinking water of mice fed a high-fat diet for 17 weeks in an effort to prevent the effects of a high-fat diet. This study showed a significant reduction in body weight and fat mass in the leucine supplemented (~55.6mg/d) high-fat fed mice when compared to the high-fat control group, however, no change was observed in mice fed a normal diet supplemented with leucine (~74.5mg/d) compared to a normal diet [7]. Taken together leucine supplementation may have a positive effect on body composition depending on the dose, species, and method of administration [8,22].

Leucine and Fatty acid metabolism

Leucine can be metabolized in muscle cells to produce acetyl CoA, a substrate for mitochondrial oxidation to produce energy. This acetyl CoA entry point into oxidative metabolism is also the same entry point by which fatty acids are utilized by the mitochondria for fuel inside the cell [24]. Leucine supplementation may be linked to fatty acid oxidation [8]. In a study conducted by Binder et al. [8], mice were fed a high-fat diet or standard chow for 8 weeks, then switched to a standard chow diet with standard water or supplemented with 1.5% leucine

(wt./vol.) in the drinking water for 21 weeks. Indirect calorimetry was used to determine the respiratory quotient, a measurement used to determine the primary source of fuels being used for energy in real time, of the mice in this study and mice supplemented with leucine had a lower respiratory quotient, indicating an increased usage of fatty acids [8]. It is important to note that no changes in the expression of genes related to fatty acid oxidation were observed in muscle tissue. Similar results were observed in another study when 1.5% leucine was supplemented in the water of mice fed a high-fat diet, no changes were observed in gene expression related to fatty acid oxidation [7]. However, in a study by Li et al.[18], leucine supplemented in the drinking water of high-fat fed mice has be shown to have an effect on fatty acid metabolism in skeletal muscle tissue. In this particular study the authors found an increase in sirtuin 1 (SIRT1), a protein associated with metabolic regulation and tolerance of oxidative stress, protein and gene expression as well as an increase fatty acid oxidation gene expression in the skeletal muscle of high-fat fed mice supplemented with leucine in the drinking water [8]. It thought that leucine supplementation stimulates the activation of AMP-activated protein kinase (AMPK) which in turn simulates SIRT1 signaling. Also, a decrease in the acetylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), a gene marker for mitochondrial biogenesis, and forkhead box O1 (FoxO1), a gene marker for negative effects on adipogenesis, was observed. The deacetylation of these PGC1α and FoxO1 allow genes associated with mitochondrial biogenesis and genes controlling energy homeostasis to be transcribed [18]. Taken together leucine supplementation has been shown to activate genes associated with fatty acid transport and oxidation in adipose tissue. However, the activation of proteins controlling fatty acid oxidation and mitochondrial biogenesis in muscle tissue requires further examination [4,7,8,16,18,25].

It is widely accepted that leucine supplementation stimulates the cellular pathway for muscle protein synthesis. The possibility of supplementing leucine in a high-fat diet to prevent obesity has not been clearly established. Therefore the objectives in this study were to: 1) determine if the supplementation of leucine to a high-fat diet, designed to induce obesity, would prevent or delay the onset of obesity in male Sprague Dawley rats and 2) to determine the role of dietary leucine supplementation in fat metabolism of rats fed a high-fat diet.

Table 1. *Leucine literature review summary*

Author	Animal Model	Supplement-ation Method	Leucine Dose	Signaling Effect	Body Weight	Body Composi-tion
X Li et al. [14]	Sprague-Dawley Rats	In water	1.5, 3, 4.5%	Increased insulin sensitivity	Increased	Increased fat mass
H Li et al. [18]	C57BL/ 6 Mice	In water	1.5%	Increased oxidative activity	Decreased	Decreased fat mass
Noatsch et al. [22]	C57BL/6 Mice	In chow	4.5g/100 g of diet	No difference due to Leu	No change	No change
Binder et al. [8]	Mouse	In water	1.5%	Increased oxidative activity	No change due to Leu	No change due to Leu
Binder et al. [7]	Mouse	In water	1.5%	Increased oxidative activity	Decreased	Decreased fat mass
Anthony et al. [6]	Rat	In water	54g/L	Increase protein synthesis	Not discussed	Not discussed
X. Tong et al. [26]	Winstar Rats	In chow	1.6%	Increase insulin sensitivity	Decreased	Decreased fat mass
T. Zampieri et al. [27]	Winstar Rats	In water	15g/L	Increase in hypothalamic genes involved in oxidation and metabolism of BCAAs	No change	Increased adiposity
T. Zampieri et al. [28]	C57BL/6 Mice	In water	1.5%	P70S6K phosphorylati on in hypothalamus.	No change	No change
A. Freudenber g et al. [29]	C57BL/6 Mice	In chow	6% w/w	No effect on genes related to lipid metabolism	Decreased	Decreased
Y. Macotela et al. [30]	C57BL/6 J Mice	In water	1.5%	FAS and ACC gene expression were returned to basal levels	No change	Decreased

Chapter 4: Specific Aims

AIM 1: Determine if the supplementation of leucine to a high-fat diet would prevent or delay the onset of obesity.

Challenge: To assess the obesigenic effects of a high-fat diet with leucine supplementation.

Approach: Animals were weighed daily to track weight gain and body composition was determined postmortem.

Impact: These measurements were used to determine the body composition of the animals in order to establish the consequences of leucine supplementation to a high-fat diet.

AIM 2: Determine the metabolic effects of leucine supplementation on a high-fat diet to prevent or delay the onset of obesity.

Challenge: To determine if leucine supplementation could stimulate the use of fatty acids as an oxidative fuel source.

Approach: Plasma glucose, insulin, and free fatty acids were measured at a fasted time point, 30 minutes postprandial, and 90 minutes postprandial to determine metabolic response to leucine supplementation.

Impact: These measurements were used to determine if leucine supplementation changes the glycemic response to a high-fat as well as the free fatty acid content.

AIM 3: Determine the mTORC1 signaling response to leucine supplementation on a high-fat diet to prevent or delay the onset of obesity.

Challenge: To determine the signaling cascade leading to muscle protein synthesis and expression of gene related to fatty acid oxidation via the mTORC1 pathway.

Approach: Phosphorylation of the downstream marker 4E-BP1 was measured to determine protein synthetic response to supplementation. PPARγ and PGC-1α gene expression was measured to determine fatty acid oxidative response, in the mTORC1 pathway.

Impact: These measurements will determine the effect leucine supplementation to a high-fat diet has on the mTORC1 pathway.

Chapter 5: Methodology

Animals

Six-week old male, Sprague-Dawley rats were divided into four treatment groups (n=30 per treatment group) totaling 120 rats. Animals were either fed a control diet (Control), control diet supplemented with leucine (Control+Leu), high-fat diet (High-fat), or a high-fat diet supplemented with leucine (High-fat+Leu).

All animals were housed individually at the University of Arkansas, Central Laboratory Animal Facility (CLAF). Rooms were maintained at 22^{o}C with a reversed light/dark cycle (12:12). All animals were weighed daily and all handling and treatment was in compliance with IACUC standards.

Diet design

Control diets were based on AIN-93G diet (Harlan Laboratories Inc., Houston, TX) and high-fat diets were based on a Harlan 60% fat diet (Harlan Laboratories Inc., Houston, TX). Thirty-two grams of leucine per kilogram of diet was supplemented into Control+Leu and High-fat+Leu diets, while 32g of glycine per kg of diet was supplemented into Control and High-fat diets. The equal amounts of each amino acid were added to ensure nitrogen balance between diets. Corn starch was removed and lard was added in the high-fat diets (**Table 2**).

Table 2. *Diet composition*

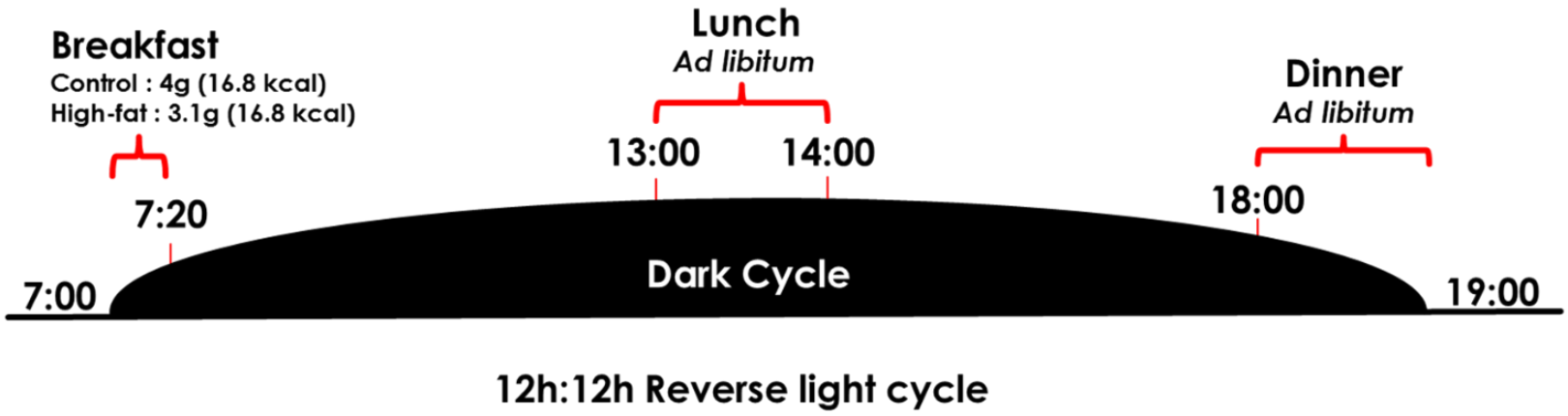
Ingredient (g/kg)	Control + Glycine	Control + Leucine	High-Fat + Glycine	High Fat + Leucine
Casein	200	200	265	265
L-Cystine	3	3	4	4
Corn Starch	397.5	397.5	0	0
Maltodextrin	100	100	128	128
Sucrose	100	100	90	90
Soybean Oil	70	70	30	30
Lard	0	0	310	310
Cellulose	50	50	65.5	65.5
Mineral Mix	35	35	48	48
Vitamin Mix	10	10	21	21
Choline Bitartrate	2.5	2.5	3	3
Calcium phosphate, dibasic	0	0	3.4	3.4
TBHQ	0.14	0.14	0	0
Glycine	**32**	**0**	**32**	**0**
Leucine	**0**	**32**	**0**	**32**

Composition of diets shows the ingredients and amounts (g/kg) used when mixing the different diets. Cornstarch was removed and lard was added to High-fat diets. Thirty-two grams of leucine was added to the leucine supplemented diets and 32g of glycine was added to non-leucine supplemented diets to maintain nitrogen-balance.

Meal-feeding

Animals were meal-fed to mimic human eating behavior, so as to model responses for human applications. Animals were fed three meals per day: breakfast, lunch, and dinner. Breakfast was an isocaloric (16.8 kcal; 4g control, 3.1g high-fat) feeding in order to assess postprandial responses to diets and animals were allowed 20 minutes to eat their meal. Lunch and dinner were ad libitum access to their designated diet. Animals were allotted 1 hour to eat their meals at lunch and dinner. Food bowls were weighed prior to and after feeding to assess food intake and all uneaten chow was discarded after weighing. Animals were fed their respective diets in a meal-fed fashion for 6 weeks and weighed daily (**Figure 3**).

Figure 3. *Meal-feeding design*



A pictorial representation of the meal feeding designed in the study. Breakfast was isocaloric, while lunch and dinner were ad libitum. Breakfast was a 20 minutes long meal, while lunch and dinner were hour long meals.

Tissue extraction

After 6 weeks all animals were sacrificed by decapitation. Animals were sacrificed at one of three time points: 0 minutes (fasted), 30 minutes postprandial, or 90 minutes postprandial. This was done to observe any changes that may occur as a result of feeding. Blood was immediately collected and plasma extracted, then snap frozen in liquid nitrogen. The gastrocnemius, soleus, and plantaris muscles were extracted and snap frozen in liquid nitrogen. The liver and epididymal fat were also extracted and snap frozen in liquid nitrogen. All samples were stored in -80^{o}C freezer for future analysis. All muscle tissue, liver, and epididymal fat were weighed and recorded prior to freezing.

Plasma glucose, insulin, and free fatty acids

Plasma glucose was analyzed using a glucose oxidiase colorimetric assay kit (Cayman, Ann Arbor, MI). Insulin was analyzed using a colorimetric kit (ALPCO, Salem, NH). Plasma free fatty acids were analyzed using an enzymatic fluorescence kit (Cayman, Ann Arbor, MI). Assays were done in duplicate in accordance with manufacturer instructions and all microplates were read using a HT-Synergy microplate reader (BioTek, Winooski, VT).

Tissue processing and sample preparation

All tissues powdered using a mortar and pestle in liquid nitrogen. Powdered tissue was then stored at -80^{o}C. Powdered tissue samples were used for all future protein and gene expression analysis.

For protein sample preparation samples were homogenized with a Polytron handheld homogenizer, in 7 volumes of homogenization buffer (20mM HEPES, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM

benzamidine, 0.5 mM sodium vanadate, and 10µl/ml protease inhibitor cocktail (Sigma-Aldrich, St. Louis MO)). Homogenate was then spun down at 4^{o}C in a centrifuge at 10,000 rpm. The supernatant layer was collected and stored at -80^{o}C for western blotting analysis.

For RNA sample preparation samples were homogenized with Polytron handheld homogenizer, in homogenization buffer from RNA extraction kit (Norgen, ON, Canada), then centrifuged at 10,000 RPM while chilled at 4^{o}C. Supernatant was pipetted off and used for RNA extraction. RNA was extracted using an RNA extraction kit (Norgen, ON, Canada) in accordance with the manufacturer instructions. RNA was stored at -80^{o}C for future gene expression analysis.

Western blotting analysis

Bio-rad western blotting apparatus was used for all western blotting analysis. Pre-cast 10% and 12% SDS-Page gels (Bio-rad, Hercules, CA) were used and gels were all ran at 200V for 45 minutes and transferred to blotting paper (Bio-rad, Hercules, CA) at 100V for 1 hour. Wester C protein plus standard with conjugate (Bio-rad, Hercules, CA) was used on all gels to determine molecular weight. All blots were placed on a rocker and incubated in 5% milk in TBST (Tris-Buffered Saline and Tween) for 1 hour. Primary antibodies were diluted 1:1000 in TBST and poured over blots, then placed on a rocker and incubated overnight at 4^{o}C. Antibodies used were p-Akt, Akt, p-4E-BP1 (ser65), and 4E-BP1 (Cell Signaling, Beverly, MA), as well as P70S6K (Bethyl, Montgomery, TX). The secondary antibody was diluted 1:1000 in TBST and poured over blot, then incubated at room temperature on a rocker for 1 hour, the secondary antibody used was anti-rabbit (Cell Signaling, Beverly, MA). All blots were rocked in electrochemiluminescence (ECL) solution for 2 minutes at room temperature prior to developing in Flourochem M imager (Protein Simple, Santa Clara, CA). Densitometry was determined using Alpha View software (Protein Simple, Santa Clara, CA).

Gene expression analysis

RNA was converted to cDNA via RNA to cDNA conversion kit (Quanta, Gaithersburg, MD). Conversion was done in accordance with manufacturer instruction in a Lightcycler 480 system (Roche, Basal Switzerland). All cDNA was stored in -20^{o}C freezer for future analysis. SYBER green master mix (Quanta, Gaithersburg, MD) was used as the reporter dye for both PGC-1α, PPARγ (**Table 2**), and 18s. All samples and controls were run in duplicate and ran in RT-PCR machine (ABI Systems, Grand Island, NY). All gene expression data was calculate using the ΔΔCT method for RT-PCR.

Statistical Analysis

Weight data was all compared using a one-way ANOVA and the means from the 4 treatment groups were used to generate graphs. Plasma glucose and insulin data was graphed using the means of the each treatment group at each time point, a two-way ANOVA was performed in order to observe any interaction between diet and time point. A two-way ANOVA was also used when comparing the protein and gene expression data across treatment groups and time points. Multiple t-tests were used to determine significance between groups within each time point, with Sidak-Bonferroni method for adjustment. All statistical calculations were performed by statistical software (GraphPad) and the graphs of the data were also made in GraphPad. Significance was determined at p-value of $p<0.05$.

Table 3. *Primer sequences*

Gene	**Primer sequence**
PGC-1α	Forward: 5'- TGA AGA GCG CCG TGT GAT T -3’
PGC-1α	Reverse: 5'- TTC TGT CCG CGT TGT GTC A -3'
PPARγ	Forward 5'- CCC ACC AAC TTC GGA ATC AG -3'
PPARγ	Reverse: 5'- GGA ATG GGA GTG GTC ATC CA -3’

Table 3 shows the PGC-1α and PPARγ primer sequences used for RT-PCR in this experiment. Forward and reverse primers are written in a 5’ to 3’ fashion.

Chapter 6: Results

Body Composition

At the end of the study rats fed a high-fat diet or high-fat diet with leucine experienced higher body weights when compared to rats fed a control diet or control diet with leucine ($p<0.05$). There was no difference in body weights between the leucine supplemented groups and their respective controls ($p<0.05$, **Figure 4A**).

Weight gain was also tracked over the course of the study. After two weeks animals fed the high-fat diets started to gain a slight but significant amount of weight when compared to the control fed groups ($p<0.05$). Animals fed the high-fat diets had a significantly higher body weight compared to the control fed groups (**Figure 4B**).

There was a reduction in the muscle weights of rats supplemented with leucine when compared to their respective controls. Rats fed a control diet supplemented with leucine showed a reduced muscle weight when compared with rats fed a control diet without leucine supplementation ($p<0.05$). Similarly, rats fed a high-fat diet supplemented with leucine had significantly lower muscle weight when compared to rats fed a high-fat diet without leucine ($p<0.05$). Additionally, rats fed a high-fat diet had the highest muscle weight across all the groups, and rats fed a control group with leucine had the lowest muscle weight across all the groups ($p<0.05$, **Figure 5A**).

Significant differences in fat mass were also detected. We observed a significant increase in the fat mass of rats fed control diet and supplemented with leucine over rats fed a control diet without leucine supplementation ($p<0.05$). Rats fed a high-fat diet supplemented with leucine tended to have more fat mass than rats fed a high-fat diet without leucine ($p<0.05$). As expected,

animals that were fed a high-fat diet, with or without leucine supplementation, had a significantly higher fat mass than animals fed a control diet ($p<0.05$, **Figure 5B**).

Western Blotting Analysis

In the fasted state, 4E-BP1 phosphorylation trended higher in the control diet without leucine supplementation than the control diet with leucine supplementation, the high-fat diet without leucine supplementation, and the high-fat diet with leucine supplementation ($p<0.097$, $p<0.087$, $p<0.053$; respectively). No differences were detected at 30 minutes postprandial or 90 minutes postprandial (**Figure 6**).

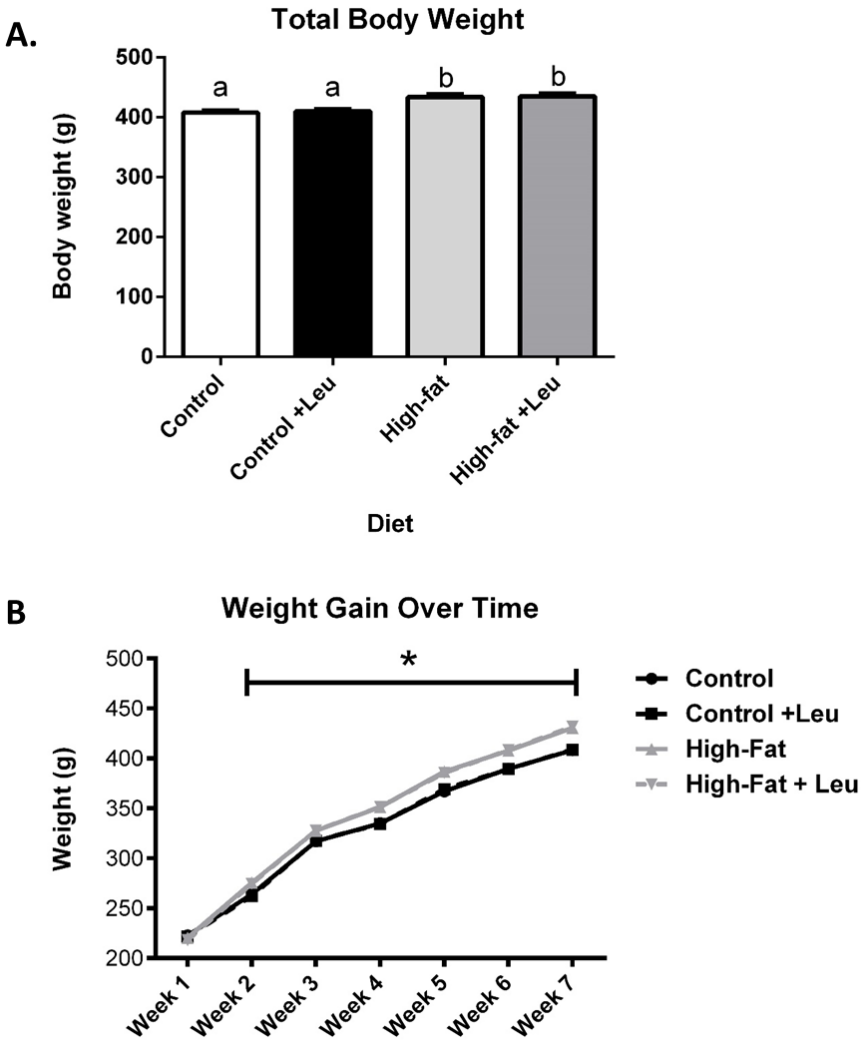
No significant differences were detected in Akt phosphorylation between groups or time points (**Figure 7**).

Gene Expression

PGC-1α expression in fasted conditions, 30 minutes postprandial, and 90 minutes postprandial did not differ between leucine supplemented groups and their respective controls. No notable interactions between time points and diets were observed were observed in this study ($p<0.05$, **Table 4**).

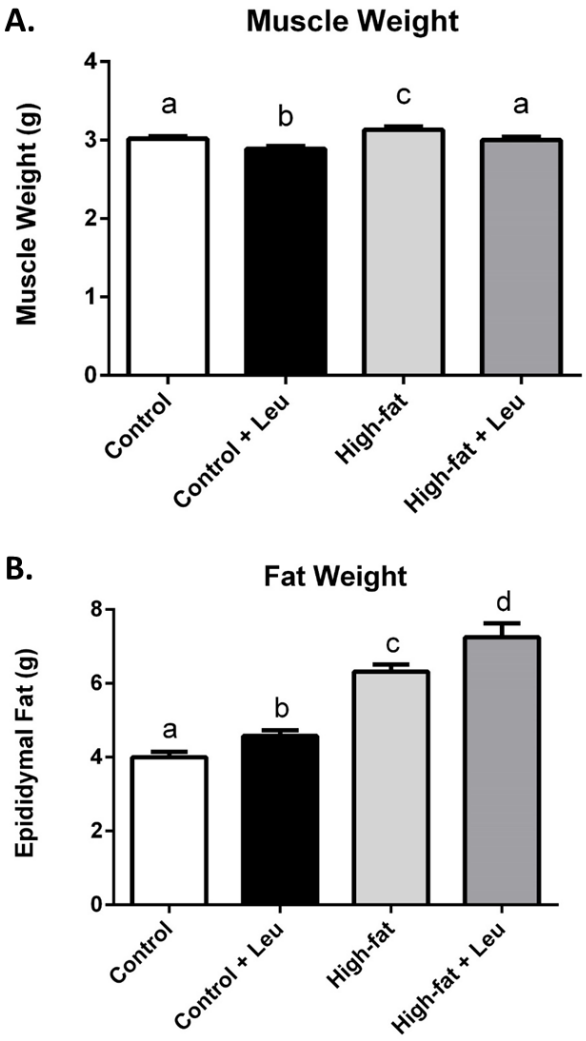
PPAR-γ expression in fasted conditions did not differ significantly between any of the groups. At 30 minutes postprandial, no significant differences were observed; however, the high-fat diet with leucine trended lower than the high-fat diet without leucine ($p<0.08$). Additionally, the high-fat diet with leucine trended lower than the control diet with leucine ($p<0.09$). At 90 minutes postprandial no differences in PPAR-γ expression were observed between the groups (**Table 4**).

Figure 4. *Total body weights and weight gain over time*



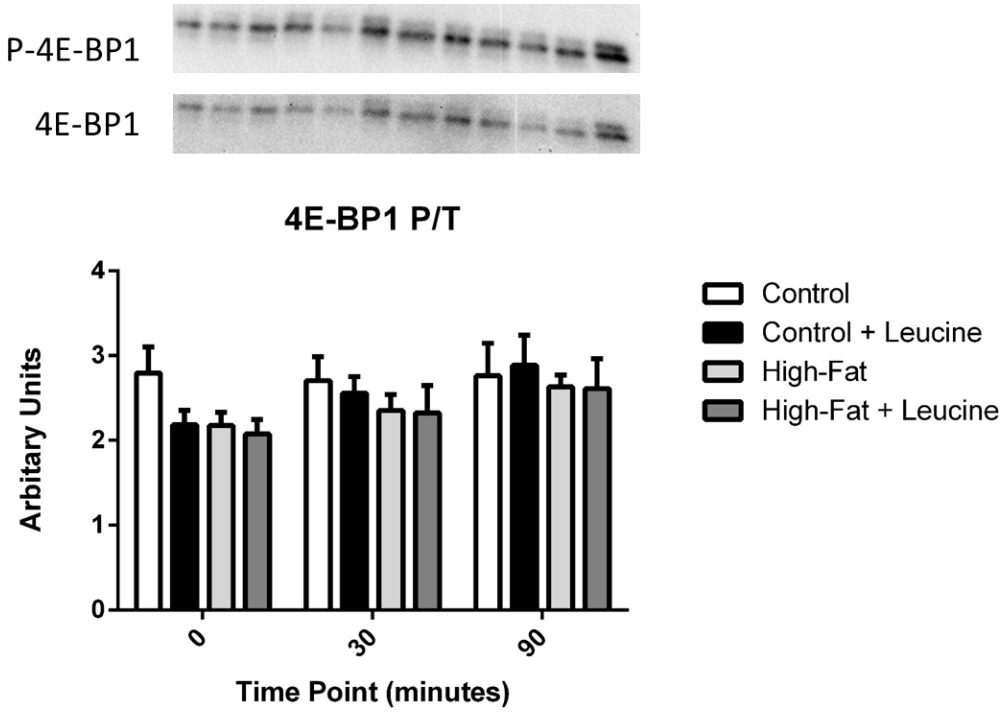
Data expressed as means ± SEM. Different letters indicate significance, $p<0.05$. (A) Shows the Total body weights from each group. Total body weights of rats taken immediately before sacrifice, but prior to breakfast on sacrifice day. High-fat fed groups experienced a higher total body weight when compared to control diets (n=30 per treatment group). (B) Tracks the weight gain of the rats in each diet group per week until the sacrifice day, annotated by week 7. On sacrifice day animals fed a high-fat diet experience significantly higher weight gain when compared to controls ($p<0.05$).

Figure 5. *Total muscle weight and fat weight*



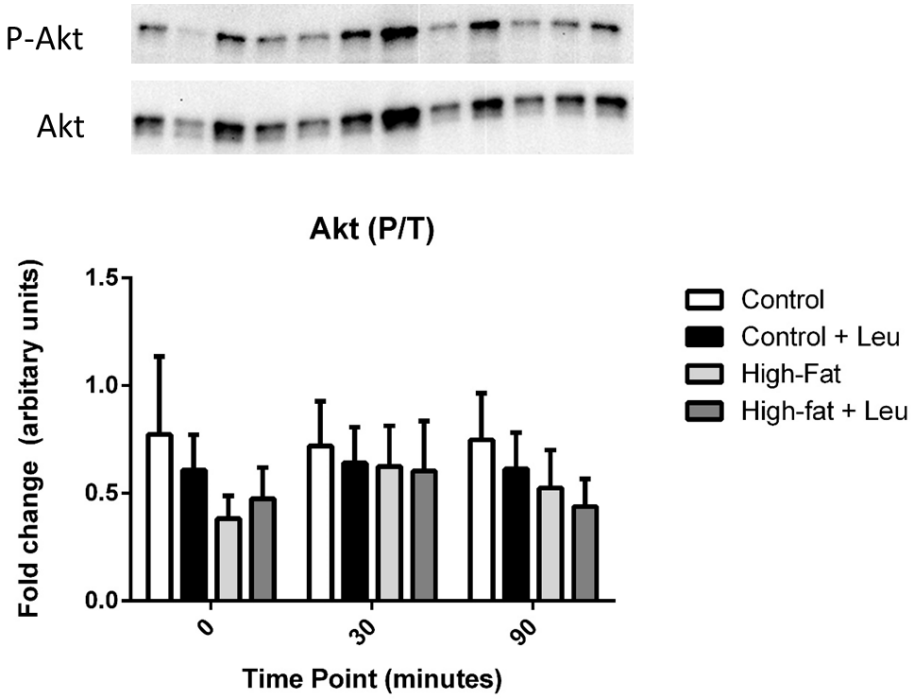
Data expressed as means ± SEM. Different letters indicate significance, $p<0.05$. (A) Total muscle wet weight was taken upon muscle extraction (n=30 per treatment group). Leucine supplemented groups experience a lower total muscle weight when compared to their respective controls ($p<0.05$). (B) Epididymal fat weight was taken upon fat tissue extraction (n=30 per treatment group). Leucine supplemented groups experience a higher total gain in fat weight when compared to their respective controls ($p<0.05$).

Figure 6. *4E-BP1 phosphorylation*



Data expressed as means ± SEM. Phosphorylation of 4E-BP1 express in a ratio of phosphorylated 4E-BP1 to total 4E-BP1(20 kDa). Changes in 4E-BP1 phosphorylation were observed in response to an isocaloric meal. Measurements were taken at fasted (0), 30 minutes postprandial, and 90 minutes postprandial (n=10 per group).

Figure 7. *Akt phosphorylation*



Data expressed as means ± SEM. Phosphorylation of Akt express in a ratio of phosphorylated Akt to total Akt (62 kDA). Changes in Akt phosphorylation were observed in response to an isocaloric meal. Measurements were taken at fasted (0), 30 minutes postprandial, and 90 minutes postprandial (n=10 per group).

Table 4. *PGC-1α and PPARγ gene expression*

	Fasted				30 min Postprandial				90 min Postprandial				Interaction
Group	Control	Control+ Leu	High-fat	High-fat + Leu	Control	Control+ Leu	High-fat	High-fat + Leu	Control	Control+ Leu	High-fat	High-fat + Leu	
PGC-1α (Expressed as a mean)	0.665	0.590	0.906	0.704	0.762	1.109	0.799	0.579	0.687	0.462	0.621	0.634	none
PPARγ (Expressed as a mean)	1.633	1.631	1.441	1.200	1.075	1.298	1.297	0.621	1.753	1.936	1.183	2.125	none

Data expressed as means ± SEM. PGC-1α gene expression was observed in response to an isocaloric meal as a maker for mitochondrial biogenesis. Measurements were taken at fasted (0), 30 minutes postprandial, and 90 minutes postprandial (n=5 per group). A two-way ANOVA was used to determine an interaction across and within time points. . PPARγ gene expression was observed in response to an isocaloric meal as a measure of lipogenesis. Measurements were taken at fasted (0), 30 minutes postprandial, and 90 minutes postprandial (n=5 per group). ($p<0.05$).

Plasma Insulin, Glucose and Free Fatty Acids

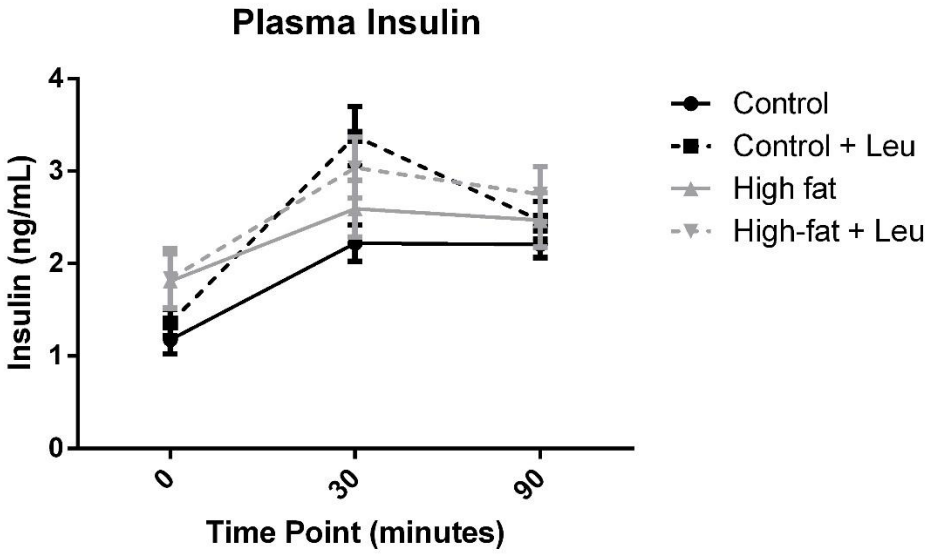
Plasma insulin levels in the fasted condition were not significant between groups; however, both the high-fat and high-fat with leucine supplementation trended higher than the control diet ($p<0.08$) in the fasted state. At 30 minutes postprandial the rats supplemented with leucine, both on a high-fat and control diet, had significantly higher insulin levels than the control group ($p<0.05$). However, no other significant differences were observed at the 30 minute time point. At 90 minutes postprandial no significance between any of the groups was observed. However, a possible relationship was observed between the control group and the high-fat with leucine supplementation group ($p<0.11$, **Figure 8**).

Glucose levels in the fasted condition did not differ between groups. At 30 minutes postprandial and at 90 minutes postprandial no differences were observed between the groups (**Figure 9**).

Free fatty levels in the fasted condition did not differ between the control diet and the control diet with leucine supplementation. However, plasma free fatty acid levels in the fasted condition were significantly higher in the high-fat with leucine group when compared to the high-fat group without leucine supplementation ($p<0.05$). Although not significant, rats fed a control diet with leucine did trend higher in fasting conditions ($p<0.10$) when compared to the high-fat fed group. At 30 minutes postprandial, we observed no difference in plasma free fatty acid levels between groups supplemented with leucine and their respective controls. However, both the control diet and the control diet with leucine had significantly lower plasma free fatty acid level when compared to the high-fat fed group without leucine supplementation ($p<0.05$). Only the control diet with leucine supplementation was significantly lower at the 30 minute time point when compared to the high-fat diet supplemented with leucine, however, the control diet

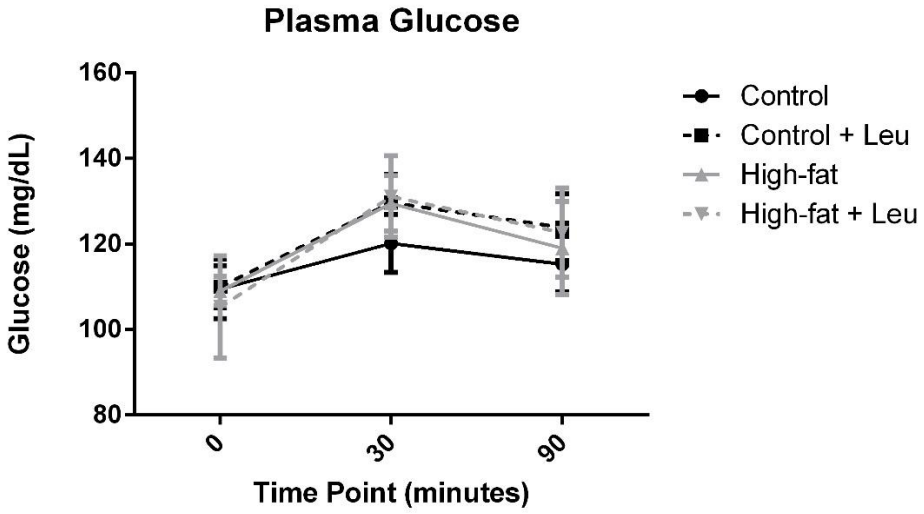
without leucine supplementation showed a trend of being lower ($p<0.08$). At 90 minutes postprandial, again we observed no difference in plasma free fatty acid levels between groups supplemented with leucine and their respective controls. However, we observed a significant decrease in plasma free fatty acids levels of rats fed a control diet or control diet supplemented with leucine, when compare to rats fed a high-fat diet without leucine supplementation ($p<0.05$). While only the control diet without leucine supplementation had significantly lower plasma free fatty acid levels when compared to the high-fat diet with leucine supplementation group, a trend was observed between the control diet with leucine supplementation and high-fat diet with leucine supplementation ($p<0.08$, **Figure 10**).

Figure 8. *Plasma insulin levels*



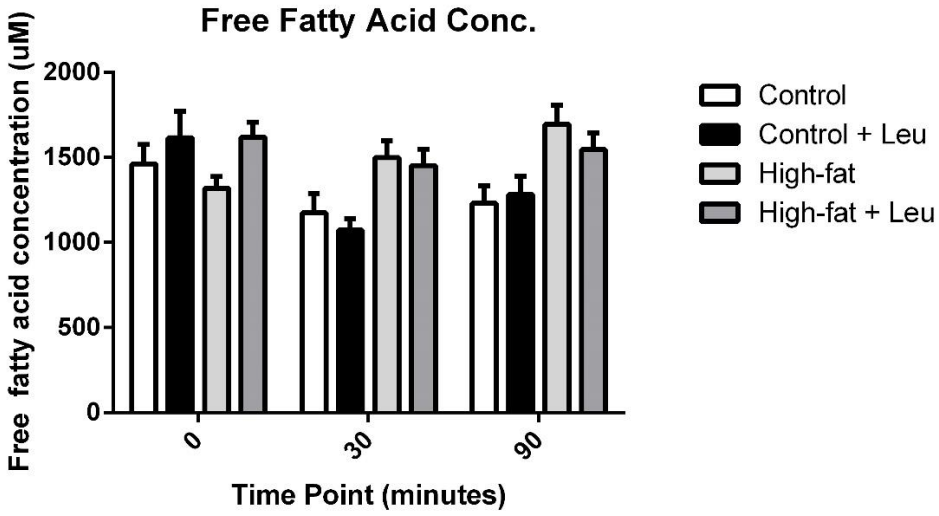
Data expressed as means ± SEM. Plasma insulin levels observed in response to an isocaloric meal. Measurements were taken at fasted (0), 30 minutes postprandial, and 90 minutes postprandial (n=10 per group). Two-way ANOVA was used within each time point and across each time point to determine significance between diets ($p<0.05$).

Figure 9. *Plasma glucose levels*



Data expressed as means ± SEM. Plasma glucose levels observed in response to an isocaloric meal. Measurements were taken at fasted (0), 30 minutes postprandial, and 90 minutes postprandial (n=10 per group). Two-way ANOVA was used within each time point and across each time point to determine significance between diets ($p<0.05$).

Figure 10. *Plasma free fatty acid levels*



Data expressed as means ± SEM. Plasma Free Fatty acid levels observed in response to an isocaloric meal. Measurements were taken at fasted (0), 30 minutes postprandial, and 90 minutes postprandial (n=10 per group). A two-way ANOVA was used to determine an interaction across and within time points, and mutiple t-tests were used within each time point to further determine the significance between diets ($p<0.05$).

Chapter 7: Discussion

The aim of the current study was to examine the ability of preventing the onset of diet-induced obesity by supplementing the branched-chain amino acid leucine to a high-fat diet in male, Sprague-Dawley rats. To our knowledge, this is the first study to look at leucine's ability to prevent, rather than treat, obesity. This study is unique, because, contradictory to current literature, this study demonstrated that male, Sprague-Dawley rats fed a high-fat diet experienced higher levels of weight gain when compared to a control diet. In addition, leucine supplementation to both the control diet and high-fat diet increased adiposity levels above that of the diet alone. When total muscle weight was compared across the diets we observed a slight but significant drop in the muscle mass of rats supplemented with leucine when compared to their respective controls. This study is not the first to show that leucine supplementation can increase adiposity above that of a high-fat diet alone [14]. Li et al. [14], showed this when leucine was supplemented to a high-fat diet via water, this resulted in an increase in body weight, primarily due to fat mass increase [14]. Additionally, another study has shown that BCAA supplementation in the food of high-fat fed rats increased weight-gain and adiposity [21]. This is thought to be as a result of BCAA metabolites competing for oxidation with fatty acid metabolites [21].

The plasma insulin levels in rats supplemented with leucine were notably higher than the control diet at 30 minutes postprandial. This could be due to the leucine stimulating insulin release from the pancreas. Although no significance was observed, we did observe a trend in both the high-fat diets as having higher plasma insulin levels in the fasted state when compared to the control diet. Although, plasma insulin levels stayed elevated after 90 minutes postprandial they appeared to be returning to basal levels. This is supported by findings in a study by Noatsch

et al.[22], where at 30 minutes postprandial insulin levels spike due to leucine supplementation and stay elevated at 60 minutes [22].

Plasma glucose levels increased in response to feeding and subsequently return to basal level. It is of note that the control diet without leucine supplementation trended lower than the other diets 30 minutes after feeding. This could indicate that the leucine supplemented in our study may have elicited a response similar to that of a high-fat diet. A similar observation was made in a study by Newgard et al [21], when BCAAs were supplemented to a high-fat diet. However, it has been show that leucine supplementation can lower blood glucose levels with supplemented to a normal diet [22] and in a high-fat diet [14]. These effects were not observed in this study.

While plasma free fatty acid levels did differ significantly between all the groups in the fasted state. In response to feeding, at 30 minutes postprandial we noticed a significant drop in plasma free fatty acid levels in the control diet group when compared to the high-fat diet groups. This pattern perpetuated at 90 minutes postprandial, further illustrating that mobilization of free fatty acids decrease in response to meal-feeding. The high-fat groups had consistently higher levels over the control diets in response to feeding probably due to the 60% fat content of the high-fat diet. Although, it seems that in the fasted state there could be more circulating free fatty acids in leucine supplemented groups. It is unclear if this mobilization is done for the storage of fatty acids in adipose tissue or for the oxidation of fatty acids in muscle tissue. Newgard et al [21], suggests that fat accumulation is in large part due to BCAAs metabolites competing for oxidation with fatty acids in the mitochondria of muscle tissue [21]. It is unclear if this is occurring in this study and further analysis must be conducted to the mechanism. It is likely that

this is the reason we are seeing an increase in weight accumulation as a result of leucine supplementation.

It has been well established that leucine activates the mTORC1 pathway and activates muscle protein synthesis. However, in this study we did not observe the phosphorylation of the downstream target of mTORC1, 4E-BP1. We only observed a trend at the fasted time point showing the control diet without leucine supplementation had higher phosphorylation. No other changes in 4E-BP1 were shown as a result of leucine supplementation. Which could indicate that the concentration of leucine within the cell never reached a sufficient level to activate mTORC1. This could be due to the metabolism of the leucine as an oxidative fuel source subsequently not causing a high enough concentration increase within the cell. However, chronic phosphorylation of IRS-1 and mTORC1 could have also occurred in the cell causing insulin resistance and increasing the fat mass of leucine supplemented groups [4,5,13-19].

We further observed that PGC-1α expression in the control diet with leucine was signifcantly higher than the high-fat diet with leucine group at 30 minutes postprandial. This could indicate that leucine supplementation stimulates the activation of PGC-1α through the mTORC1 pathway is occurring, but is blunted in the presence of a high-fat diet, however this effect seems to drop to basal levels at 90 minutes postprandial. It is unclear why a similar trend was not observed in the high-fat fed group with leucine.

PGC-1α expression has been shown to increase with leucine supplementation in the water and have a positive effect on delaying obesity [18]. In the current, we observed no significant increase in PGC-1α expression was detected that could be solely attributed to leucine supplementation. However, in the current study leucine was mixed into the diet and fed in a meal

fashion. This method of delivery and timing could be a contributing factor in the effectiveness of leucine supplementation [7,18].

It is interesting to note that while no significant changes were observed in PPARγ gene expression across any of the time points, we did observe a trend at in the PPARγ expression in rats fed a high-fat diet with leucine. We observed a decreased trend in the PPARγ expression in this group. This, is interesting since this group seemed to gain the most weight as a result of a high-fat diet with leucine supplementation. Although we observed no changes PPARγ, it is activate through the mTORC1 pathway [13]. However, it is not uncommon to see no response in PPAR activation [8,21]

It is important to note that the age of the rats in our study was 6 weeks old. This could be a source of some discrepancies, since other studies usually start at 8 weeks with a rat model and supplement for longer than 6 weeks [14]. This could be a reason for some differences we see. However, the goal of this study was to observe the onset of obesity which usually happens around 6 weeks of supplementation [14]. Additionally, we supplemented leucine in the chow and not the drinking water. This method of supplementation is not as common as supplementing leucine in the drinking water, but has been shown to be an effective means of delivering leucine [22, 26-30]

In conclusion, we have demonstrated that the supplementation of leucine to a high-fat diet does not prevent the onset of diet-induced obesity in rats fed a high-fat diet. In fact, groups supplemented with leucine experience higher levels of adiposity than their respective controls. While the mechanisms underlying this are still unclear we have examined targets downstream in the mTORC1 signaling pathway to determine if this pathway has been stimulate. However, we have currently been unable to find any significant activation in this pathway due to our treatment.

This area warrants further investigation in to the mechanism of this apparent leucine-induce adiposity.

Chapter 8: References

1. Centers for Disease Control and Prevention. "Overweight and Obesity." Centers for Disease Control and Prevention. N.p., 13 Aug 2012.

2. World Health Organization. “Obesity and Overweight.” World Health Organization. N.p., May 2014.

3. Layman, Donald K., et al. "A Reduced Ratio of Dietary Carbohydrate to Protein Improves Body Composition and Blood Lipid Profiles during Weight Loss in Adult Women." The Journal of nutrition 133.2 (2003): 411-7. Print.

4. Petzke, Klaus J., Anne Freudenberg, and Susanne Klaus. "Beyond the Role of Dietary Protein and Amino Acids in the Prevention of Diet-Induced Obesity." *International journal of molecular sciences* 15.1 (2014): 1374-91. Print.

5. Norton, Layne E., et al. "Leucine Content of Dietary Proteins is a Determinant of Postprandial Skeletal Muscle Protein Synthesis in Adult Rats." *Nutrition & metabolism* 9.1 (2012): 67. Print.

6. Anthony, J. C., et al. "Leucine Stimulates Translation Initiation in Skeletal Muscle of Postabsorptive Rats Via a Rapamycin-Sensitive Pathway." The Journal of nutrition 130.10 (2000): 2413-9. Print.

7. Binder, Elke, et al. "Leucine Supplementation Protects from Insulin Resistance by Regulating Adiposity Levels." *PloS one* 8.9 (2013): e74705. Print.

8. Binder, Elke, et al. "Leucine Supplementation Modulates Fuel Substrates Utilization and Glucose Metabolism in Previously Obese Mice." Obesity (2013)Print.
9. Guo, Juen, et al. "Persistent Diet-Induced Obesity in Male C57BL/6 Mice Resulting from Temporary Obesigenic Diets." PLoS One 4.4 (2009): e5370. Print.

10. Layman, D. K. "The Role of Leucine in Weight Loss Diets and Glucose Homeostasis." The Journal of nutrition 133.1 (2003): 261S-7S. Print.

11. Layman, Donald K., et al. "Increased Dietary Protein Modifies Glucose and Insulin Homeostasis in Adult Women during Weight Loss." The Journal of nutrition 133.2 (2003): 405-10. Print.

12. Wu, Guoyao. Amino Acids: Biochemistry and Nutrition., 2013. Print

13. Laplante, M., and D. M. Sabatini. "MTOR Signaling at a Glance." Journal of cell science 122.Pt 20 (2009): 3589-94. Print.

14. Li, Xiang, et al. "Chronic Leucine Supplementation Increases Body Weight and Insulin Sensitivity in Rats on high-fat Diet Likely by Promoting Insulin Signaling in insulin-target Tissues." *Molecular nutrition & food research* 57.6 (2013): 1067-79. Print.

15. Kimball, S. R., and L. S. Jefferson. "Signaling Pathways and Molecular Mechanisms through which Branched-Chain Amino Acids Mediate Translational Control of Protein Synthesis." *The Journal of nutrition* 136.1 Suppl (2006): 227S-31S. Print.

16. Léger, Bertrand, et al. "Akt Signalling through GSK-3β, mTOR and Foxo1 is Involved in Human Skeletal Muscle Hypertrophy and Atrophy." *The Journal of physiology* 576.3 (2006): 923-33. Print.

17. Sugawara, Takayuki, et al. "Regulation of Muscle Protein Degradation, Not Synthesis, by Dietary Leucine in Rats Fed a Protein-Deficient Diet." *Amino acids* 37.4 (2009): 609-16. Print.

18. Li, H., et al. "Leucine Supplementation Increases SIRT1 Expression and Prevents Mitochondrial Dysfunction and Metabolic Disorders in High-Fat Diet-Induced Obese Mice." *American journal of physiology.Endocrinology and metabolism* 303.10 (2012): E1234-44. Print.

19. Layman, D. K., and D. A. Walker. "Potential Importance of Leucine in Treatment of Obesity and the Metabolic Syndrome." The Journal of nutrition 136.1 Suppl (2006): 319S-23S. Print.

20. Layman, D. K., and J. I. Baum. "Dietary Protein Impact on Glycemic Control during Weight Loss." The Journal of nutrition 134.4 (2004): 968S-73S. Print.

21. Newgard, Christopher B., et al. "A Branched-Chain Amino Acid-Related Metabolic Signature that Differentiates Obese and Lean Humans and Contributes to Insulin Resistance." Cell metabolism 9.4 (2009): 311-26. Print.

22. Noatsch, Anne, et al. "Body Weight and Energy Homeostasis was Not Affected in C57BL/6 Mice Fed High Whey Protein Or Leucine-Supplemented Low-Fat Diets." *European journal of nutrition* 50.6 (2011): 479-88. Print.

23. Morino, Katsutaro, et al. "Reduced Mitochondrial Density and Increased IRS-1 Serine Phosphorylation in Muscle of Insulin-Resistant Offspring of Type 2 Diabetic Parents." *Journal of Clinical Investigation* 115.12 (2005): 3587-93. Print.

24. Berg JM, Tymoczko JL, Stryer L, Gatto GJ. Biochemistry. Ed. Lisa Samols. 7 Vol. , 2012. Print.

25. Sun, Xiaocun, and Michael B. Zemel. "Leucine Modulation of Mitochondrial Mass and Oxygen Consumption in Skeletal Muscle Cells and Adipocytes." Nutr Metab (Lond) 6 (2009): 26. Print.

26. Tong, Xing, et al. "Effects of Whey Protein and Leucine Supplementation on Insulin Resistance in Non-Obese Insulin-Resistant Model Rats." Nutrition (2014)Print.

27. Zampieri, Thais T., et al. "L-Leucine Supplementation Worsens the Adiposity of Already Obese Rats by Promoting a Hypothalamic Pattern of Gene Expression that Favors Fat Accumulation." Nutrients 6.4 (2014): 1364-73. Print.

28. Zampieri, Thais T., et al. "Oral Leucine Supplementation is Sensed by the Brain but neither Reduces Food Intake nor Induces an Anorectic Pattern of Gene Expression in the Hypothalamus." PloS one 8.12 (2013): e84094. Print.

29. Freudenberg, Anne, Klaus J. Petzke, and Susanne Klaus. "Comparison of High-Protein Diets and Leucine Supplementation in the Prevention of Metabolic Syndrome and Related Disorders in Mice." The Journal of nutritional biochemistry 23.11 (2012): 1524-30. Print.

30. Macotela, Yazmin, et al. "Dietary Leucine-an Environmental Modifier of Insulin Resistance Acting on Multiple Levels of Metabolism." PLoS One 6.6 (2011): e21187. Print.

Appendix A:

RT-PCR protocol

1. **Reconstitute Primers**: xx nm X 10 = xx ul of H2O (pure and autoclaved water)
2. Dilute primers to "**Working Primer Solutions**": Add 20ul of **Reconstituted Primer** to 180ul of H2O (pure and autoclaved water); yielding a total volume of 200ul of **Working Primer Solution**. Ex. 20ul of **Reconstituted Primer** + 180ul of H2O (pure and/or autoclaved water) = 200ul **Working Primer Solution**
3. Next, make "**Master mix**" by adding : n X 10ul of sybergreen mix = xx ul of sybergreen mix
   n X 1ul of **Reconstitute Primer** F (forward)= xx ul of primer
   n X 1ul of **Reconstitute Primer** R (Reverse)= xx ul of primer
   n X 8ul of H20 (pure and autoclaved) = xx ul of H20
   n = number of samples or wells per gene (e.g. FAS, ACC, 18s) always make a little more than you need. So, if n=50 make n=55.
4. Add **5ul** of **Diluted cDNA** sample in to well. **NOTE**: if you have not diluted the samples from your cDNA plate, do so now. *Diluting cDNA*: Label micro centrifuge tube and place 18-20ul of sample in tube with 180ul of water. **IMPORTANT**: Ensure you have extracted the same amount of cDNA from each sample for dilution. EX. If you only extract 18ul from one well then get 18ul from all the wells.
5. Next add **20ul** of primer specific **Master mix** (e.g. FAS, ACC, 18s) to desired wells.
6. Place plastic cover over plate and seal thoroughly, then centrifuge to pull contents of each well to the bottom of the well.
7. Place in PCR machine and run desired program depending on primers.

Example plate layout
in duplicate

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
B	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12
C	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18
D	S19	S19	S20	S20	S21	S21	S22	S22	S23	S23	S24	S24
E	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
F	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12
G	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18
H	S19	S19	S20	S20	S21	S21	S22	S22	S23	S23	S24	S24

S#= SAMPLE NUMBER

Housekeeping/reference gene (e.g. 18s, GAPDH)

Target gene (e.g. FAS, ACC)

Run Parameters (may vary; this is base on FAS and 18s primers; 58°C could vary to 60°C):
Initial hold- **50°C** for **2** minutes
95°C for **10** minutes
40 cycles of- **95°C** for **15 SECONDS**
58°C for **1** minute

Appendix B:

Western Blotting Protocol

1. Set heat block to 98oC and wait for heat block to come to temperature.
2. Once heat block has come to temperature remove desired western blotting samples from -80oC freezer and apply lid locking caps to each tube.
3. Heat samples in heat block for 10 minutes.
4. While samples are heating assemble gel electrophoresis tank.
5. Remove pre-cast gels from packaging and remove comb and seal on the bottom of the gel.
6. Place gels in interior chamber frame with the short plates facing each other. Note: If running one gel use buffer dam.
7. Secure interior chamber frame clamps around gels.
8. Fill interior chamber with 1x Running buffer.
9. Once samples are finished heating remove from heat block and vortex each sample. Note: Never remove lid locking caps while samples are still hot.
10. Remove protein marker standard from -20oC freezer and allow to come to room temperature.
11. Using gel loading tips and micro-pipette, pipette 3ul of protein marker standard into one of the wells. Then 15ul of your sample into an empty well, repeat until all samples have been loaded in their own well or no more empty wells are left.
12. Place interior chamber with newly loaded gels inside the electrophoresis tank. Ensure you match the black electrode with the black mark on the tank and the red electrode with the red mark on the tank.
13. Fill the electrophoresis tank with 1x Running buffer to the appropriate level for the number of gels. Note: if running only one gel fill tank to the 2 gel mark, if running 3 gels fill tank to the 4 gel mark.
14. Attach the lid of the electrophoresis tank ensure the black electrode is connected to the black cable and the red electrode is connected to the red cable.
15. Plug cables into power pack ensuring that the black cable is attached to the black outlet and the red cable is attached to the red outlet.
16. Set the power pack to maintain constant voltage and set to 200V.
17. Press the run button. The gel should take about 45 minutes to run, but this could vary based on percentage of gel. Once the blue marker dye runs out of the bottom of the gel discontinue the run and begin the transfer process.
18. To begin the **Transfer** process tank a gel electrophoresis tank place a small stir bar at the bottom of the tank and place a red/black transfer chassis into the tank. Ensure the red side of the transfer chassis is matched up with the red mark on the tank and the black side of the transfer chassis is matched to the black mark on the tank.
19. Assemble the transfer sandwich

a) Open a clear/black transfer sandwich using the black side as the bottom of the sandwich.

b) Saturate a transfer sponge in 1x Transfer buffer and place it on the black side of the transfer sandwich.

c) Next, saturate a sheet of filter paper in 1x Transfer buffer and place on top of transfer sponge.

d) Break open plastic gel cassette using gel cassette tool and cut-off gel lane dividers with gel knife. Float gel in 1x Transfer buffer to remove from plastic cassette. Note: this may require gently using the gel knife to break the gel free from plastic cassette. Place gel on-top-of saturated filter paper in the transfer sandwich.

e) Wet blotting paper in methanol to activate blotting paper, then equilibrate in 1x Transfer buffer for 1-2 minutes. Note: prepping the blotting paper can be done prior to assembly of sandwich. Place blotting paper on-top-of the gel in the transfer sandwich.

f) Ensure there are no bubbles between the gel and blotting paper. If bubble are present smooth them out.

g) Saturate a 2nd sheet of filter paper in 1x Transfer buffer and place on-top-of blotting paper in the transfer sandwich.

h) Lastly, Saturate a 2nd transfer sponge in 1x Transfer buffer and place on-top-of 2nd sheet of filter paper in the transfer sandwich.

i) Close and lock transfer sandwich.

j) Repeat until all gels are in transfer sandwiches.

20. Place newly assembled transfer sandwich in transfer chassis. Ensure the black side of the transfer sandwich faces the black side of the transfer chassis and the clear side of the transfer sandwich faces the red side of the transfer chassis.
21. Place ice pack into electrophoresis tank and fill tank to blotting level with refrigerated 1x Transfer buffer.
22. Place gel electrophoresis tank on stir plate and turn stir plate on.
23. Attach the lid of the electrophoresis tank ensure the black electrode is connected to the black cable and the red electrode is connected to the red cable.
24. Plug cables into power pack ensuring that the black cable is attached to the black outlet and the red cable is attached to the red outlet.
25. Set the power pack to maintain constant voltage and set to 100V.
26. Set the power pack timer to 60 minutes and press the run button.
27. After 1hour, turn off the power supply and remove the blotting paper from the transfer sandwich.
28. Place blotting paper, with the side that was in contact with the gel facing up, in a plastic blot washing container.
29. Rinse blot with 1x TBST

30. To block: pour ~10ml of 5% milk in TBST into blot washing container. Place on rocker for 1 hour at room temperature. Note: If you need to block longer than an hour place blot washing container on a rocker in 4^{o}C.
31. Prepare primary antibody
    a) Dilute primary antibody to 1:1000 in 1x TBST
32. After blot has blocked for at least 1 hour, rinse blot 2 times in 1x TBST.
33. Then wash blot for 5 minutes in 1x TBST at room temperature using a rocker.
34. Drain off TBST.
35. Pour ~5 ml of diluted primary antibody on blot and place on a rocker in 4^{o}C refrigerator overnight.
36. After the blot as probed overnight, pour off primary antibody.
37. Rinse blot twice in 1x TBST, then wash for 10 minutes in 1x TBST at room temperature.
38. Prep secondary antibody
    a) Dilute secondary antibody to 1:1000 in 1xTBST
    b) Add marker conjugate at 1:20000 to secondary antibody mixture.
39. Drain off TBST.
40. Pour ~5 ml of diluted secondary antibody mixture on blot and place on rocker for 1 hour at room temperature.
41. After 1 hour pour off secondary antibody mixture.
42. Rinse blot twice in 1x TBST
43. Perform at least 2-3 ten minute washes in 1x TBST
44. Drain off TBST
45. Pour 4ml of mixed ECL on blot and rock at room temperature for 2 minutes.

Alternatively, if running low on ECL place blot on a flat surface that can be placed in imager. Then pipette 600ul of mixed ECL on the area of the blot where the bands you probed for are (use the colored marker as a guide). Let the blot set for 1 minute. Next, flip the blot over and let it set for 1 more minute. Flip blot back over.

46. Place blot in imager and image.

Appendix C:

Harsh Striping Protocol

1. Rinse blot in methanol
2. Rinse blot 2x in pH_2O
3. Wash blot for 5 minutes in 1xTBST
4. Drain off TBST
5. Add a thin layer of Harsh Stripping Buffer to blot
6. Incubate blot for 30 minutes at room temperature
7. Drain off Harsh Stripping Buffer
8. Rinse blot 3x in pH_2O
9. Rinse blot 4x in 1xTBST
10. Wash blot for 10 minutes in 1xTBST
11. Repeat western blotting steps 26-42

Appendix D:

IACUC approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Jamie Baum

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: February 8, 2012

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date : **February 20, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#12019- "DIETARY LEUCINE - ROLE IN METABOLISM AND OBESITY PREVENTION**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing [Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **02-20-2015,** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian