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The Effect of Leucine Supplementation on Mitochondrial Biogenesis and Mitochondrial Protein Synthesis in Rats Fed a High-Fat Diet

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Abstract

This study aims to illuminate interaction between leucine supplementation and mitochondrial proteins concerned with synthesis and biogenesis. We hypothesized that supplementation with leucine will attenuate the weight gain of the high fat diet in comparison with the normal chow group, by enhancing mitochondrial biogenesis and content. We conducted a laboratory experiment using Western Blotting techniques to determine protein expression. Proteins of interest in this study include PGC-1α, COX-IV, TUFM, TFAM, and mtIF2. The findings from the research show a main effect of an increase in the expression of PGC-1 α in high fat diets. There was no difference in the expression of COX-IV with regard to either leucine supplementation or diet. There was a main effect of an increase in the expression of TUFM in high fat diets. A main effect of an increase in mtIF2 was seen in NC groups when compared with HF diet groups. The findings of this study support the current model that high fat diets cause dysregulation of mitochondria in that PGC-1 α protein content was upregulated and yet COX-IV protein content was unchanged.

1. Introduction

Obesity is a metabolic disease defined as having a body mass index greater than or equal to 30 kg/m^2. Obesity is associated with various comorbidities such as type 2 diabetes, gall bladder disease, high blood pressure, and osteoarthritis (Must et al., 1999).There has also been an increase in the occurrence of obesity surrounding the turn of the 21st century (Baskin, Ard, Franklin, & Allison, 2005). This increase in obesity has been shown to be the result of increased amounts of food intake along with decreases in physical activity (Wing et al., 2001). Obesity is also associated with an increase in intramuscular triglycerides, and this has been associated with insulin resistance, lipotoxicity, and incomplete β-oxidation in muscle (Holloway, Bonen, & Spriet, 2009). Obesity is a rapidly growing metabolic disease that is deteriorating the quality of life of millions of people.

Mitochondria supply the body and more specifically skeletal muscle with the ability to undergo aerobic metabolism in the form of oxidative phosphorylation. Skeletal muscle uses this organelle as a means of synthesizing the energetic substrate ATP (Ernster & Schatz, 1981). In addition to this function, the electron transport chain, intracellular calcium regulation, fatty acid oxidation, and regulation of apoptosis, all occur within the mitochondria of skeletal muscle (Green & Reed, 1998; Kennedy & Lehninger, 1949; Vasington & Murphy, 1962). Recent studies suggest that obesity impairs mitochondrial quantity and oxidative capacity (Chanséaume & Morio, 2009). Impairment in oxidative capacity has been linked to increases in fatty acid accumulation within skeletal muscle. Additionally, during a high-fat diet, mitochondrial protein synthesis is disrupted as evidenced by decreased cytochrome c oxidase activity and inhibited mitochondrial respiration (Greene et al., 2014). Another detriment to oxidative capacity comes in the form of impaired mitochondrial biogenesis. Mitochondrial biogenesis is the process by which new mitochondria are created. PGC-1α, the master regulator of mitochondrial biogenesis, is decreased due to high fat diets, obesity, and lipid infusion (Greene et al., 2014; Richardson

et al., 2005; Sparks et al., 2005). These impairments to mitochondrial function then lead to decreased quality of life from cardiovascular disease, diabetes, and weight gain.

Mitochondrial protein synthesis includes transcription, the process by which mRNA transcript is synthesized from DNA. Then, in translation, the mRNA transcript is brought to the mitoribosomal complex and synthesized into protein. A specific protein of importance in transcription of mitochondrial DNA is mitochondrial transcription factor A (TFAM). TFAM is one of the two transcription factors that must be present for mitochondrial transcription to take place (Falkenberg et al., 2002; Tiranti et al., 1997). TFAM also directly regulates mitochondrial DNA (mt-DNA) copy number in mammals (Ekstrand et al., 2004). When TFAM was knocked out in mice, mt-DNA copy number decreased. Reduced mt-DNA copy number results in decreased survival rates and reduced mitochondrial messenger RNA (mt-mRNA) transcript (H. Li et al., 2000). Two proteins that are specific to the process of mitochondrial translation are mitochondrial translation initiation factor (mtIF2) and mitochondrial translation elongation factor (TUFM). mtIF2 is responsible for bringing the fMet-tRNA to the small, 28S subunit of the ribosome and initiating translation (Liao & Spremulli, 1990). TUFM is responsible for effective and efficient translation elongation of mt-mRNA transcript (Valente et al., 2007a). In both transcription and translation, TFAM, mtIF2, and TUFM are crucial to the process of mitochondrial transcription and translation (Smits, Smeitink, & van den Heuvel, 2010).

The nonpolar, branched-chain amino acid leucine, has been shown to increase the energy partitioning from adipocytes to muscle cells. This change in energy partitioning or energy usage due to leucine supplementation has been shown to increase usage of fats in muscle, increased insulin utilization, improved glucose and cholesterol metabolism, and increased muscle protein synthesis via the mammalian target of rapamycin(mTOR) pathway (Xu, Kwon, Cruz, Marshall, & McDaniel, 2001; Y. Zhang et al., 2007).The metabolic enhancements that have been associated with leucine make it a prime

substrate to be used in studying metabolic syndromes such as obesity. In addition to these findings, leucine is unique among the other nonpolar, branched chain amino acids, valine and isoleucine, since leucine is the only one of the three that is sufficient to stimulate muscle protein synthesis, fat utilization, mitochondrial biogenesis, and decreased protein degradation (Anthony, Anthony, & Layman, 1999; Liang, Curry, Brown, & Zemel, 2014; Mitch & Clark, 1984). While there is well documented research showing that leucine has beneficial effects on protein synthesis, its effects on mitochondrial protein synthesis and biogenesis need to be elucidated (Anthony et al., 1999; Liang et al., 2014). Additionally, leucine's effects on these processes in rats on a high fat diet is not understood. Therefore, the purpose of this study is to determine the effects of leucine supplementation on mitochondrial biogenesis in the soleus muscle of Sprague-Dawley rats fed a high fat diet. We hypothesize that supplementation with leucine will attenuate the weight gain of the high fat diet in comparison with the normal chow group, by enhancing mitochondrial biogenesis and content.

2. Review of Literature

This review of literature will be separated into five parts discussing leucine supplementation, obesity, mitochondria, high fat diets, and mitochondrial protein synthesis.

2.1. *Leucine*

With the rapid rise in obesity, researchers have looked for a substrate that will either nullify or ameliorate the effects of a high-fat diet. The detrimental metabolic effects of high-diet include increased skeletal intramuscular triglycerides, mitochondrial dysfunction, and insulin insensitivity. Recently, however, research into the metabolic effects of the amino acid leucine have shown to increase skeletal muscle protein synthesis and mitochondrial biogenesis, may be involved in fatty acid mobilization, and may also increase insulin sensitivity in skeletal muscle(Dodd & Tee, 2012; Liang et al., 2014; Sun & Zemel, 2007; Xu et al., 2001).

In addition to these effects, leucine also impacts thermogenesis and satiety (Devkota & Layman, 2010). Leucine has been shown to increase thermogenesis, and an increase in thermogenesis necessarily increases energy expenditure. Additionally, leucine enhances satiety. Satiety, or the feeling of fullness, can be directly attributed to weight loss. This is to say that a diet containing leucine may have positive benefits in preventing metabolic syndrome through the enhancement of thermogenesis and satiety (Devkota & Layman, 2010).

Leucine has been shown to increase skeletal muscle protein synthesis through activation of the mammalian target of rapamycin (mTOR) pathway. In addition to this function of leucine, it also acts against the other side of protein turn over by reducing proteolysis. So leucine not only increase muscle protein synthesis, it also decreases proteolysis(Dodd & Tee, 2012; Paddon-Jones & Rasmussen, 2009) .In addition to the function of increasing skeletal muscle protein synthesis, leucine has also been shown to

increase mitochondrial biogenesis (Liang et al., 2014). The specific method of action for increasing mitochondrial biogenesis is through activation of PGC-1α via sirtuin-1(SIRT-1).

In the past two to three years, interest in the study of SIRT-1 has increased dramatically due to findings in mouse models that show that SIRT-1 is not only an activator of PGC-1α but also an inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Herranz & Serrano, 2010). The inhibition of NF-κB is important to health specifically in an obese state because it specifically activates pro-inflammatory cytokines that are associated with obesity (Ganai, Khan, Malik, & Farooqi, 2015).

Leucine has also been shown to increase translation initiation in skeletal muscle of food-deprived rats. Anthony et al. have shown that leucine, among the other branched-chain amino acids isoleucine and valine, is a more potent activator of skeletal muscle protein synthesis (Anthony, Anthony, Kimball, Vary, & Jefferson, 2000). Leucine was shown to act primarily through hyperphosphorylation of 4E-BP1 leading to increased availability of eIF4 to form the active eIF4G-eIF4E complex. Anthony also showed that in addition to being an activator of translation initiation, leucine also acts through a rapamycin-sensitive pathway.

Furthermore, leucine has been shown to be of interest for post exercise recovery due to its ability to increase translation initiation of protein synthesis in skeletal muscle following exercise. This moves the state of the muscle cell from one that is primarily catabolic during exercise, to one that is of a positive balance for protein turn over (Norton & Layman, 2006).

2.2. *Obesity*

In the United States alone, obesity is a major health concern. Studies done at the turn of the 21st century show that nearly 1/3 of the population of adults in the United States are obese (Hedley et al., 2004). This metabolic syndrome is defined as having a Body Mass Index > 30 kg/m^2 (World Health

Organization, 2000).Obesity has many effects on health that include adipose tissue dysfunction, insulin resistance, mitochondrial dysfunction, basal levels of inflammation, and muscle dysfunction.

a. *Economic Concerns Surrounding Obesity*

In addition to the health concerns associated with obesity, the cost of healthcare on average for an obese individual is higher as well. In 1995 the total cost attributable to obesity was $99.2 billion (Wolf & Colditz, 1998). From this total, $51.64 billion is direct medical cost, and the remaining sum is due to a combination of lost productivity and lost days of work. In 2001, it was estimated that the average healthcare costs for an obese individual per year was $1,069 higher than for a normal weight individual (Wang, Beydoun, Liang, Caballero, & Kumanyika, 2008). Furthermore, in a study spanning from 1998-2006 estimated that in 2006, obese individuals paid an average of $1,429 more than normal weight people; therefore, obesity-related healthcare costs were expected to increase total healthcare cost by $147 billion in 2008 (Finkelstein, Trogdon, Cohen, & Dietz, 2009).

b. *Obesity and Health Status*

The rising pandemic of obesity is a global health concern. The World Health Organization (WHO) characterizes obesity as a state of unusual fat mass gathered in adipose tissue, which may cause extreme health consequences (World Health Organization, 2000). In the United States, obesity is currently prevalent in nearly two-thirds of the adult population, with an increased prevalence expected. Research has also shown that obesity is a key predictor of high blood pressure, type 2 diabetes, cardiovascular disease, and premature death (Ogden et al., 2006).

Body mass index (BMI) is an indirect measure of obesity with a correlation to body fat mass in children, and adults (World Health Organization, 2000). A BMI value greater than or equal to 30 kg/m^2 is considered obese (World Health Organization, 2000). Among adults, a greater proportion of women

(33.2%) are obese when contrasted with men (31.1%). Complications of obesity in adults include dyslipidemia, type 2 diabetes, coronary illness, hypertension, and premature death (Weisberg et al., 2003). Alongside the expansion in rate of obesity in adults, is the increase of obesity in the pediatric populace. Due to this, the younger generation is experiencing increased levels of type 2 diabetes, hypertension, cardiovascular ailment, and metabolic syndrome. These elevated comorbidities associated with obesity have contributed to a decreased quality of life. It has likewise been recognized that when people struggle with their weight at an early age, they encounter more trouble in losing this weight later in life.(Ogden et al., 2006).

Obesity has also been shown to prompt insulin and leptin resistance, which brings about dysfunctional adipose and skeletal tissue (Boyle, Zheng, Anderson, Neufer, & Houmard, 2012). When this happens, metabolic dysregulation occurs with increased levels of circulating free fatty acids (FFAs), and increased generation and discharge of pro-inflammatory adipokines (Weisberg et al., 2003). If left untreated, elevated FFA levels can lead to lipotoxicity, chronic inflammation, hypertension, atherosclerosis, and cardiovascular disease. An obese individual normally exhibits a debilitated lipid profile, where triglycerides are raised, high-density lipoprotein (HDL) cholesterol is lessened, and low density lipoprotein (LDL) is increased (Dyer & Elliott, 1989; Stamler, Stamler, Riedlinger, Algera, & Roberts, 1978). It is likewise vital to note that visceral fat amassing is more risky for the advancement of cardiovascular ailment than fat on extremities (Matsuzawa, Nakamura, Shimomura, & Kotani, 1995). Additionally, there is a link between obesity and advancement of type 2 diabetes with an increased risk of developing pre-diabetic conditions as an after effect of impeded glucose tolerance, and insulin resistance in the tissues.

2.3. *Mitochondrial Function*

Mitochondria are ubiquitous organelles that are found in aerobic cells (Altmann, 1894). At the time of their discovery by Altmann, they were called “bioblasts”, and later renamed to be called a mitochondria. The name “mitochondria” comes from the Greek “mitos” meaning “thread” and “chondros” meaning “granule.” This renaming was in reference to the appearance of the structure of the mitochondria during spermatogenesis (Benda, 1898). Altmann hypothesized that the mitochondria carried out vital functions within the cell but it would not be until almost 50 years later until the discovery of the mitochondrial role in adenosine triphosphate production (ATP).

Study of the mitochondria began with using redox dyes to observe the staining of mitochondria using Janus Green B. Janus Green B is a basic dye that is used to supravitally stain mitochondria and determine oxygen content. It was used by its inventor Leanor Michaelis to stain mitochondria in 1899 (Michaelis, 1899). Following the studies of Michaelis, in 1925 Keilin described the cytochromes as a series of catalysts that were dehydrogenases (Keilin, 1925). Then, in 1937 the Krebs cycle or citric acid cycle was discovered and one of the products of this cycle, among others, is nicotinamide adenine dinucleotide (NADH) (Krebs & Johnson, 1980). NADH is a coenzyme that has either an oxidized (NAD+) or reduced form (NADH). NADH, the protonated form, is oxidized by the catalytic dehydrogenases that are the cytochromes (Kennedy & Lehninger, 1949). The importance of this dehydrogenation became clearer when the superstructure of the mitochondria was determined. In 1952 Palade, using electron micrographs, ascertained that the mitochondrion is a dual membrane organelle with a rounded outer membrane and a folded, ridged inner membrane called *cristae mitochondriales* (Palade, 1952). The outermost partition between the outer membrane and inner membrane space is called the intermembrane space and the area within the inner membrane is called the matrix (Racker, 1970). The structure of the mitochondria in combination with the electron transport chain is then used to create an

electrochemical gradient between the intermembrane space and the mitochondrial matrix. This gradient is created by the transport of protons from NADH through the cytochromes, and into the intermembrane space by the electron transport chain. Then, the electrons move from subunit one through Q, then to subunit III. After moving from subunit III the electrons move through cytochrome C and then back into the mitochondrial matrix to form water. This transfer of electrons through the electron transport chain is used to create the electrochemical and pH gradient between the intermembrane space and mitochondria matrix. Following electron transport, the energy and proton motive force generated from the gradient is used to drive ATP synthase and produce ATP (Mitchell, 1966). In addition to ATP, water is also generated through the combination of the proton that moved through ATP synthase and oxygen.

2.4. *Mitochondrial Biogenesis and Content*

The molecular mechanisms of mitochondrial biogenesis have only recently been discovered. Mitochondrial dysfunction is important to discuss in context of mitochondrial biogenesis and is discussed further in this review of literature. This section is dedicated to current knowledge concerning the creation and regeneration of mitochondria in the cell.

Mitochondrial biogenesis is defined as the growth and division of already existing mitochondria. It is of popular scientific belief that the mitochondria originated from an endosymbiotic relationship within a host cell. The so believed origins of mitochondria come due to the fact that mitochondria replicate by themselves and contain their own circular genome. From this double stranded, circular, roughly 16.5 kilobase strand, 37 genes are encoded. These 37 genes contribute in the creation of 13 of the subunits of the electron transport chain that include complexes I, III, IV, and V. In addition to these proteins, other gene products include 22 tRNAs (transfer RNAs) and two rRNAs (ribosomal RNAs) needed for translation of the previously mentioned subunits (Baker, Frazier, Gulbis, & Ryan, 2007). Several notable

ways to induce mitochondrial biogenesis include exercise, caloric restriction, low temperature, oxidative stress, cell division and renewal and differentiation (Bonnard et al., 2008; Civitarese et al., 2007). Mitochondrial biogenesis includes both the replication of mitochondria and also the size of mitochondria.

Important to remember however, is the fact that the majority of proteins that function within the mitochondria are encoded within the cellular nucleus. This means that the majority of the proteins that make up the mitochondria come not from its own DNA, but the DNA of the parent cell. These proteins must pass through specific translocases. There are four major translocases and they include the translocase of the outer membrane (TOM), translocase of the inner membrane (TIM), presequence translocase-associated motor (PAM), and sorting and assembly machinery (SAM). TOM is the starting place for all proteins that pass in and out of the mitochondria, and following TOM, TIM sorts matrix targeted precursors. Following sorting by TIM, PAM regulates heat shock protein 70(Hsp70) to further translocation into the matrix. SAM then inserts β-barrel proteins within the bilayer of the outer membrane. Each of these proteins and their regulatory properties are integral to the success of mitochondrial biogenesis.

As for signaling cascade mechanisms, there is no known protein of greater importance for the success and initiation of mitochondrial biogenesis than peroxisome proliferator-activated receptor-γ (PPAR) coactivator-1 α (PGC-1 α) (Puigserver et al., 1998). Work done by Puigserver et al. in mice exposed to cold (4 degrees Celsius) induced increased expression of PGC-1 α mRNA expression in tissues responsible for thermogenesis. Specifically in brown fact and skeletal muscles. The specific function of PGC-1 α is the co-transcriptional regulation of other transcription factors including nuclear respiratory factors one and 2(NRF-1 and NRF-2) and transcription factor of activated mitochondria (TFAM). NRF-1

and NRF-2 are vital in the transcription of mitochondrial enzymes and they work together with TFAM, a main force in the transcription and replication of mitochondrial DNA (Virbasius & Scarpulla, 1994).

PGC-1β is very similar in molecular structure to PGC-1 α and shares a similar function. It regulates mitochondrial biogenesis through activation of transcription factors as seen in ectopic expression in L6 myoblasts. PGC-1β also increases basal oxygen consumption. However, the importance between PGC-1 α and PGC-1β is where they differ. Specifically, PGC-1β is not upregulated in brown fat or skeletal muscle due to cold or due to exercise. Based on this, it appears that PGC-1 α and PGC-1β both contribute to mitochondrial biogenesis, but they are induced into function through different means (Meirhaeghe et al., 2003).

2.5. *Mitochondrial dysfunction*

Mitochondria provide many cells of the body with the ability to produce ATP through aerobic respiration, regulate intracellular calcium and apoptosis (Green & Reed, 1998; KENNEDY & LEHNINGER, 1949; Sun & Zemel, 2007). It is due to these important functions, that having numerous, healthy mitochondria are important for health and wellness. Therefore, it is no wonder that there are a growing number of publications concerning mitochondria in relationship to disease state. Specifically, mitochondrial dysfunction is associated with obesity and cardiovascular disease (Krieger & Duchen, 2002; Schon & Manfredi, 2003; D. Zhang et al., 2003). Poorly functioning mitochondria manifest themselves as an increase in overall oxidative stress caused by reactive oxygen species (ROS), and abnormal values for mitochondrial biomarkers (Murphy, 2009). While there are many biomarkers and regulatory proteins for mitochondrial health, notable biomarkers that measure mitochondrial biogenesis and content include PGC-1 α, COX-IV, TFAM, TUFM, and mtif2.

PGC-1α is a major regulatory protein of mitochondrial biogenesis in skeletal muscle. Specifically, it is a coactivator of PPARγ, an activator of mitochondrial biogenesis (Puigserver et al., 1998). PGC-1 α is

inducible by exercise training in both rodents and in humans, and expression of PGC-1α increases oxidative capacity (Baar et al., 2002; Lin, Handschin, & Spiegelman, 2005). PGC-1α knockout mice fatigue very easily and are unable to complete exercise (Leone et al., 2005).

Cytochrome c oxidase (COX) IV is one of the three enzyme complexes that make up the structures required for oxidative phosphorylation (Keilin & Hartree, 1939). COX-IV is the oxidative machinery responsible for the final step of oxidative phosphorylation. In this step, the fourth and final enzyme in the process takes one proton from each of the other subunits, combines them with oxygen, and forms water (Y. Li, Park, Deng, & Bai, 2006) .When the protons pass from the intermembrane space across the electrochemical gradient to combine with oxygen, the energy given off in this process is used to form ATP from ADP and inorganic phosphate.

Mitochondrial transcription factor A (TFAM) is a marker of mitochondrial protein synthesis. TFAM is necessary to insure the proper transcription of mitochondrial DNA (Virbasius & Scarpulla, 1994). This protein biomarker is associated with PGC-1α through the activation of nuclear respiratory factors one and two (NRF-1 and NRF-2) (Evans & Scarpulla, 1990). It is due to these associations and functions in the process of mitochondrial DNA transcription that it is commonly used to measure mitochondrial transcription.

Mitochondrial translation elongation factor (TUFM) has been implicated in studies of mitochondrial health. TUFM has been shown to be specifically associated with the improper development of new mitochondrial protein in obese Zucker rats (Greene et al., 2014). Additionally, TUFM is associated with the insulin cascade and the reduction in insulin sensitivity in a high fat diet (Matsuzawa-Nagata et al., 2008; Mercader et al., 2012).

Mitochondrial translation initiation factor 2 (mtIF2) is a biomarker associated with TUFM in the synthesis of mitochondrial DNA. mtIF2 is currently believed to attach to the small subunit of the

mitochondrial ribosome and initiate translation. Then, following the attachment of the large subunit to the small subunit, and GTP hydrolysis, mtIF2 is then released so that elongation can occur (Gaur et al., 2008).

2.6 *Animal Modeling of High-Fat Diets*

Obesity and its associated comorbidities such as insulin resistance, dyslipidemia, and type 2 diabetes are important in science and clinical research. In order to study obesity and its associated metabolic syndromes there must be a suitable, analogous model to study them in animals. The model must be such that it parallels the pathogenesis of obesity and metabolic syndrome in humans. The model must also resemble the human phenotype and must be replicable.

Researchers have mainly used two different approaches to metabolic syndrome in animals. On one side, obesity has been modeled using fa/fa Zucker (obese) and fa/- Zucker (lean) rats. In these rats, obesity is induced genetically and as such may not be attributable directly to the diet set for them. This model is mainly used to study the effects of obesity and not the actual process of becoming obese through a high fat diet.

Another approach to model that has been adopted for the modeling of obesity and metabolic syndrome is research using high fat diets to induce obesity (Oakes, Cooney, Camilleri, Chisholm, & Kraegen, 1997). The first research that induced obesity through diet dates back to the 1940s (SAMUELS, REINECKE, & BALL, 1942). This research used a diet that was %70 fat and is the building block that modern high fat diet modeling is based off of. And while a diet that is 70% by mass of fat is not biologically relevant to humans due to the average American fat intake being near 33%, it was a starting place for the study of high fat diets (National center for health statistics.2014). Samuels et al. showed that rats fed a high fat diet exhibited abnormal glucose tolerance, and later studies have shown that

high-fat diets lead to insulin resistance, and hyperglycemia as well. Because of this convention found in rats, it is accepted that high fat diet feeding can be used to induce and study metabolic syndrome.

2.6. *Mitochondrial Protein Synthesis*

a. Transcription

In addition to the cellular genome, humans also have unique mitochondrial DNA (mtDNA) that is separately partitioned within the mitochondria. Mitochondrial DNA is circular, and contains essential subunits of the electron transport chain and the rRNAs and tRNAs necessary for translation within the mitochondrial matrix. In order for transcription of mitochondrial DNA to occur, mitochondrial RNA polymerase(POLRMT) and two transcription factors, mitochondrial transcription factor B2(TFB2M), and mitochondrial transcription factor A(TFAM) must be present to produce basal levels of RNA transcription.

POLMRT is responsible for polymerizing ribonucleotides into RNA transcript using DNA as a template. Mitochondrial RNA polymerases are single subunit structures that are highly similar to the RNA polymerases found in T3 and T7 bacteriophages (Masters, Stohl, & Clayton, 1987). Like in T7 bacteriophages POLMRT can generate primers to initiate DNA replication, linking both transcription and replication. The primary mitochondrial transcription initiation factor is TFB2M (Falkenberg et al., 2002). TFB2M facilitates the change in DNA promotor from closed to open needed for initiation to occur, and for POLMRT to bind (Litonin et al., 2010). While TFB2M does initiate breakdown of the promotor, it does not appear to increase binding recognition for POLMRT. In addition to the effects of the promotor region of DNA, TFB2M also acts on the catalytic site for RNA polymerization (Sologub, Litonin, Anikin, Mustaev, & Temiakov, 2009).

TFAM was the first mitochondrial transcription factor identified and is most notable for its ability to bend and wrap DNA using its High Mobility Group (HMG domains). It is one of the two transcription factors required for mitochondrial transcription (Litonin et al., 2010). TFAM's role in transcription involves binding and bending mtDNA upstream of mtDNA promotors (Fisher & Clayton, 1985). This DNA bending was later found to be a TFAM imposing a 'U-turn' in the DNA promotor where both of the HMGs turn the DNA 90 degrees (Ngo, Kaiser, & Chan, 2011; Rubio-Cosials et al., 2011). In addition to DNA bending, TFAM also contributes to transcriptional activation through interaction with TFB2M at its C-terminal tail, and it is that that the DNA bending imposed by the HMG promotes the TFAM-TFB2M interaction.

b. Translation

Translation factors are highly conserved proteins that are necessary for successful translation of mRNA transcript. Bacteria have 3 translation initiation factors Initiation Factors 1-3. Even though mitochondria are very similar to bacteria in a number of ways, unlike in bacteria, human mitochondria only require 2(Koc & Spremulli, 2002; Ma, Farwell, Burkhart, & Spremulli, 1995). Those two mitochondrial translation initiation factors are mtIF2 and mtIF3. It is believed that mtIF2 can replace IF1 through a 37 amino acid insertion and then assume the role of both IF1 and IF2 found in bacteria.(Gaur et al., 2008). As for the other necessary translation factor in humans, mtIF3, it is involved in initiation of translation by binding the fMet tRNA and contributes to subunit dissociation (Christian & Spremulli, 2009). Upon successful initiation, mitochondrial translation elongation factor (TUFM) recognizes properly aminoacylated tRNA molecules and delivers them to the ribosome in a complex with the GTP necessary for translocation (Hunter & Spremulli, 2004). The interaction is important for the integrity of the translational process (Nagao, Suzuki, & Suzuki, 2007). The importance of TUFM can be seen in

studies showing that a single mutation in the TUFM gene can cause infantile encephalopathy from decreased mitochondrial translation (Valente et al., 2007b).

3. Specific Aims

Mitochondrial dysfunction occurs specifically in metabolic disease states such as in a high-fat diet, obesity and diabetes (Chanséaume & Morio, 2009; Mantena et al., 2009). The mitochondria is a vital structure, and its function is crucial to proper cell signaling, maintenance, and energy metabolism. The processes involved in mitochondrial regulation and function are numerous, and many of the specific signaling pathways have yet to be determined. However, it is through these signaling pathways and protein interactions that mitochondrial function takes place. The transcriptional co-activator peroxisome proliferator-activated receptor γ co-activator 1 alpha (PGC-1α) is the master regulator of mitochondrial biogenesis in skeletal muscle (Puigserver et al., 1998; Wu et al., 1999). This protein is highly expressed in oxidative skeletal muscle such as the soleus muscle, and, in an obese state, PGC-1α expression is lower than in lean subjects (Greene et al., 2014).

Factors that contribute to regulation of mitochondrial biogenesis and oxidative phosphorylation include cytochrome c oxidase subunit IV(COX-IV), transcription factor of activated mitochondria (TFAM), mitochondrial translation initiation factor 2 (mtIF2), and mitochondrial translation elongation factor (TUFM). COX-IV is a marker of mitochondrial density and has been shown to increase in obese subjects, it has been hypothesized that this because of impaired autophagy or due to mitochondrial biogenesis with the former being the current assumption (Greene et al., 2014).TFAM is a transcription factor that functions in the same pathway as PGC-1α, and is a marker of mitochondrial biogenesis. TFAM is critical in the process of mitochondrial transcription. mtIF2 and TUFM and both nuclear encoded proteins that are translated in the cytoplasm and are subsequently translocated into the mitochondria. They are vital pieces of the process of translation in the mitochondrial matrix, and normal regulation of both mtIF2 and TUFM have been deemed to be necessary for mitochondrial translation (Gaur et al., 2008; Valente et al., 2007a).

Aim 1. To examine the effect of leucine supplementation on proteins dealing with mitochondrial biogenesis in high fat diet fed rats. We hypothesize that a high fat diet supplemented with leucine will induce increased mitochondrial content and density via elevated PGC1-α and COX IV levels. We will: 1) Examine the function of leucine on mitochondrial biogenesis through measurement of PGC-1α expression in high fat diet and control groups; 2) Examine the COX-IV content in the soleus muscle of each group as a measure of mitochondrial volume between groups: control, control + leucine, high fat, high fat + leucine.

Aim 2. To examine markers of mitochondrial transcription and translation in rats fed a high fat diet. We hypothesize that a high fat diet supplemented with leucine will induce transcription and translation activity via elevated TFAM, mtIF2, and TUFM levels. Experiments will examine the transcription factors that are co-activated by PGC-1α including TFAM, mtIF2, and TUFM. This will allow investigators to determine the effect of high fat diets on muscle protein synthesis and where this effect occurs. We will use Western blotting to measure protein content of TFAM, mtIF2, and TUFM from the soleus tissue.

We will: 1) Examine mitochondrial transcription by protein expression of TFAM between groups with normal diets and high fat diets; 2) Examine mitochondrial translation by protein expression of TUFM and mtIF2 between groups fed a high fat diet and those fed a high fat diet with leucine.

4. Methods

4.1. *Animals used for testing and diet*

Eight-week-old male Sprague-Dawley rats (Harlan-Teklad), weighing 220 ± 2.0g at the beginning of the experiment, were singly housed in a secure, temperature and humidity-controlled environment. Animals were kept on a 12:12 h reversed light-dark cycle (light 1900 –0700), and were fed commercial pelleted diet and water ad libitum for 9 days. Once the experiment began, rats were randomly assigned into treatment groups (n = 4-5/group): 1) control+ glycine; 2) control + leucine; 3) high fat + glycine; 4) high fat + leucine. Rats were fed macronutrient contents of 60% carbohydrate (CHO), 23.7% protein (PRO), 15.9% fat (control; Table 2) or 16.9% CHO, 23.4% PRO, 60% fat (HF; Table 2) containing 32 g of either glycine or leucine per kg of feed. A customized daily feeding schedule as previously reported by Baum et al. (2006), was used to mimic human eating behavior. Throughout the experimental period, rats had ad libitum access to water (Baum et al., 2006)(Figure 4A and 4B).

Body mass (BM) and food intake were monitored daily. Before breakfast, rats were placed in a container on a scale and measurements were recorded in grams (g). Rats were given a set portion of either a chow diet (4g) or high-fat (3.1g). After 42 days of feeding, rats were sacrificed and tissue was collected. Soleus muscles were removed and snap-frozen in liquid nitrogen. Samples were stored at -80°C for later analysis.

4.2. *Homogenization and Western Blotting*

Tissue was homogenized in Mueller Buffer, and protein concentration was measured using the Qubit 2.0 (LifeTechnologies, Grand Island, NY). Muscle homogenate (30mg/µL) was fractionated into 6-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Gels were transferred over- night to polyvinylidene difluoride (PVDF) membranes. Membranes were Ponceau stained before blotting to verify equal loading of the gels. Membranes were blocked in 3-5% bovine serum albumin (BSA), in Tris-

buffered saline with 0.1% Tween-20 (TBST), for 2 h. Primary antibodies for PGC-1α, TUFM, TFAM, mtIF2, COX-IV (Cell Signaling, Boston, MA, 4850P) were diluted 1:2000–1:8000 in 3-5% BSA or nonfat milk, in TBST, and incubated at 4°C overnight. Anti-goat, and anti-rabbit secondary antibodies(Santa Cruz, Santa Cruz, CA) were diluted 1:1,000 in 3-5% BSA or nonfat milk, in TBST, and then incubated at room temperature for 1 h. Enhanced Chemiluminescence (ECL) was performed using Fluorochem M imager(Protein Simple, Santa Clara, CA) to visualize antibody- antigen interaction. Blotting images were quantified by densitometry using AlphaView software (Protein Simple). The Ponceau-stained membranes were digitally scanned, and the 45-kDa actin bands were quantified by densitometry and used as a protein loading correction factor for each lane.

4.3. *Data Analysis*

The independent factors in this study were diet (C vs. HF), and treatment (leucine vs. no leucine). Dependent variables of interest included protein content of PGC-1α, TFAM, mtIF2, TUFM, and mitochondrial target protein COX-IV. A diet by treatment (2x2) ANOVA was used to analyze data regarding each dependent variable of interest. Statistical significance was set with an alpha value of $p < 0.05$. When significant F ratios are found, a two-tailed Student's *t* test post hoc analysis will be used to distinguish among means. All data will be analyzed using commercial software (SAS, version 9.3, Cary, NC) and all results will be reported as mean ± SEM.

5. Results

5.1. *Body weight and muscle mass*

The rats on a HFD gained significantly more weight ($p < .0001$) than those fed a normal chow diet with values of (409.07 ± 3.01 vs. 434. 52 ± 3.01), respectively (Figure 5A).There was a decrease in the soleus wet weight in NC+Leucine and HF+Leucine groups compared to their respective NC and HF groups ($p<0.05$, Figure 5B). Beginning approximately day 20, HF and HF+Leucine rats weighed significantly more than the NC or NC+Leucine fed rats ($p<0.05$) for the remainder of the trial(434.52±3.01 vs. 409.07±3.01) (Figure 5A). Muscle mass to body mass ratios were not significantly different.

5.2. *Expression concerning markers of mitochondrial biogenesis and content*

This study used PGC-1 α as a marker of mitochondrial biogenesis in skeletal muscle. There was no difference in PGC-1 α protein content in the rats supplemented with leucine on the NC diet compared to rats who were not supplemented. There was a main effect of the HF diet to increase PGC-1 α protein content regardless of leucine supplementation (Figure 5C). This study used COX-IV as a marker of mitochondrial content in skeletal muscle. There was no effect of leucine supplementation on COX-IV protein content regardless of leucine supplementation. Diet had no effect on COX-IV protein expression. ($p>0.05$)(Figure 5D).

5.3. *Protein expression of markers involved in mitochondrial translation and elongation*

This study used TUFM as a measure of mitochondrial translation elongation. No differences were found in the expression of TUFM with regard to leucine supplementation. There was a main effect of high fat diet to increase expression of TUFM in groups fed a HF diet when compared with those fed a NC diet (Figure 5E). This study used mtIF2 as a measure of mitochondrial translation initiation in skeletal

muscle. No significant differences were found in the expression of mtIF2 with regard to leucine supplementation. There was a main effect of NC diet to increase mtIF2 expression when compared with those fed a HF diet ($p<0.05$) (Figure 5F).

6. Discussion

The goal of this study was to examine the role of leucine supplementation on mitochondrial biogenesis and biomarkers of mitochondrial transcription and translation on rats fed a high fat diet. It is important to note that these rats were not in fact obese at the time of testing; they were becoming obese. However, they were gaining fat mass due to their high fat diet (60% fat). Additionally, the focus of this study was to determine if leucine would counteract the detrimental effects a high fat diet has on markers of mitochondrial quantity, biogenesis, and translation. We hypothesized that a high fat diet supplemented with leucine would yield increased mitochondrial biogenesis and content via PGC-1α and COX-IV. We also hypothesized that a high fat diet supplemented with leucine would yield increased mitochondrial transcription and translation via TUFM, and mtIF2.

6.1. *Mitochondrial Content and Biogenesis*

Peroxisome proliferator-activated receptor-γ coactivator -1α is the "master regulator of mitochondrial biogenesis (Puigserver et al., 1998). PGC-1α functions through many transcriptional coactivators, and is a measure of mitochondrial biogenesis. In this study, we found that there was an increase in PGC-1α expression in the group of rats fed a high fat diet. This is congruent with the literature in that Greene et al. have found that PGC-1 α protein expression is increased in obese rats (Greene et al., 2014). It may seem counter intuitive that PGC-1 α would be upregulated in both obese animals and those fed a high fat diet. PGC-1 α, all expression being normal, should increase the overall oxidative capacity of the skeletal muscle due to an increase in mitochondrial biogenesis through activation of transcription pathways (Finck & Kelly, 2006). Increased PGC-1 α is also in line with findings from Holloway et al. that showed a loss of association between PGC-1α and fatty acid oxidation in obese humans (Holloway et al., 2008). Additional explanation for this finding can also be due to dysregulation of PPARδ. PPARδ is a fatty acid sensor and is key in protecting against reduced mitochondrial oxidation

from fatty acid-induced dysfunction (Ravnskjaer et al., 2010).PGC-1 α was being upregulated in the high fat diet group, but it is a coactivator of many transcriptional factors including transcription factor of activated mitochondria(TFAM), nuclear respiratory factors (NRF-1 and NRF-2) and PPARs (Finck & Kelly, 2006; Puigserver & Spiegelman, 2003). Due to PGC-1 α being a coactivator of many other transcription factors, it may be that there is a decrease in the other ligands such as PPARγ and TFAM in the PGC-1 α pathway leading to mitochondrial dysregulation. The conclusion therefore is that in this situation, there are other factors that are at play in regulation of mitochondrial biogenesis other PGC-1 α. While PGC-1 α is the most important regulator of mitochondrial biogenesis, it appears that there are outside factors as well.

COX-IV is a measure of mitochondrial content and density. This indicates that the mitochondrial density of the groups is not significantly different from one another, and that leucine does did not have an effect on COX-IV expression. This finding does come with some interest though. This study showed that PGC-1α, a marker of mitochondrial biogenesis, is upregulated with high fat diets. Biogenesis should necessarily create more mitochondria and increase COX-IV. There may be no change in the expression of COX-IV at the protein level, but what the mitochondria is doing with this protein is important. During a high fat diet (60% fat as in this study) Yuzefovych et al. have shown that there is an increase in the release of COX-IV into the cytosol. This release of COX-IV, a mitochondrial encoded protein, into the cytosol is indicative of apoptosis. Therefore, the overall measure of COX-IV content is not significantly different between groups based upon diet or leucine supplementation, but differing regulation of COX-IV may be more of an issue than solely expression (Yuzefovych, Musiyenko, Wilson, & Rachek, 2013).

6.2. *Mitochondrial Transcription and Translation*

Mitochondrial translation elongation factor, or TUFM, aids in elongation after the mRNA transcript is attached to the ribosome (Smits et al., 2010). This study showed that there was a main

effect increasing the expression of TUFM in a high fat diet. This finding is consistent with literature regarding rats fed a high fat diet in another study done by Gutierrez-Aguilar et al. measuring an increase in the expression of TUFM in skeletal muscle (Gutierrez-Aguilar, Kim, Woods, & Seeley, 2012). An additional study from Greene et al. have shown an increase in TUFM mRNA expression in obese animals as well (Greene et al., 2014). To the current date, TUFM is the main protein involved in regulation of translation elongation in the mitochondrial genome, and is vital to proper function.

The expression of mitochondrial translation initiation factor (mtIF2) showed no difference with regard to leucine supplementation. mtIF2's role is to bring the mRNA transcript to the small subunit of the ribosome and initiate translation. This study showed that mtIF2 expression was higher in the NC group when compared with the HF group. In a previous study Davis et al. showed an increase in the expression of mtIF2 expression with regard to mRNA in high fat diets (Davis et al., 2014).While gene expression does not always lead to protein expression, this novel finding is one that leads to the conclusion that there is a dysfunction with regard to the translation of mtIF2 preventing it from being expressed as in the normal diet group. An increase in mtIF2 mRNA expression in a high fat diet is the cell signaling for more mtIF2 to be generated. However, this study showed that the relative mtIF2 protein expression on a high fat diet is less than the expression from that of the NC diet. It is from this that the conclusion can be drawn that there are other signaling pathways or factors involved in preventing the mtIF2 mRNA from being translated.

Based on these results, further areas of interest and research can focus on specifically mitochondrial biogenesis and translation as these areas saw a significant change from the NC group with regard to diet. While overall it did not appear that leucine had an effect on mitochondrial biogenesis, content, or translation, there was a trending relationship between it and biogenesis in this study ($.06<p<.065$).

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Figure 4A

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, diabasic	0	0	3.4	3.4
TBHQ	0.14	0.14	0	0
Glycine	32	0	32	0
Leucine	0	32	0	32

Figure 4B

Macronutrient Component	Control Group		High-Fat Group	
	Kcal/Kg	% Energy	Kcal/Kg	% Energy
Carbohydrate (CHO)	2390	60	872	16.9
Protein (PRO)	940	23.7	1204	23.4
Fat	630	15.9	3060	60

Figure 5A

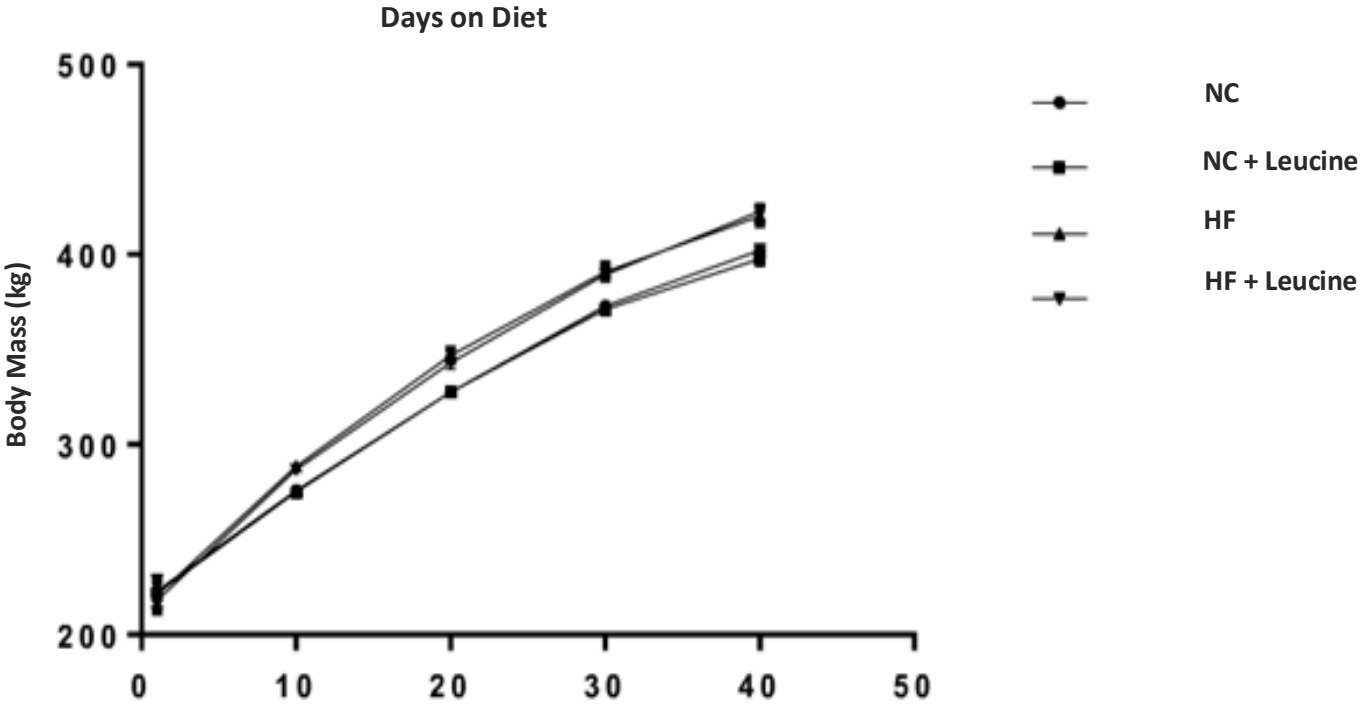


Figure 5B

	Normal Chow		High Fat	
	No Leucine	Leucine	No Leucine	Leucine
Soleus (mg)	166.3 ± 2.4	161.2 ± 3.1	180.2 ± 2.9	173.8 ± 2.8
Body Mass (g)	408.1 ± 3.5	410.1 ± 3.9	433.9 ± 4.7	435.2 ± 4.8
Muscle Mass/Body Mass (mg/g)	0.41 ± 0.01	0.39 ± 0.006	0.42 ± 0.004	0.40 ± 0.006

Note. Data are means ± SEM. The HFD contained 60% fat.

Figure 5C

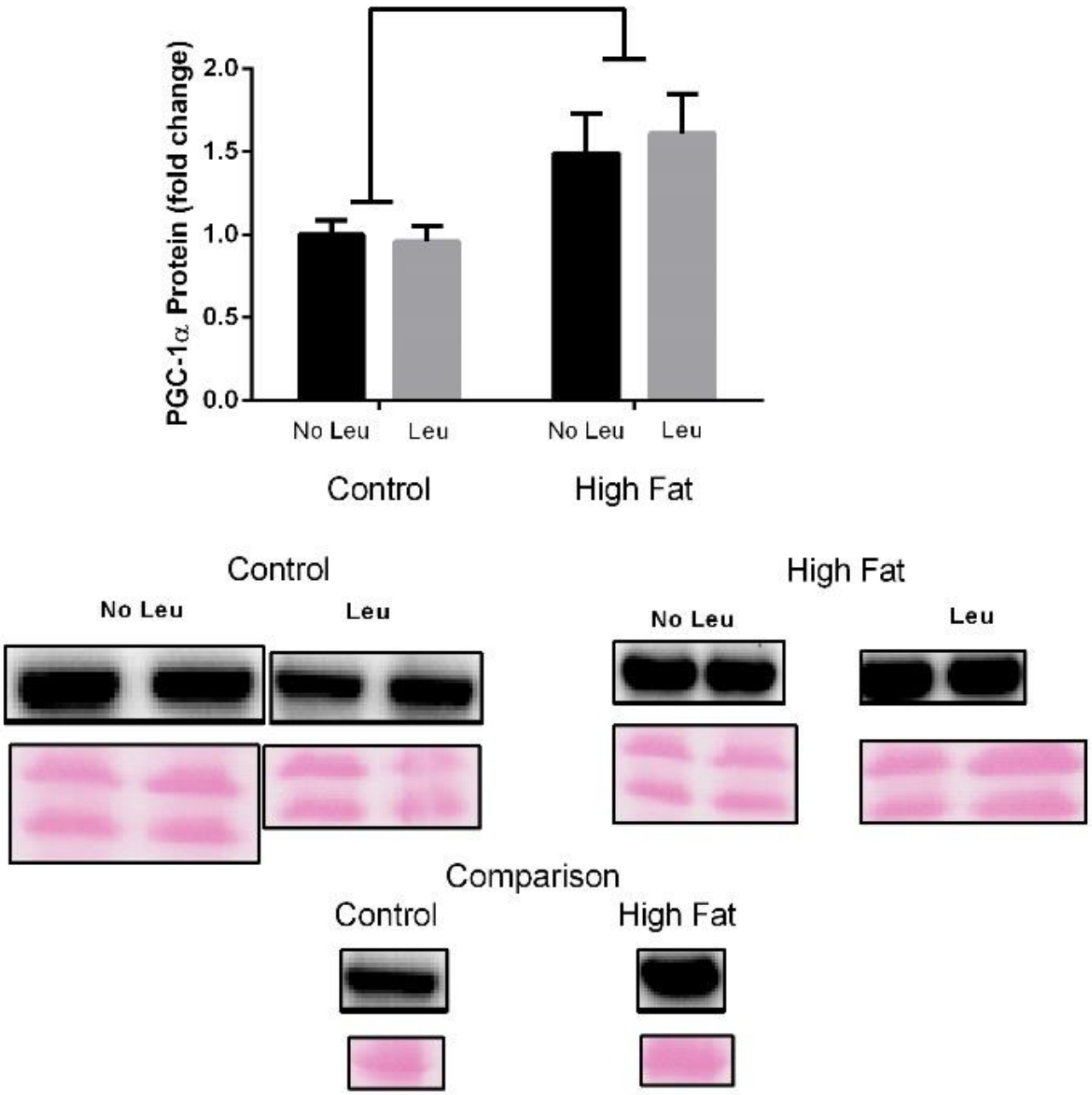
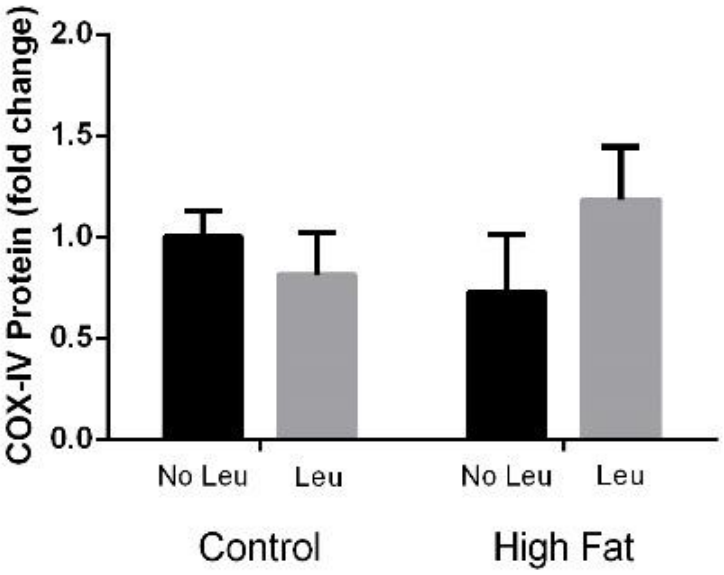

PGC-1α Protein (fold change)
2.0
1.5
1.0
0.5
0.0
No Leu
Leu
No Leu
Leu
Control
High Fat
Control
High Fat
No Leu
Leu
No Leu
Leu
Comparison
Control
High Fat

Figure 5D


COX-IV Protein (fold change)
2.0
1.5
1.0
0.5
0.0
No Leu
Leu
No Leu
Leu
Control
High Fat

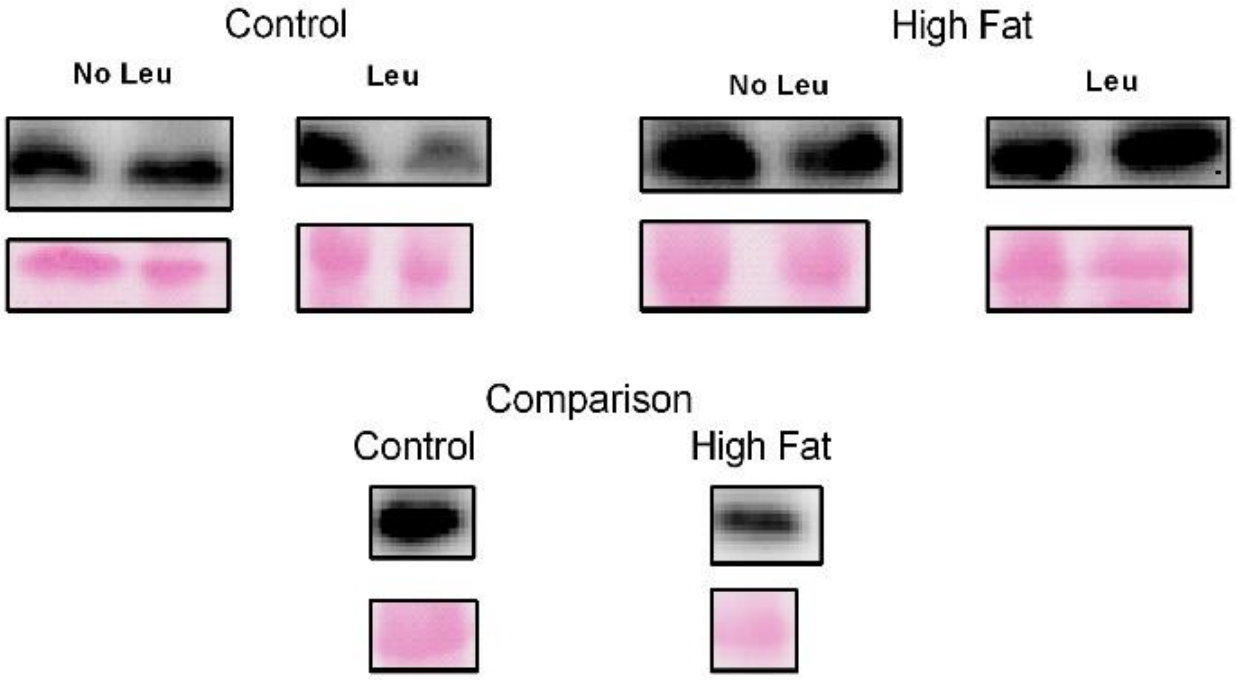

Control
No Leu
Leu
High Fat
No Leu
Leu
Comparison
Control
High Fat

Figure 5E

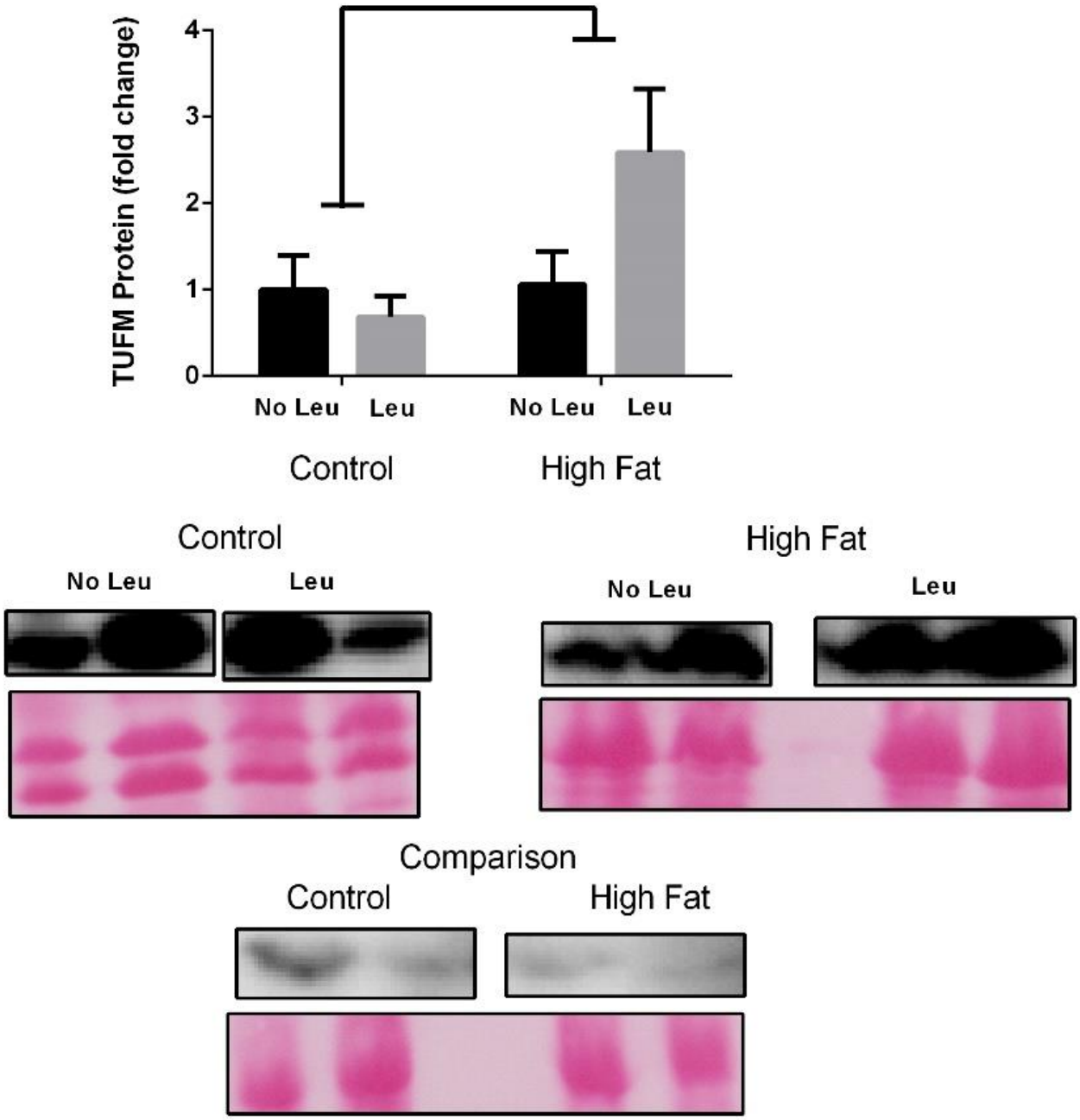
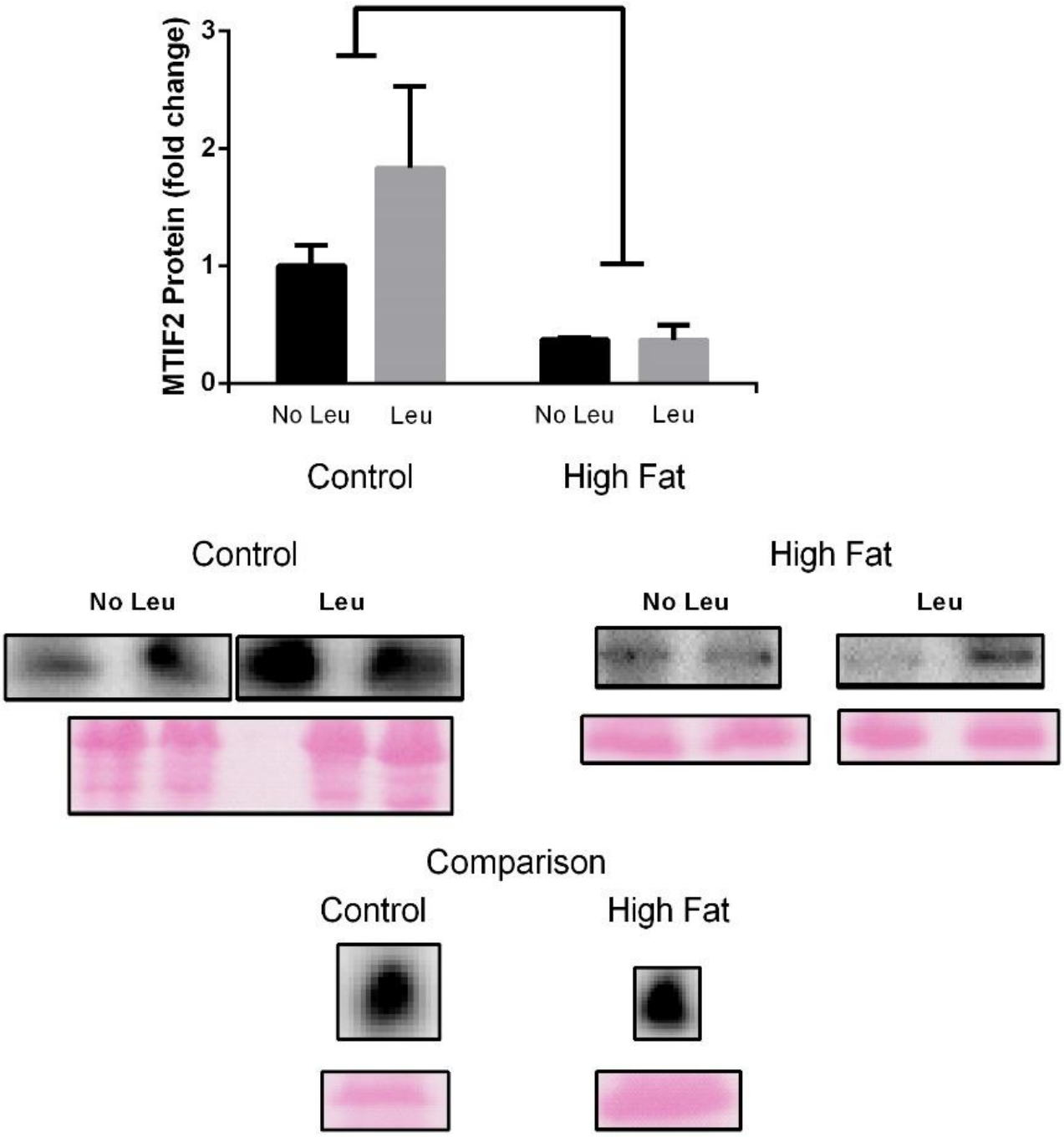

TUFM Protein (fold change)
4
3
2
1
0
No Leu
Leu
No Leu
Leu
Control
High Fat
Control
No Leu
Leu
High Fat
No Leu
Leu
Comparison
Control
High Fat

Figure 5F


MTIF2 Protein (fold change)
3
2
1
0
No Leu
Leu
No Leu
Leu
Control
High Fat
Control
High Fat
No Leu
Leu
No Leu
Leu
Comparison
Control
High Fat