ABSTRACT

Effect of Pre-Exercise Nutrition on Human Skeletal Muscle UCP-3 Expression

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Uncoupling proteins have been proposed as potential therapeutic target for treating obesity and its related diseases such as type II diabetes mellitus. Free fatty acids (FFA) have been shown to be a possible regulator of UCP-3 expression in skeletal muscle, however, studies have shown conflicting results. Using a crossover design, untrained participants performed an endurance exercise session (350 kcal working at 70% of their VO_{2peak}) after two experimental conditions, after the consumption of a multi-macronutrient shake or after a fasting period of 8 h. Results show a significant increase in FFA in the fasting condition as well as a significant increase in UCP-3 mRNA and protein expression at 1 h and 4 h respectively in the shake condition. It is concluded that UCP-3 mRNA and protein expression might be controlled in a stronger way by factors other than FFA. Variables like the protein and fat content from the multi-macronutrient shake, as well as the insulin levels in the shake condition, could have played a key role in the significant increase in UCP-3 mRNA and protein expression in the shake condition; however, further research is needed to confirm this hypothesis.

Effect of Pre-Exercise Nutrition on Human Skeletal Muscle UCP-3 Expression

by

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A Thesis

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ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BMI	Body Mass Index
CPT-1	Carnitine Palmitoyltransferase I
EPOC	Excess Post-Exercise Oxygen Consumption
ETC	Electron Transport Chain
$FADH_2$	Reduced Flavin Adenine Dinucleotide
FEE	Factorial Energy Expenditure
FFA	Free Fatty Acids
HIRT	High-Intensity Interval Resistance Training
NADH	Reduced Nicotinamide Adenine Dinucleotide
OD	Optical Density
O_2	Oxygen
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PRM16	Protein β and PR Domain Containing 16
RMR	Resting Metabolic Rate
RT-PCR	Real Time Polymerase Chain Reaction
UCP	Uncoupling Protein
UCP-1	Uncoupling Protein-1
UCP-3	Uncoupling Protein-3
WAT	White Adipose Tissue

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DEDICATION

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CHAPTER ONE

Introduction

According to the World Health Organization (2014), obesity rates have nearly doubled since 1980, increasing the prevalence of chronic comorbidities and causing around 2.8 million of deaths per year (Hall et al., 2010; Kenchaiah et al., 2002). Among other important factors, obesity development is believed to be generated mainly by energy unbalances, whether through increased energy consumption, decreased energy expenditure or both (Denova-Gutierrez et al., 2010; Pereira-Lancha, Campos-Ferraz, & Lancha, 2012; Wirfalt et al., 2001). Nutrition and physical activity are environmental factors that play a vital role in health status maintenance. Both are part of the first line of obesity treatment. It has been shown that the decrease of domestic duties, along with the increase in sedentary activities has considerably lowered the actual energy expenditure. According to Speakman and Selman (2003), in order to achieve the energy expenditure of our primitive ancestors, it would be necessary for humans to engage in 90 min of intense physical activity daily (Speakman & Selman, 2003). Moreover, it has been shown that high levels of physical activity decrease mortality rates, an effect that is further amplified when the exercise is performed at high intensity (Samitz, Egger, & Zwahlen, 2011). The physiological effects of physical activity have been known for decades; however, it was not until some years ago that the biochemical and molecular effects started to receive special attention from scientists. One of the genes affected by exercise is the gene controlling uncoupling proteins (UCP). UCPs are mitochondrial proteins that dissipate the inter-membrane electrochemical potential as heat by transporting protons into the

mitochondrial matrix (Busiello, Savarese, & Lombardi, 2015). UCPs are classified in five isoforms, UCP-1, UCP-2, UCP-3, UCP-4 and UCP-5 (Adams, 2000). Of these, UCP-3 is the predominant isoform expressed in skeletal muscle (Busiello et al., 2015; Yoshitomi, Yamazaki, Abe, & Tanaka, 1998). This mitochondrial protein has been shown to protect against reactive oxygen species (ROS), lipid peroxidation, FFA accumulation in skeletal muscle and insulin resistance, at the same time of increasing metabolic rate in skeletal muscle (Busiello et al., 2015; Choi et al., 2007). Two of the transcription factors that have been proposed as important regulators of UCP expression activation are PGC1-α and PPARα, however, evidence is lacking (Azzu, Jastroch, Divakaruni, & Brand, 2010; Huh et al., 2012). Both transcription factors play a central role in the response to external stressors, such as fasting and exercise (Cluberton, McGee, Murphy, & Hargreaves, 2005; Leone, Weinheimer, & Kelly, 1999; Pilegaard, Ordway, Saltin, & Neufer, 2000). Exercising at fasting conditions induces UCP-3 mRNA expression in skeletal muscle, which is decreased by glucose consumption before and during the exercise session. The explanation to this effect was a possible involvement of free fatty acids (FFA) controlling UCP-3 expression, nevertheless, conflicting results have been found (Kusuhara, Tobe, Negoro, & Abe, 2005; Noland et al., 2003; Schrauwen et al., 2002). Additionally, it seems that the consumption of diets with different macronutrient composition seem to have the opposite effect on exercise-induced UCP-3 mRNA expression in skeletal muscle. While both high-protein and high-fat diets have shown to increase UCP-3 expression, a high-carbohydrate diet has been shown to have the opposite effect (Brun et al., 1999b; Frier, Jacobs, & Wright, 2011; Petzke, Riese, & Klaus, 2007). Until now studies assessing the effect of exercise on UCP-3 expression in skeletal muscle have used

only glucose as energy source. Additionally, the glucose provided was consumed both before and during the exercise session (Cluberton et al., 2005; Schrauwen et al., 2002). Most individuals typically ingest multi-nutrient meals prior to exercise. As a result, ingesting only carbohydrates as the sole energy source during an exercise session might not represent a typical, practical scenario. Assessing the effect of a multi-macronutrient shake right before the exercise seems to be a more suitable approach to better understand the involvement of the transcription factors PGC1-α and PPARα, as well as the effect of FFA on UCP-3 expression.

Purpose of the Study

The primary purpose of the study is to assess the effect of pre-exercise nutrition on human skeletal muscle UCP-3 mRNA and protein expression. The secondary goal of the study is to assess the mRNA expression and possible involvement of the transcription factors PPARα and PGC-1α on UCP-3 expression.

Hypotheses

H_1: Overall, there will be a significantly higher glucose concentration in the shake condition.

H_2: There will be a significant increase in serum glucose concentration after the shake ingestion.

H_3: There will be a significantly higher serum FFA concentration in the fasting condition.

H_4: There will be a significantly higher PPARα mRNA expression in the fasting condition.

H_5: There will be a significant increase in PPARα mRNA expression after exercise in both conditions.

H_6: There will be a significantly higher PGC-1α mRNA expression in the fasting condition.

H_7: There will be a significant increase in PGC-1α mRNA expression after exercise in both conditions.

H_8: There will be a significantly higher UCP-3 mRNA expression in the fasting condition.

H_9: There will be a significant increase in UCP-3 mRNA expression after exercise in both conditions.

H_{10}: There will be a significantly higher UCP-3 protein levels in the fasting condition.

H_{11}: There will be a significant increase in UCP-3 protein levels after exercise in both conditions.

Delimitations

- Twelve male participants between the ages of 18-30 years who were recreationally active (involved in < 3 h per week of endurance exercise in the last 6 months) participated in the study.
- Participants from all ethnicities were recruited from Baylor and surrounding areas.
- Participants were excluded from the study if they consumed supplements other than vitamins within the past 6 months.
- Participants were allowed to join the study only if they were considered to be at low to moderate risk for cardiovascular disease, with no contradictions to exercise according to the American College of Sports Medicine.
- Eligible participants were cleared to participate in the study after completing a mandatory medical screening process performed in the Exercise Nutritional Biochemical Laboratory (EBNL) and the Baylor Laboratory for Exercise Science and Technology (BLEST) and after providing informed consent in accordance with the Institutional Review Board for human subjects and the Helsinki Code.

Limitations

- Since this study involved a convenience sampling within the city of Waco, as well as the small simple size (n = 12), the age range (18-30 years) and the gender selection, external validity may be decreased; although, it is unlikely to be a significant concern.
- Each participant likely had different circadian rhythms that might interfere with the hormonal response to exercise. Effect that was minimized by testing participants during the morning hours (7:00 – 10:00 am).
- The training status might cause a different response to exercise. To decrease this variability the selected population was required to perform less than 3 h per week of low to moderate intensity exercise.
- Nutritional intake can modify the gene regulation performed by exercise. To minimize this external variable participants were asked to fill out a 24 h food log before coming to the lab and to maintain a fasting period of 8 h. Moreover, participants were asked to keep a consistent diet throughout the duration of the study.
- Results were limited to the time points at which samples were collected.

Assumptions

- Trained laboratory personnel minimized interpersonal variances in the study protocol.
- Participants performed at their best in the VO_{2peak} testing.
- Participants adhered to guidelines provided at the beginning of the study.

- Participants maintained their dietary habits throughout the duration of the study and adequately completed their 24 h food log before the exercise dates.
- Participants refrained from partaking in any endurance or resistance exercise 48 h before each exercise session.
- Participants slept adequately (7 - 8 h) before each testing session.
- Volunteers arrived at each testing session after a fasting period of 8 h.
- Participants truthfully answered all questions regarding medical history, nutritional supplement and drug consumption, exercise training status, exercise performed during the duration of the study, and sleeping patterns.

Definitions

DEXA is the gold standard method for body composition screening. It uses two X-ray beams of different energy levels. It is a rapid (6-7 min) and relatively inexpensive test that requires a low dose radiation (10% of a chest radiograph) (Rowe, 2005).

mRNA are cytoplasmic molecules that transfer genetic information from the DNA to the protein synthesis machinery. They are formed by multiple changes performed on the pre-mRNA molecule, which is from 10 to 50 times longer than the mature mRNA molecule (Murray et al., 2009).

Myokine is a cytokine produced and secreted by skeletal muscle that can affect downstream cytokine-mediated signaling pathways (Makowski, 2012).

Thermogenesis is the heat production resulting from the presence of uncoupling proteins (thermogenin) in the inner mitochondrial membrane (Murray et al., 2009).

Total Energy Expenditure is formed by Resting Energy Expenditure (REE) plus Thermic Effect of Food (TEF) and Energy Expenditure during Physical Activity. It is usually measured by using oxygen consumption and carbon dioxide production (indirect calorimetry) (Gropper & Smith, 2008).

UCP from uncoupling proteins are mitochondrial inner-membrane anion carriers that decrease the electrochemical gradient in the inter-membrane space (Busiello et al., 2015).

VO_{2peak} is a common test accepted as a criterion of cardiorespiratory fitness level. It is the product of the maximal cardiac output Q (liters of blood per minute) and arterial-venous oxygen difference (mL of oxygen per L of blood), and it is expressed in mL/kg/min. It can be calculated directly by measuring oxygen consumption or indirectly by using heart rate (Thompson, Medicine, Gordon, & Pescatello, 2010).

CHAPTER TWO

Literature Review

Obesity Rates and Physical Activity

Unites States has one of the highest obesity rates in the world. Approximately 34.4% of the American population is considered to be within the obesity range (McGuire, 2011; Preston & Stokes, 2011). According to the results of the analysis performed by Preston and Stokes (2011), no other country is estimated to gain as much benefit from the elimination of obesity as the United States. A proper program of physical activity and healthy nutrition are known as the first line of treatment against obesity. The actual recommendation of physical activity is 2.5 h of moderate aerobic exercise per week with muscle-strengthening activities on at least two days per week (CDC, 2011). According to Samitz and colleagues (2011), the mortality reduction for this amount of physical activity (150 to 300 min per week of moderate to vigorous intensity) would be about 14 to 26%.

Role of Exercise on Energy Expenditure

During exercise, the increased metabolic demands produce a higher rate in the respiratory chain and the oxidative phosphorylation machinery to improve adenosine triphosphate (ATP) production. The mitochondrial electron transport chain (ETC) moves electrons from an electron donor (e.g. reduced nicotinamide adenine dinucleotide or reduced flavin adenine dinucleotide, NADH and $FADH_2$ respectively) to a terminal electron acceptor (Oxygen, O_2), while simultaneously pumping protons from the matrix to the intermembrane space. These coupled reactions produce a gradient potential that

will be further utilized to produce ATP and H_20 (Murray et al., 2009). The O_2 consumption by the mitochondria in order to form H_20 molecules is the reason why it is commonly stated that the real respiratory machinery is not located in the lungs but within the mitochondria itself. This would indicate that the higher the ATP demand, the greater the necessity of O_2, which would consequently increase the breathing frequency during physical activity. In addition, once the exercise session is finished, the increased O_2 consumption would continue during the post-exercise phase. In the past, it was believed that this excess in O_2 consumption was caused by glycogenesis from the lactate produced during physical activity. However, it seems that there is no such relationship between lactate metabolism and O_2 consumption after exercise. According to Gaesser & Brooks (1984), less than 20% of the lactate is re-converted to glycogen while around 55 to 70% is oxidized, which would indicate that oxidation is the primary post-exercise pathway of exercise-produced lactate. Also, it has been shown that the rest of the O_2 consumed after exercise is explained by numerous factors such as: recovery from decreased muscle glycogen levels, creatine-phosphate restoration, increased sympathetic tone and circulation of stress hormones (Gaesser & Brooks, 1984; Knab et al., 2011). In fact, Gaesser and Brooks (1984) proposed to change the terms "lactacid", "alactacid" and "O_2 debt" to "excess post-exercise oxygen consumption" (EPOC). An increased EPOC means that there is extra energy consumption above the resting metabolic rate after the cessation of the exercise session. According to "The Institute of Medicine," when calculating the energy expenditure of physical activity, an additional 15% of the actual energy consumption during the exercise session should be added due to EPOC effects (IOM, 2005). This adjustment to the factorial energy expenditure (FEE) formula has been shown

to be more effective than the estimation formula alone (Warwick, 2006). It is worth noting that both aerobic and resistance exercise have been shown to increase energy expenditure, during both the exercise session and the post-exercise/recovery phase (Kirk et al., 2009; Knab et al., 2011; Paoli et al., 2012; Sevits et al., 2013).

Effect of Aerobic Exercise on Energy Expenditure

Using a metabolic chamber, Knab et al. (2011) demonstrated a 37% increase in the energy expenditure during the post-exercise phase of a cycling session. In other words, burning around 519 kcal per exercise session caused an increase of 190 kcal in the recovery phase, generating a final energy expenditure of around 700 kcal per day (Knab et al., 2011). Intensity and duration of the exercise session are critical factors determining energy expenditure after exercise. A single bout of sprint (225 kcal) has shown to increase energy expenditure up to 2 to 3 h post-exercise while a 45 min cycling session at 70% of $VO_{2\ max}$ caused a rise in the energy expenditure of up to 14 h in the recovery phase (Knab et al., 2011; Sevits et al., 2013). As such, it can be suggested that the larger duration and higher intensity of the exercise session may elevate the post-exercise energy expenditure.

Effect of Resistance Exercise on Energy Expenditure

Resistance training may also prevent weight gain because of its long-term increase in energy expenditure. Kirk et al. (2010) showed a significant increase in resting metabolic rate (120 kcal) after a training period of 6 months in a group of sedentary, overweight, young adults. These effects occurred even when the volume of the training program was relatively low at 11 min per session with a frequency of three times per

week (Kirk et al., 2010). Importantly, the increase in metabolic rate was not taken in the post-exercise period but 72 h after the last exercise session. This indicates that resistance training is able to increase the energy expenditure during resting conditions and not just during the recovery phase as endurance exercise does (Kirk et al., 2009; Scharhag-Rosenberger, Meyer, Walitzek, & Kindermann, 2010). This could be the result of an increased fat free mass within the resistance-training group (Kirk et al., 2010). In resistance exercise, intensity also plays a crucial role. Paoli et al. (2012) observed a 5% increase in the resting metabolic rate on a traditional resistance-training group after 22 h of the last exercise session while a 23% increase was observed in the high-intensity interval resistance training (HIRT) group even when the duration of the exercise sessions of the last group were shorter (62 versus 32 min in the traditional and HIRT training groups, respectively). Likewise, it was demonstrated that when aerobic exercise is performed after 24 to 48 h of unaccustomed resistance exercise, the energy expenditure increases 13% above a normal aerobic session. This could be the result of increased re-synthesis of damaged muscle fibers or increased muscle fibers recruitment to couple the metabolic demands (Burt, Lamb, Nicholas, & Twist, 2014). In summary, it seems that the combination of both resistance and endurance exercise might be the best strategy to increase metabolic rate in the short and long term setting.

Nutrition and the Metabolic Response to Exercise

The nutrition before and after the workout has shown to modify the gene expression normally affected by exercise. To this respect, it has been demonstrated that a low carbohydrate diet within 48 h after a glycogen-depleting exercise can lead to a twofold increase in FFA concentrations along with increases in fatty acid translocase,

UCP-3, carnitine palmitoyltransferase-1 (CPT-1), β-hydroxyacyl-CoA dehydrogenase and hormone sensitive lipase (Arkinstall, Tunstall, Cameron-Smith, & Hawley, 2004). On the other hand, a high-carbohydrate diet in the post-exercise period can lead to a 300% increase in muscle glycogen content, along with increases in the transcription of the glucose transporter isoform 4 and glycogenin (Arkinstall et al., 2004). The above results could be used in either way: 1) a high-carbohydrate diet in the post-exercise period could be useful for athletes and people engaged in endurance sports in order to improve their performance, while, 2) a low carbohydrate diet in the post-exercise period could be used if an increase in energy expenditure is the desired result.

White and Brown Adipose Tissues

Adipose tissue is a major metabolic organ traditionally classified as either white (WAT) or brown (BAT) adipose tissue. WAT and BAT cell types are anatomically different and have antagonistic functions. On one side, WAT is characterized by single lipid spherical droplets with low mitochondria concentration and almost undetectable UCP-1 content. In contrast, BAT is characterized by multiple small vacuolae, abundant mitochondria, high UCP-1 content and higher capillary and nerve supply than WAT, thus, producing the brown color. The UCP content on the internal membrane of the mitochondria can completely change its function. The mechanism through which mitochondria normally works is by letting the protons flow down the mitochondrial membrane through the ATP synthase. However, when the protons run back to the matrix through UCP (without ATP formation), the stored energy is dissipated as heat. While WAT function is mainly to store the energy consumed in excess, BAT function is to dissipate energy through heat production. It is interesting to note that besides being

derived from the same mesenchymal stem cell, WAT and BAT have different cell precursors. While WAT is originated from an adipogenic lineage, BAT is derived from the myogenic factor 5 lineage, the same precursor of myocytes. The transformation of this myogenic factor into BAT has shown to be controlled by the growth factors: bone morphogenetic protein-7 and myostatin, as well as the transcriptional factors: CCAAT/enhancer binding protein β and PR domain containing 16 (PRDM16). Brown adipocytes and muscle cells are specialized in catabolism rather than anabolism, both cells are sympathetically innervated, with high amounts of mitochondria and possess adaptive thermogenesis (Saely, Geiger, & Drexel, 2012). It was not until some years ago that BAT was still being considered as irrelevant in adults. However, new research has shown the positive correlation between BAT and energy expenditure, as well as the negative correlation between BAT and body fat percentage. As such, new research is being done in this area with BAT becoming in a major attractive target for obesity treatment. Synthetic chemicals that activate the transcription factor PRDM16 or produce a similar action are also of potential interest as anti-obesity drugs (Saely et al., 2012).

Beige Adipose Tissue

Recently, a new type of adipose tissue was identified, beige adipose tissue also known as brite or brown such as adipose tissue. This new adipocyte type resembles WAT at basal conditions, but under certain stimulation is able to increase heat production and thus energy expenditure by changing its gene expression. The adult human body has little depots of BAT, located mainly in the inter-capsular, axillary, paravertebral, and peri-renal areas. However, the discovery of this type of adipose tissue responsive to environmental changes that is able to resemble the function of BAT has captured the

attention of scientists. Beige adipocytes are expressed within WAT, which is present throughout the body in two representative locations: visceral WAT and subcutaneous WAT. The former one is located mainly around the organs providing padding, while the latter one is found under the skin and its main purpose is to isolate the body from extreme temperatures. Besides sharing some similarities with WAT at basal conditions and with BAT under β-adrenergic stimulation or cold exposure, beige adipose tissue also possesses its own gene expression pattern. The characteristic genes of BAT are: UCP-1, epithelial V-like antigen 1, pyruvate dehydrogenase kinase isozyme 4, early B-cell factor 3 and heat shock protein B7; while for WAT these genes are: adiponectin, resistin, liproprotein lipase, and glycerol-3-phosphate dehydrogenase and lastly for beige adipose tissue: transmembrane protein 26, tbox1 and short stature homeobox 2 (Park, Kim, & Bae, 2014). The transformation of the beige adipose tissue from large lipid droplets and lack of UCP-1 content into multiple small, multi-locular droplets with high UCP-1 concentration is known as the "browning" of WAT. However, this process is reversible, after the removal of the external stimulus the beige adipose tissue will start a re-conversion into white like cells known as "whitening" (Puigserver et al., 1998).

Mitochondria

Mitochondria are the main regulators of cellular energy. Reduced factors such as $FADH_2$ and NADH, obtained from macronutrients metabolism, provide the electrons needed to reduce O_2 and form water molecules at the fourth complex of the ETC. Along with electron transportation through the ETC, protons are translocated from the matrix to the inner membrane space, thus, creating an increased electrochemical potential that will be later used to produce ATP by the ATPase. Although highly crucial, ATP production is

not perfectly coupled leading to energy lost as heat by the re-entry of protons into the matrix, which will decrease the electrochemical potential. This proton leak is the result of two processes: a basal and an inducible leak. The inducible leak of protons is conducted by membrane proteins such as UCPs (Busiello et al., 2015) (Figure 1).

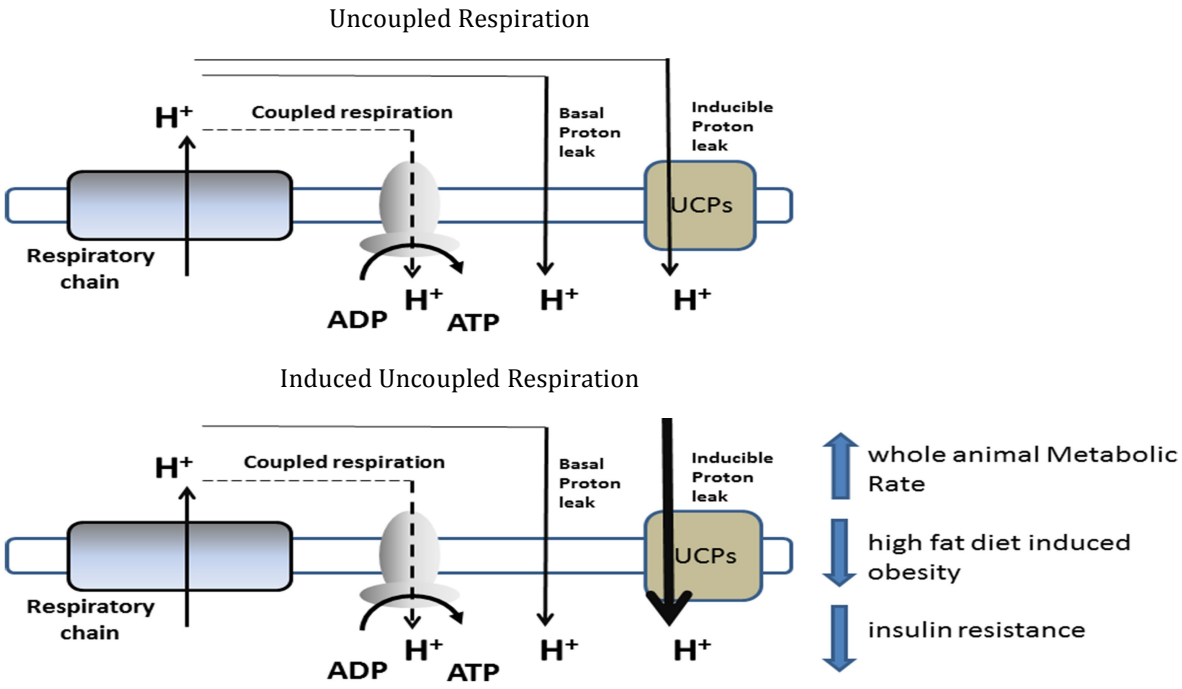


Figure 1. Induced Proton Leak Through UCP. First image shows a normal-basal UCP activity while lower image shows an increased UCP activity. Increased UCP activity by internal and external induction could have the potential to increase metabolic rate, thus, providing resistance to high-fat diet induced obesity and insulin resistance (Image modified from: Busiello et al., 2015)

Molecular Understanding of the Metabolic Response to Exercise

Uncoupling Proteins

Uncoupling proteins are mitochondrial inner membrane anion carriers that decrease the electrochemical gradient in the inter-membrane space without an increase in ATP production. Futile protein conductance produces heat and accounts for 20 to 70% of the resting metabolic rate depending on the cell type (Busiello et al., 2015; Rolfe &

Brand, 1997; Schrauwen et al., 2002). The first uncoupling protein discovered was UCP-1, which is predominantly expressed in BAT, accounting for as much as 10% of the mitochondrial proteins within this tissue. UCP-1 is overexpressed after cold exposure and overeating, having a half-life in the order of hours to days (Busiello et al., 2015). Its ablation in thermoneutral conditions is able to cause obesity even in mice fed a control diet (Feldmann, Golozoubova, Cannon, & Nedergaard, 2009). The second isoform, UCP-2, has shown to cause cytoprotection, immune cell modulation and regulation of glucose sensing in the brain and the pancreas, having a half-life of just 1 h. Lastly, UCP-3 is mainly expressed in skeletal tissue but is also present in BAT and the heart, having a half-life of 1 to 4 h. The described roles for this isoform include protection against ROS and increased FFA oxidation. Initially, it was thought that UCP-3 could play a role in thermogenesis since it is also induced by cold exposure; however, it was later discovered that its absence does not lead to a change in body temperature. Nonetheless, there is evidence that specific UCP-3 deletion from BAT leads to the impairment of the non-shivering thermogenesis. UCP-3 mutations have shown to be associated with significant changes in fat mass within humans (Azzu et al., 2010; Busiello et al., 2015). Transgenic over-activation of UCP-3 has shown to have exercise-like effects, increasing energy expenditure, causing complete fatty acid oxidation, and decreasing oxidative stress (Aguer et al., 2013). Moreover, a positive association between metabolic rate and lifespan has been demonstrated in rodents. Mice in the upper quartile of energy expenditure showed 17% higher O_2 consumption and 36% longer lifespan compared with mice from the lowest quartile. Mitochondria isolated from the group with the highest energy expenditure presented increased proton leak through UCP-3 and adenine

nucleotide translocase (Speakman & Selman, 2003). Another effect that UCP-3 could regulate is glucose sensitivity. The protein levels of UCP-3 in the human vastus lateralis of patients with type 2 diabetes has shown to be 50% of that from their healthy counterparts (Schrauwen et al., 2002). UCP-3 activation is induced by different molecules including FFA, superoxide and lipid peroxidation products. The expression of this mitochondrial protein seems to be controlled by PPARs, thyroid hormone, and the myogenic differentiation 1 protein, as well as by different conditions such as fasting, cold exposure, and a high-fat diet (Azzu et al., 2010). In conclusion, even though increasing thermogenesis might not be the main role of UCP-3 in the cell, its activation by the proper effectors can lead to thermogenesis (Azzu et al., 2010)(Figure 2).

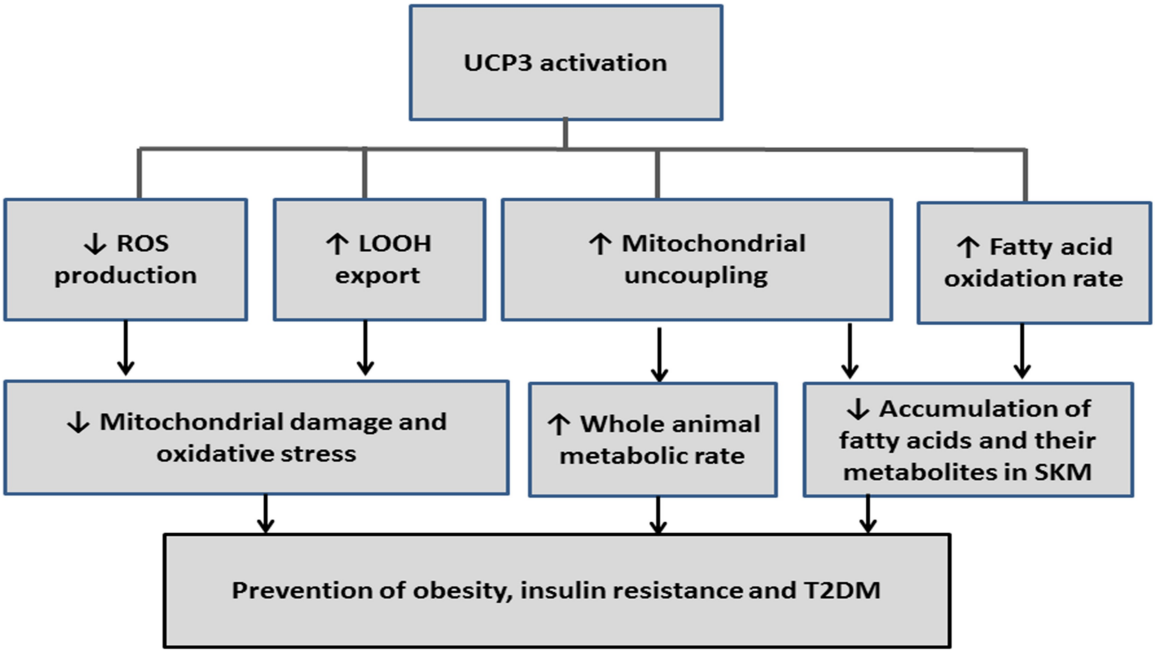


Figure 2. Potential Therapeutic Effect of Uncoupling Protein 3 in Obesity, Insulin Resistance and T2DM Prevention. UCP-3 has shown to 1) decrease mitochondrial damage by providing protection against ROS and transporting LOOH out of the mitochondrial matrix; 2) rise metabolic rate by increasing mitochondrial uncoupling and 3) prevent accumulation of fatty acids in SKM by increasing fatty acid oxidation. Abbreviations: ROS: Reactive Oxygen Species, LOOH: Lipid Hydroperoxide, T2DM: Type 2 Diabetes Mellitus, SKM: Skeletal Muscle (Image retracted from: Busiello et al., 2015).

Peroxisome Proliferator-Activated Receptor Gamma Co-Activator 1 Alpha

PGC-1α is a protein of 795 amino-acids with a molecular mass of 92 kDa that contains three phosphorylation sites for protein kinases. It is mainly expressed on brown adipose tissue, heart, kidneys and brain (Huh et al., 2012; Puigserver et al., 1998). PGC-1α is involved in a considerable number of cellular responses to external energy demanding factors. It controls body fat regulation, the thermogenic response to cold conditions, mitochondrial biogenesis and the expression of the electron transport chain, oxidative phosphorylation and lipogenic proteins (Leone et al., 2005). Its deletion in mice is able to produce a significant increase in body fat percentage that is independent of food consumption or physical activity; moreover, null mice show lower muscle performance and exercise capacity (Leone et al., 2005). PGC-1α is able to co-activate the UCP-1 gene promoter by interacting the transcription factors peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ) (Bostrom et al., 2012; Huh et al., 2012; Puigserver et al., 1998).

Peroxisome Proliferator-Activated Receptor Alpha

PPARs are transcription factors that locally tune gene expression to satisfy metabolic demands and contribute with inter-organ communication (Yessoufou & Wahli, 2010). PPARα is a ligand activated transcriptional factor that belongs to the nuclear hormone receptor family. PPARα possess a wide range of metabolic effects depending on the expressing tissue. In skeletal muscle, a tissue with high PPARα expression, this transcription factor is able to switch substrate utilization from glucose to fatty acids, increasing their uptake and oxidation. In liver, besides increasing fatty acid uptake and oxidation, PPARα raises the production of high-density lipoprotein cholesterol while

subsequently decreasing acute phase reactants and very low-density lipoprotein cholesterol. In starvation, PPARα is able to increase lipolysis in adipose tissue and fatty acid oxidation and insulin sensitivity in pancreas (Yessoufou & Wahli, 2010). Ureido-fibrate-5, a PPARα agonist that is 200-fold more potent than fenofibric acid, was able to decrease serum triglycerides by 70%, as well as inducing a significant increase in mitochondrial CPT-1 expression and β-oxidation in both liver and skeletal muscle (Minnich, Tian, Byan, & Bilder, 2001). Furthermore, it has been demonstrated that PPARα null mice develop myocardial and hepatic steatosis, hypoglycemia, and an inadequate ketogenic response, which demonstrates the important metabolic effect of PPARα in regulating fatty acid oxidation, glycemic control, and the ketogenic response in the fasting condition (Leone et al., 1999). Besides its effect in fatty acid oxidation, PPARα has also shown to be able to control UCP-3 expression. WY 14643, a specific PPARα agonist, has shown to induce UCP-3 expression in a time- and dose-dependent manner, while thiazolidinediones, specific ligands for PPARϒ, does not show any effect in the expression of this mitochondrial protein. It is interesting to mention that WY 14643 treatment does not have any effect on FFA, indicating that, although FFA could play a role in UCP-3 expression, they are not the only mediators regulating its expression (Brun et al., 1999a). Furthermore, it has been shown that AMPK could play a role in PPARα activation. As an example, the 5-aminoimidazole-4-carboxamide ribonucleotide, also known as AICAR, an activator of the AMP-activated protein kinase, has been shown to be able to increase the mRNA expression of PPARα and PGC-1α (Lee et al., 2006).

Free Fatty Acids Role on UCP-3 Expression

A single bout of aerobic exercise increases the adipose tissue expression of PGC-1α, UCP-1 (adipose tissue) and UCP-3 (skeletal muscle), whereas training (3 weeks) does not seem to have an effect on their expression when enough time (12 h) is left between the last exercise bout and the sample collection (Norheim et al., 2014; Pilegaard et al., 2000; Roca-Rivada et al., 2013). Interestingly, it has been proposed that exercise does not directly affect UCP-3 expression in skeletal muscle but rather due to the exercise-induced FFA release. In other words, if enough glucose supply is provided during physical activity, exercise does not have any effect on UCP-3 expression due to a blunted response in FFA levels (Schrauwen et al., 2002). These findings could mean that the longer the exercise duration and the higher the intensity, the more pronounced the effect on skeletal UCP-3 expression could be, as long as energy supply is not being provided. However, further research in this area is necessary in order to elucidate the entire physiologic and metabolic pathway needed to activate UCP expression in both skeletal and adipose tissues.

β-Aminoisobutyric Acid

β-aminoisobutyric acid (BAIBA), a small molecule produced by PGC-1α, is a myokine that has recently been shown to produce browning of white adipose tissue and also increase β-oxidation in hepatocytes by acting through PPARα. *In vitro*, BAIBA treatment has shown to increase mitochondrial respiration and the expression of UCP-1 and cell death-inducing DFFA-like effector A (CIDEA) using cultured adipocytes from WAT. The results have been consistent in vivo, with BAIBA treatment increasing the expression of UCP-1, CIDEA, PGC-1α and cytochrome c in the same tissue. The above

results were accompanied by improvements in glucose tolerance, increased whole body energy expenditure and decreased body fat percentage, effects that occurred without significant modifications in activity or food intake. It seems that BAIBA acts through a PPARα dependent manner since BAIBA treatment on PPARα null mice failed to increase the expression of thermogenic genes in WAT (Roberts et al., 2014). Furthermore, it has been shown that three weeks of exercise training in rodents increases by 25 fold the gene expression of UCP-1 in WAT and by 5 fold the BAIBA concentrations in the gastrocnemius and quadriceps (Roberts et al., 2014). These results were replicated in humans, finding an increase in BAIBA concentrations after 20 weeks of exercise training (Figure 3). Lastly BAIBA concentration in humans is inversely associated with metabolic risk factors (Roberts et al., 2014).

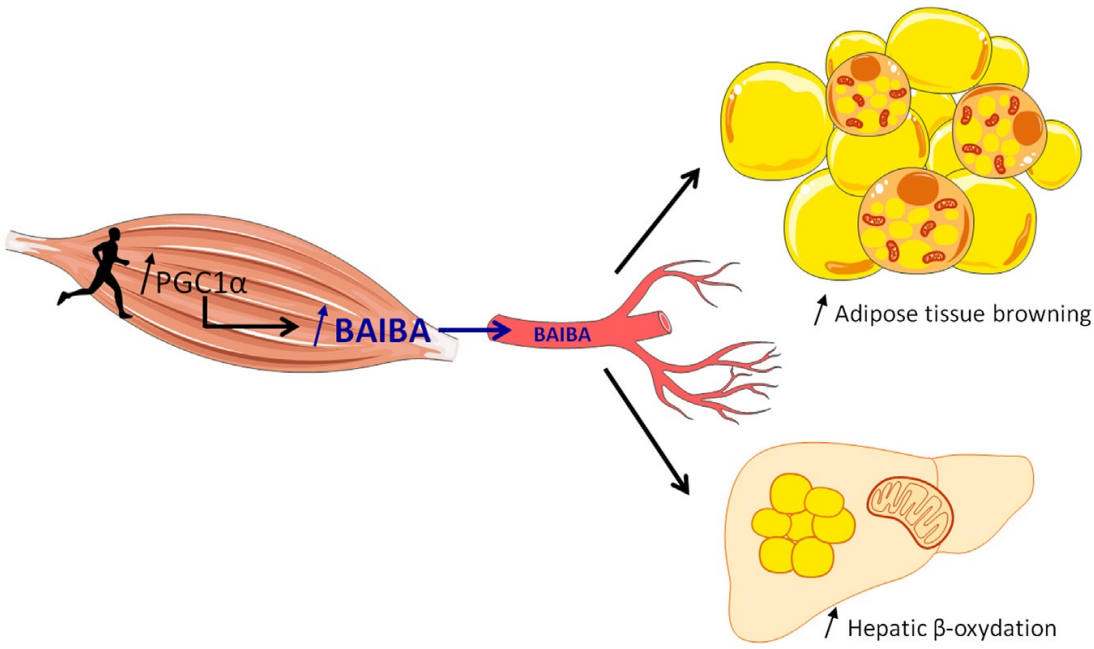


Figure 3. BAIBA Effects on Adipose Tissue and Liver. Exercise increases BAIBA concentrations through PGC-1α activation, producing browning of WAT and increased β-oxidation (Kammoun & Febbraio, 2014)

Another mechanism of action that has been proposed for BAIBA's effect is by acting through leptin. This hypothesis was corroborated by conducting studies on ob/ob (a homozygous recessive mice with a mutation in the Ob gene that causes total leptin deficiency), on ob/+ mice (heterozygous mice that produces decreased leptin production) and wild type. BAIBA treatment in ob/ob mice failed to decrease body adiposity; however, it significantly decreased plasma HDL, hepatic necroinflammation, and the number of apoptotic nuclei in these genetically-modified mice. BAIBA treatment in ob/+ mice receiving a high-calorie diet has been shown to limit increases in body fat percentage by 40%. In addition, protective effects against such conditions as steatosis, necroinflammation, glucose intolerance, and hypertriglyceridemia were associated with elevated leptin levels. Additionally, ob/+ mice showed an increase in CPT-1 concentration in liver and WAT as well as an increased phosphorylation of acetyl-CoA carboxylase. Interestingly enough, the changes observed in ob/+ mice were present despite any significant changes in food consumption. Wild type mice presented most of the effects observed in the heterozygous model, but in a less marked fashion (reduction of body fat mass of 27%). These results suggest that leptin may be a possible mechanism of action through which BAIBA performs its effects; however, the implication of leptin's effects caused by BAIBA does not rule out the existence of other potential mechanisms of action since wild type mice, with normal leptin levels, were also able to decrease body fat mass (Begriche et al., 2008).

Conclusion

The biochemical and molecular effects of exercise are now being better understood. Exercise, especially at higher intensities, increases energy expenditure even after the exercise session has finished. Increasing BAT seems to be a promising way to increase energy expenditure, improve metabolic outcomes and assist in obesity treatment. In this regard, it has been shown that nutrition in the pre-exercise period is able to change the gene expression normally activated in response to exercise. Specifically, it has been demonstrated that when enough glucose is provided before and during the exercise session, the normal increase in UCP-3 expression observed after the exercise session is totally blunted, which could be the result of a null increase on serum fatty acids. This increased UCP-3 expression in the glucose condition could be seen as a detrimental effect if an increase in the metabolic rate is the desire outcome. Therefore, the goal of the present study was to assess from the molecular standpoint the effect of pre-exercise nutrition on UCP-3 expression in skeletal muscle; furthermore, important metabolic transcription factors will also be evaluated under these circumstances.

CHAPTER THREE

Methods

Experimental Approach

In a cross-over design participants visited the laboratory on three different occasions. The first visit involved a familiarization and testing session while the visits two and three focused mainly on the impact of pre-exercise nutrition on gene expression. Each exercise session was performed in the morning, after a fasting period of 8 h and was separated by each other for at least five days. A meal replacement shake was randomly assigned before one of the exercise sessions (Figure 4).

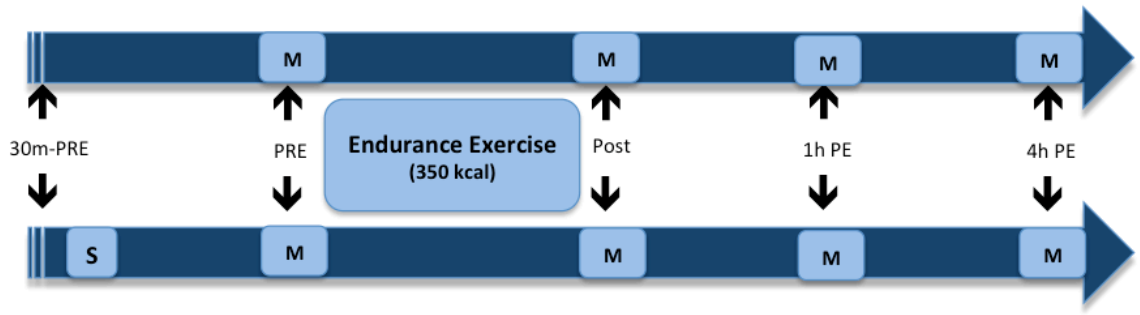


Figure 4. Experimental Design. The arrows and the squared M's represent the times at which blood samples and muscle biopsies were taken, respectively. (S: shake, M: muscle biopsy, 30m-PRE: 30 min pre-exercise, PRE: immediately pre-exercise, Post: immediately post-exercise, 1h-PE: 1 h post-exercise and 4h-PE: 4 h post-exercise).

The sampling time at 4 h was selected in order to avoid overpassing UCP-3 half-life time, which is located between 1 to 4 h, at the same time of allowing enough time for protein transcription and translation to occur (Azzu et al., 2010). A total of 12 apparently healthy, young males with normal Body Mass Index (BMI between 18.5 and 24.99 kg/m^2) between the ages of 18 to 30 years partaking in recreational, non-competitive

training (less than 3 h per week during the last 6 months) were recruited to perform two exercise sessions. Enrollment was open to men of all ethnicities. Participants were required to complete a medical screening before enrollment to ensure clearance to exercise according to the American College of Sports Medicine (ACSM) criteria. Volunteers were excluded from participating in this study if they did not satisfy the BMI, if they were currently consuming nutritional supplements. Finally, all eligible participants signed an informed consent form approved by Baylor University. Approval was granted by the Institutional Review Board for Human Subjects and all experimental procedures involved in this study conformed to the ethical considerations of the Declaration of Helsinki.

Study Site

All exercise testing and exercise sessions were conducted in the Baylor Laboratory for Exercise Science and Technology (BLEST). Blood and muscle samples, as well as lab work were performed at the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Independent and Dependent Variables

The independent variables were fasting and shake conditions. The dependent variables were serum glucose and FFA, as well as UCP-3 protein levels and UCP-3, PGC-1α and PPARα mRNA expression.

Entry and Familiarization Session (Visit 1)

Volunteers who showed interest in participating in the study were invited to attend to an entry-familiarization session. Once in the lab, participants were familiarized

with the study design via a verbal and written explanation, signing afterwards a university-approved informed consent form. Participants were then required to complete a medical screening form before enrollment to ensure clearance to do exercise according to the American College of Sports Medicine (ACSM) criteria. Volunteers were excluded from participating in this study if they didn't satisfy the BMI or if they were currently consuming supplements. Participants who satisfied these criteria went through anthropometric, body composition and peak O_2 uptake ($VO_{2\ peak}$) assessments. Participants arrived to the lab in the morning after a fasting period of 8 h, refrained from any type of exercise 48 h before each exercise session and recorded their dietary intake 24 h before the testing day (Table 1). Approval for this study was granted by the Institutional Review Board for Human Subjects of Baylor University. Furthermore, all experimental procedures involved in this study conformed to the ethical considerations of Helsinki Code.

Anthropometric Measurements

Total body mass (kg) was determined using a calibrated electronic scale with an attached stadiometer (Detecto, Webb City, MO). Participants were weighed with minimal clothing and with no shoes, standing on the scale with weight evenly distributed on both feet, arms placed down at sides, standing completely up-right and looking straight ahead. The reading for total body was recorded to the nearest 0.1 kg and height was measured to the nearest centimeter. Waist and hip circumference was then measured with a flexible, non-stretchable measuring tape (Baseline Evaluation Instrument, Bolingbrook, IL) at the umbilicus level and at the maximal protuberance of the buttocks, respectively.

Table 1
Overview of Research Design

Familiarization	Exercise Session 1	Exercise Session 2
Explanation of Study	24h Dietary Intake Form	24h Dietary Intake Form
Informed Consent Form	Heart Rate and Blood Pressure	Heart Rate and Blood Pressure
Pre-Screening Form	Muscle Biopsies (4)	Muscle Biopsies (4)
Health History Form	Blood Sampling (5)	Blood Sampling (5)
Determination of	Meal replacement shake (randomly assigned)	Cycle Ergometer
-Anthropometric Measurements	Cycle Ergometer	
-Body Composition		
-Resting Heart Rate and Blood Pressure		
-Assessment of $VO_{2\ peak}$		

Body Composition Assessment (Visit 1)

Fat mass and fat-free mass along with body fat percentage, were assessed by a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA) within the familiarization session. During the DEXA test the participant laid motionless in a supine position for a period of 6 min wearing only a t-shirt and shorts. The participants were exposed to a low dosage radiation of approximately 1.5 mR during the scan, which is about 10% of the radiation produced by a chest radiograph (Rowe, 2005).

Heart Rate and Blood Pressure

In all visits, heart rate and blood pressure was assessed directly before, during and after the exercise session. The values taken at the first session determined eligibility to enter the study. Heart rate was determined by using a POLAR® FT7 heart rate monitor (Polar Electro Inc. Lake Success, NY) while blood pressure was assessed after a resting period of 5 min using an aneroid sphygmomanometer.

Assessment of $VO_{2\ peak}$ (Visit 1)

During the entry session participants performed a maximal cycling test. In brief, each exercise session started with 1 min collection of resting data, followed by a warm up period of 3 min at 25 W and increases of 25 W every minute until the participants reached their tolerance level. The test was terminated once the participant achieved volitional fatigue. The pedal rate was maintained at 50 rpm throughout the test as confirmed by a metronome. Participants were verbally encouraged by study personnel to provide a true maximal effort. The $VO_{2\ peak}$ test was considered valid if the participant achieved at least one of the following criteria: a respiratory exchange ratio above 1.1, an age predicted HR_{max} within 10 beats per min or a plateau in their $VO_{2\ peak}$ with an increased workload. The highest VO_2 achieved was defined as the $VO_{2\ peak}$. O_2 uptake was measured by an open-circuit sampling system with a low-resistance valve (Parvo Medics, Provo, UT). Heart rate was monitored through the duration of the exercise session using a Polar® FT7 heart rate monitor strap with a wrist receiver (Polar Electro Inc. Lake Success, NY). Blood pressure was measured before and after the exercise session using an aneroid sphygmomanometer.

Meal Replacement Shake

The exercise session in which the pre-exercise meal was provided was randomly selected based on the "drawn from the hat" procedure. The meal replacement shake chosen was carefully selected in order to provide similar nutritional information than a regular college breakfast, which consists of one cup of non-sugary cereal (aka. corn flakes), 1 cup of milk and a half of a small (6" to 6 7/8" long) banana. Providing a total of 342 kcal, 53 g of carbohydrates (29 g sugar), 10 g of fat and 13 g of protein (Food Processor, EHSA Research, Salem, OR). As such, the selection of a commercial meal replacement shake provided an easy to digest and absorb meal that was consistent among participants. According to the above mentioned, the commercially-available Milk Chocolate Ensure Plus® meal replacement drink was chosen for the purpose of this study. Ensure contains 350 kcal, 50 g of carbohydrate (22 g of sugar), 13 g of protein and 11 g of fat (Figure 5).

Nutrition Facts

Serv. Size 1 bottle
(8 fl oz)

Calories 350

Calories from Fat 100

Amount Per Serving		%DV§	Amount Per Serving		%DV§
Fat	11g	17%	Sodium	220mg	9%
Saturated Fat	1g	5%	Potassium	540mg	15%
Trans Fat	0g		Carbohydrate	50g	17%
Polyunsaturated Fat	4.5g		Dietary Fiber	<1g	<4%
Monounsaturated Fat	5g		Sugars	22g	
Cholesterol	10mg	3%	Protein	13g	26%

Figure 5. Nutritional Information of Milk-Chocolate Ensure Plus.

Exercise Protocol (Visits 2 and 3)

In a cross-over fashion, participants engaged in two exercise sessions; each one separated from each other for at least a week; one involved the ingestion of a meal replacement shake and the other a fasting condition throughout the exercise session. An illustration of the exercise protocol can be seen in Figure 1 and Table 1. At each exercise session, participants arrived to the laboratory after a fasting period of 8 h between 7:00 to10:00 am. They turned in their dietary logs and had an indwelling venous catheter inserted to obtain their first blood samples. During the session in which the meal replacement shake was ingested, the shake was provided right after the baseline blood draw. Participants under both conditions underwent a 30 min resting period after the baseline sampling. At the end of this period, participants had their second blood draw and first muscle biopsy, directly before the exercise session. Since participants were untrained individuals and the exercise sessions were designed to workout at a high intensity (70% of $VO_{2\ peak}$), the YMCA cycling protocol (Figure 6) was chosen to start the workout in stages according to their heart rate response (Akalan & Robergs). In brief, resting O_2 consumption was measured the first minute, after which a 3 min warm up at 25 W was performed. Resistance was then increased every 3 min according to their heart rate response at the last 15 s of every stage (Figure 6). Heart rate and respiratory gas analysis values were checked at 1 min intervals throughout the exercise session to verify intensity (70% of the $VO_{2\ peak}$) and estimate energy expenditure. The exercise bout was standardized to an energy expenditure of 350 kcal, which is equivalent to the ingested kilocalories from the shake. Heart rate was assessed using a Polar® FT7 heart rate monitor (Polar Electro Inc. Lake Success, NY). At the end of the exercise session,

participants had their second muscle biopsy and third blood draw, after which they rested, having the fourth and fifth blood draw and their third and fourth muscle biopsies at 1 and 4 h post-exercise, respectively.

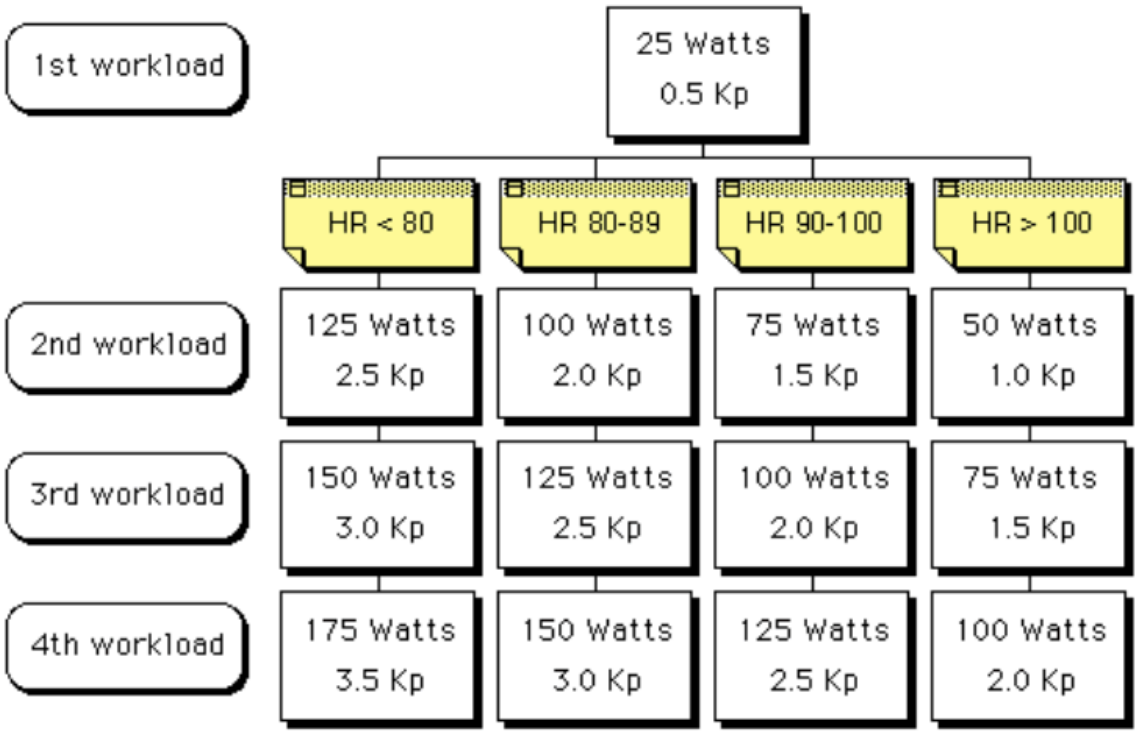


Figure 6. An Illustration of the YMCA Bike Protocol Used for the Exercise Sessions

Muscle Biopsies (Visits 2 and 3)

Percutaneous muscle biopsies (15 to 20 mg) were obtained from the middle portion of the vastus lateralis at the midpoint between the patella and the greater trochanter of the femur. The leg biopsied for the first exercise session was chosen at random with the opposite leg being used for the second exercise session. The biopsy area was shaved and cleaned with alcohol and Betadine (fluid antiseptic). Participants were anesthetized with 1.0 mL of a subcutaneous injection of Lidocaine at 1%. Once local anesthesia had taken effect (approximately 5 min), the biopsy was performed using a 16-

gauge fine needle aspiration biopsy (Tru-Core I Biopsy Instrument, Medical Device Technologies, Gainesville, FL) inserted into the skin at an approximate depth of 1 cm. The remaining biopsies were made to extract tissue from 0.5 cm to the former from medial to lateral. After sample obtention, adipose tissue was trimmed and skeletal muscle was frozen in liquid nitrogen and then stored at -80°C for later analyses. Four muscle samples were taken every exercise session, with a total of eight muscle samples per participant by the end of the study. The muscle samples were taken immediately before, immediately after, and at 1 and 4 h after the exercise session. The 60 min post-exercise sample was selected since it has been shown that 1h is enough time to assess changes in UCP-3 mRNA expression within muscle (Noland et al., 2003). The 4 h post-exercise muscle biopsy was chosen in order to avoid overpassing UCP-3 half-life time (1 to 4 h), at the same time of allowing enough time for protein transcription and translation to occur (Azzu et al., 2010).

Blood Sampling (Visits 2 and 3)

Venous blood samples were obtained into 10 mL vacutainer tubes from a 20 gauge intravenous catheter inserted into the antecubital vein. Immediately after taking each blood sample, the catheter was flushed with 10 UI/mL of sodium heparin to prevent from clotting. Vacutainers were kept at room temperature for 10 min to allow blood clot formation and were then centrifuged at room temperature for 15 min at 1300 RCF in a fixed angle centrifuge. The serum was collected in micro-tubes and stored at -80°C for their later analysis. Five blood samples were obtained at each exercise session, with a total of ten blood samples being obtained during the course of the study. At each exercise

session, blood samples were obtained: at resting conditions, immediately before and after exercise, and at 1 and 4 h post-exercise.

Dietary Analysis (Visits 2 and 3)

Participants recorded their dietary intake 24 h before each exercise session. Diets were not standardized, however, participants were asked to keep their normal dietary habits through the duration of the study. The 24 h recall was evaluated using Food Processor dietary assessment software (EHSA Research, Salem, OR) to determine the consumption of macronutrients throughout the study.

Blood Serum Analyses

Serum Fatty Acids

FFA content was determined spectrophotometrically using a FFA quantification kit (Bio Vision #K612-100, Mountain View, CA) at a wavelength of 570 nm. After preparing the ACS Reagent, the Enzyme Mix, and the Reaction Mix buffers according to manufacturer instructions, the standard and the samples (50 µL) were added to each well. Then, 2 µL of ACS Reagent were added into each sample and standard well incubating at 37 °C for 30 min. Lastly, 50 µL of Reaction Mix were added to each well incubating again at 37 °C for 30 min. The assay was read in duplicates using the microplate reader xMark microplate absorbance spectrophotometer (Bio-Rad, Hercules, CA). Data analysis was analyzed using data reduction software (Microplate Manager 6, Bio-Rad, Hercules, CA).

Serum Glucose

Serum glucose levels were measured enzymatically (catalog number 439-90901, Wako Diagnostics) according to manufacturer instructions. Briefly, after reagents were prepared, 3 µL of saline solution (blank), standard and sample were added into each well. After the plate was filled by the samples and the standard curve, 350 µL of working color reagent were added into each well. Plate was first incubated at 37°C for 5 min and then read with a microplate absorbance spectrophotometer (xMark, Bio-Rad, Hercules, CA) at an absorbance of 570 nm. Serum glucose concentrations were determined using data reduction software (Microplate Manager 6, Bio-Rad, Hercules, CA).

Skeletal Muscle Analysis

Total RNA Isolation

Approximately 10 to 15 mg of muscle tissue were used for biochemical analysis. Total cellular RNA was extracted from homogenate of biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate contained within the TRI-reagent (#T9424 Sigma Co., St. Louis, MO). In brief, 500 µL of TRI-reagent were added to each tube followed by homogenization of the muscle samples. After letting the samples sit at room temperature for 5 min, 100 µL of chloroform were added to each tube. The tubes were then vortexed and allowed to sit for 15 min at room temperature. Samples were then centrifuged at 12,000 x g for 15 min at 2 to 8 °C. This process separated the samples into three distinct phases, a lower (pink) organic phase, which contains the protein, a middle (gray) interphase containing the DNA, and an upper (clear) aqueous phase containing the RNA. Using a sterile transfer pipette, the clear aqueous

phase was transferred into a new microtube. The remaining phases (interphase and organic) were stored in the ultra-low freezer at -80°C. Subsequently, 250 µL of 100% isopropanol were added to each tube and allowed to sit at room temperature for 10 min. Samples were then centrifuged at 12,000 x g for 10 min at 2 to 8°C, allowing for the formation of a RNA pellet. The supernatant was discarded, followed by addition of 500 µL of 75% ethanol. Samples were then vortexed to wash the pellet and centrifuged at 12,000 x g for 10 min at 2 to 8°C, discarding the supernatant at the end. The washing procedure with ethanol at 75% was repeated. The pellet was finally allowed to air dry for 10 min, then 50 µL of nuclease free water were added to the microtube. Total RNA concentration was determined spectrophotometerically (SmartSpec Plus, Bio-Rad, Hercules, CA, USA) by optical density (OD) at 260 nm using an OD_{260} equivalent to 40 µg/µl and the final concentration was expressed relative to muscle wet-weight.

Reverse Transcription and Complementary DNA (cDNA) Synthesis

A microgram of total skeletal muscle RNA was reverse-transcribed to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Each reverse transcription reaction mixture was incubated at 25°C for 5 min, then at 42°C for 30 min, heated to 85°C for 5 min, and finally hold at 4°C. The cDNA concentration was determined by using an OD_{260} equivalent to 50 µg/µl. Starting cDNA template concentration was standardized by adjusting all samples to 200 ng prior to amplification.

Oligonucleotide Primers for Real Time Polymerase Chain Reaction (RT-PCR)

The primers access number and mRNA sequence of UCP-3, PGC-1α, PPARα and β-actin are described in Table 2. PCR Primers sequences were determined by getting the

mRNA of the target protein from the NCBI Nucleotide database (www.ncbi.nlm.nih.gov) and by using the BLAST designing web page from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and then commercially synthesized (Integrated DNA Technologies, Coralville, IA).

β-actin was used as the control gene due to its consideration as a constitutively expressed "housekeeping gene," and the fact that it has been shown to be an appropriate external reference standard in human skeletal muscle using RT-PCR (Mahoney et al., 2004).

Table 2
Sequences of Primers Used for RT- PCR

Gene name	Accession number	Primer sequences
PGC-1α	NM_013261.3	F: 5'-TTGACTGGCGTCATTCAGGAG- 3' R: 5'-ACTTGAGTCCACCCAGAAAGC-3'
UCP-3	NM_003356.3	F: 5'-CCCTCCTTTTTGCGTTTGGG-3' R: 5'-CGTTAGCTACCAGTGGCCTT-3'
PPARα	AY206718	F: 5'-TCGGCGAGGATAGTTCTGGA-3' R: 5'-AGGAAACGGCACACTTACCC-3'
β-actin	NM_001101	F: 5'-CTCACCATGGATGATGATATCGC- 3' R: 5'-AGGAATCCTTCTGACCCATGC- 3'

RT-PCR Amplification and Quantitation

Two μL of cDNA template, 10 μL of iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA), 1.0 μL of the reverse primer reaction mixture, 1.0 μL of the forward primer reaction mixture, and 6.0 μL of nuclease-free water were added to each well.

Each reaction was amplified using RT-PCR (iCycler IQ RT-PCR Detection System, Bio-Rad, Hercules, CA, USA). The amplification profile was run for 40 cycles employing a denaturation step at 95°C for 30 s, primer annealing at 58°C for 30 s, and extension at 72°C for 30 s. Fluorescence was measured after each cycle resulting from the incorporation of SYBR green dye into each amplicon. The expression of mRNA was determined from the ratio of the C_T values relative to β-actin. The specificity of the PCR was demonstrated with an absolute negative control reaction containing no cDNA template, and a single gene product was confirmed using DNA melt curve analysis (Chemi-Doc XRS, Bio-Rad, Hercules, CA, USA). The expression of mRNA was determined as the percent change in delta C_T values relative to β-actin for each target gene.

Total Muscle Protein Isolation

Proteins obtained after DNA isolation were precipitated from the phenolethanol supernatant with 1.5 mL of 2-propanol per milliliter of TRI Reagent used in Sample Preparation. Samples stood at room temperature for 10 min to be later centrifuged at 12,000 x g for 10 min at 2 to 8°C. Supernatant was then discarded and pellet was washed three times in 0.3 M guanidine hydrochloride dissolved in 95% of ethanol solution, using 2 mL per milliliter of TRI reagent used in sample preparation. During each wash, samples were stored in wash solution for 20 min at room temperature and then centrifuged at 7,500 x g for 5 min at 2 to 8°C. After the three washes, 2 mL of 100% ethanol per milliliter of TRI Reagent used in Sample Preparation were added to dissolve the protein pellet through vortex use. Tubes were allowed to stand at room temperature for 20 min, after which they were centrifuged at 7,500 x g for 5 min at 2 to 8°C. Protein pellets were

then dried for 5 to10 min. The protein pellet was dissolved in 1% SDS by working a pestle. Insoluble material was removed by centrifugation at 10,000 x g for 10 min at 2 to 8°C. Supernatant was transferred into a new tube and immediately stored at -80°C (TRI-Reagent Technical Bulletin, T9424, Sigma-Aldrich).

Total Muscle Protein Quantitation

Total protein concentration was measured by following the DC Protein Assay (Bio-Rad Hercules, CA, USA). The DC Protein Assay is a colorimetric assay for protein concentration following detergent solubilization. The reaction of protein with an alkaline copper tartrate solution (Reagent A) and a Folin reagent (Reagent B) produces a blue color. The microplate assay protocol was utilized in order determine the protein concentration for skeletal muscle UCP-3 protein content. DC protein reagent S (20 µL) was added to each mL of reagent A making working reagent A. Protein standards curve were developed ranging from 46 µg to 1,500 µg. Standards and samples (5 µL) were pipetted into a dry microtiter plate. Reagent A (25 µL) was added into each well, followed by reagent B (200 µL). The plate was allowed to mix for five seconds, removing bubbles that with a clean pipet tip. After 15 min, the absorbances were read at 750 nm.

UCP-3 Protein Levels

UCP-3 (long and short isoforms) was analyzed by duplicate according to manufacturer instructions, using a commercially-available ELISA kit (catalog number MBS945861, MyBioSource, San Diego, CA). After all reagents were prepared, blank wells were filled with all components but sample, standard wells were filled with 100 µL

of standard solution and test sample wells were filled with 100 µL of sample dilution (80 µL of sample diluent and 20 µL of sample). Samples were incubated for 2 h at 37 °C. Liquid was then removed and 100 µL of biotin antibody (1x) were added to each well, incubating for 1 h at 37 °C. Three washes followed the incubation period, after which 100 µL of avidin conjugated horseradish peroxidase were added to the wells, incubating the plate for 1 h at 37 °C. The plate was washed for five times to remove avidin residues. Next, 90 µL of TMB substrate were added to the wells, incubating the plate in the dark for 15 to 30 min at 37 °C. Color was maintained by adding 50 µL of stop solution. The plate was read at 450 nm using a microplate absorbance spectrophotometer (xMark, Bio-Rad, Hercules, CA), within 5 min after adding the stop solution. The intensity of the color was directly proportional to the amount of UCP-3 bound in the initial step (UCP-3 ELISA Kit, MBS812533, MyBiosource). Data analysis was performed using Microplate Manager 6 Software (Bio-Rad, Hercules, CA), and the final concentration was expressed relative to muscle weight.

Statistical Analyses

Paired samples t-tests were performed to determine differences for dietary intake. Statistical analyses for muscle protein and mRNA expression variables were performed by utilizing a 2 x 4 [condition (meal/no meal) x time point] ANOVA test. Analyses of serum variables were performed by utilizing a 2 x 5 [condition (meal/no meal) x time point] ANOVA test. Significant within-condition and within-time differences were determined using a Tukey HSD post-hoc test. Significant main effects for time were further evaluated by one-way ANOVA with repeated measures. Effect size was assessed using partial eta-squared ($\eta 2$), and was determined to be: weak = 0.17, medium = 0.24,

strong = 0.51, very strong = 0.70 (O'Connor et al., 2007). Based on prior research an a-priori power calculation showed that 11 participants per group is adequate to detect a significant difference between groups in the dependent variable of serum FFA (Ahlborg, Felig, Hagenfeldt, Hendler, & Wahren, 1974; Essen, Hagenfeldt, & Kaijser, 1977; Head, Jakeman, Kendall, Cramb, & Maxwell, 1993; Jeukendrup, Borghouts, Saris, & Wagenmakers, 1996; Kaminsky, Knowlton, Perkins, & Hetzler, 1986; Phillips et al., 1996; Sherman, Peden, & Wright, 1991; Wahren, Felig, Ahlborg, & Jorfeldt, 1971). In order to minimize the probability of making type II error, 13 males were recruited for this study, from which 12 were included in the data analysis. All statistical procedures were performed using IBM SPSS Statistics 21.0 software. A probability level of < 0.05 was always adopted throughout.

CHAPTER FOUR

Subject Demographics

Twenty-six participants were initially recruited for the study, completed consent forms, and participated in an initial familiarization session. Of the twenty-six participants, half of them completed the research study, as twelve participants dropped out before the first exercise session and another was unable to continue due to difficulties getting his blood. A total of thirteen apparently healthy, young males with normal BMI (18.5 and 24.99 kg/m^2) between the ages of 18 to 30 years completed the study. Of them, 12 participants were utilized in data analysis, as one participant's fasting baseline glucose results were considered to be abnormally high (176 and 102 mg/dL on the shake and fasting days, respectively). The average age of the 12 participants was 20.2 ± 3.3 years. Average BMI and $VO_{2\ peak}$ were 22.7 ± 1.9 kg/m^2 and 41.8 ± 7.4 mL/kg/min respectively. Body composition was measured during the familiarization session. Table 3 shows the average body composition and anthropometric information at the onset of the study.

Dietary Analysis

All participants recorded their food intake 24 h prior to each of the testing sessions. All participants were instructed to keep a consistent diet the day before both of the exercise sessions. Nutritional information of the meal replacement shake provided the morning of the testing session was not included in the nutritional analysis. Table 4 provides the average values (± SD) for total kilocalories and macronutrients before each testing session. Paired-samples t tests revealed no significant differences for kilocalories

(t = 0.905), fats (t = 0.256), carbohydrates (t = 0.096), or protein (t = 0.565), intake between experimental conditions.

Table 3
Body Composition and Anthropometric Information at the Onset of the Study

Variable	Mean ± SD
Weight (kg)	73.2 ± 9.8
Height (m)	1.8 ± 0.1
Waist circumference (cm)	82.4 ± 5.6
Hip circumference (cm)	97.7 ± 4.7
Waist-to-hip ratio	0.8 ± 0.1
Body fat mass (kg)	9.83 ± 2.5
Body fat percentage (%)	14.9 ± 2.7
Lean mass (kg)	54.88 ± 7.62

Note: Body fat mass and body fat percentage excludes the head

Table 4
Dietary Intake of Participants

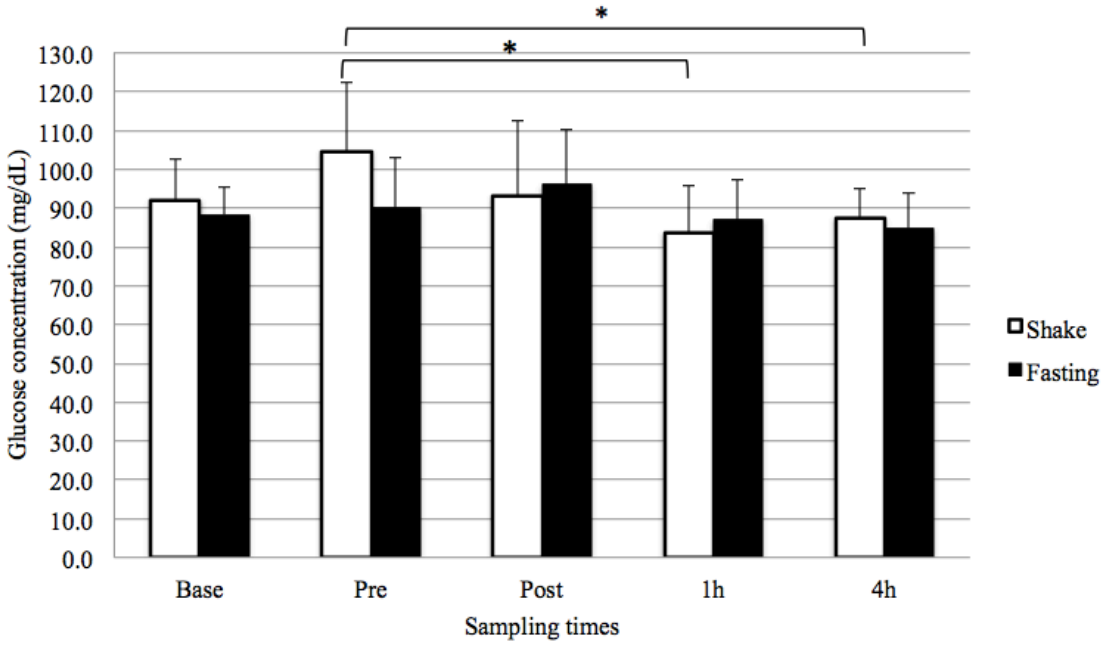
Variable	Shake	Fasting	t-value
Kilocalories (kcal/day)	2570.90 ± 984.37	2638.13 ± 825.50	0.811
Fat (g/day)	122.27 ± 67.62	103.39 ± 39.47	0.348
Carbohydrate (g/day)	263.74 ± 82.44	312.83 ± 98.47	0.110
Protein (g/day)	106.58 ± 45.99	117.49 ± 56.33	0.510

Note: Data are presented as mean ± SD

Serum Glucose

There was no statistically significant condition by time interaction in serum glucose concentration (p = 0.112, effect size = 0.066). There was also no significant main effect for condition indicating no difference in serum glucose concentration

between both experimental conditions (p = 0.212, effect size = 0.014). However, the time main effect demonstrated a statistically significant difference in glucose concentration between time points (p = 0.005, effect size = 0.128). Post-hoc analyses revealed a significant decrease in glucose concentration at 1 h (p = 0.007) and 4 h (p = 0.033) compared with pre-exercise in the shake condition. In the fasting condition, no significant (p = 0.161, effect size = 0.112) differences were observed (Figure 7).



Sampling time	Shake	Fasting
Base	92.1 ± 10.7	88.4 ± 7.2
Pre	104.7 ± 17.7	90.0 ± 13.2
Post	93.2 ± 19.2	96.2 ± 14.2
1h	83.7 ± 12.3	86.9 ± 10.6
4h	87.4 ± 7.7	84.9 ± 9.2

Figure 7. Serum Glucose Concentration in Response to Fasting and Shake Conditions. The asterisk (*) indicates a significant main effect for time (p< 0.05). Within the shake condition, a significant decrease in glucose concentration was observed at 1h (0.007) and 4h (0.033) compared with pre-exercise, while no significant differenes were noted for the fasting condition (p= 0.161, effect size= 0.112). Data expressed as means ± SD.

Serum Glucose Hypotheses Conclusion

- H_1: There will be a significantly higher glucose concentration in the shake condition. Since no significant statistical differences were observed, this hypothesis is rejected.

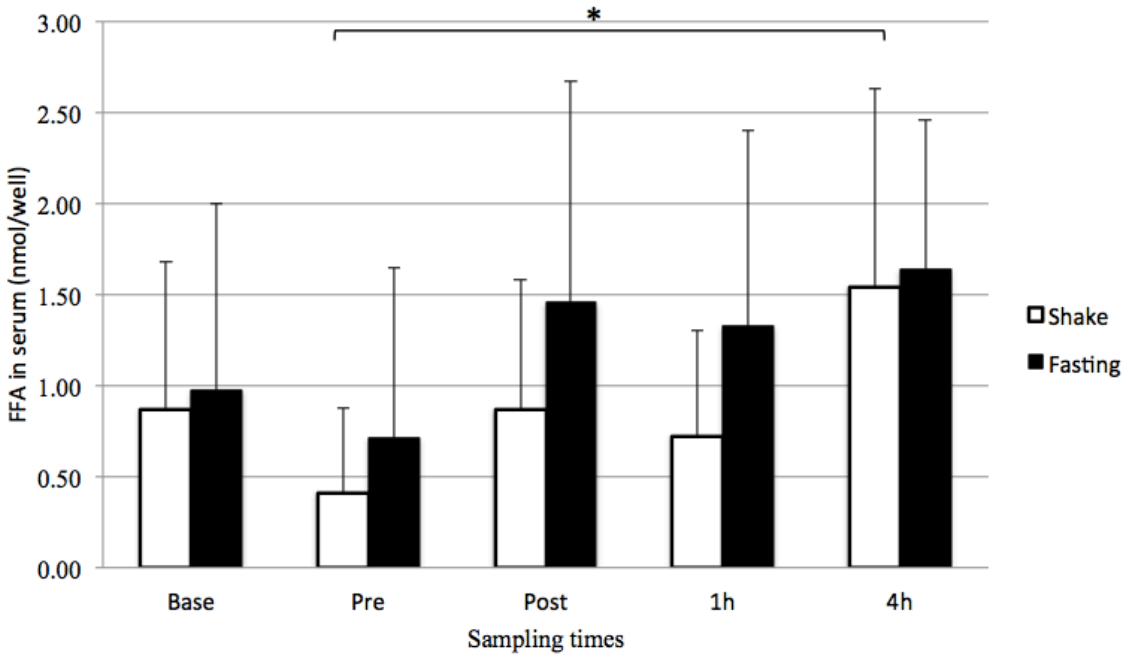
- H_2: There will be a significant increase in serum glucose concentration in response to the shake ingestion; therefore, this hypothesis is rejected.

Free Fatty Acids

There was no statistically significant condition by time interaction for serum FFA concentration ($p = 0.769$, effect size = 0.017). The condition main effect showed a statistically significant higher serum FFA concentration in the shake condition ($p = 0.046$, effect size = 0.037). The main effect time also demonstrated a statistically significant difference in serum FFA concentration between time points ($p = 0.004$, effect size = 0.129). Post-hoc analyses revealed a significant increase in FFA concentration at 4 h compared with pre-exercise ($p = 0.005$) in the shake condition, however, in the fasting condition, no significant differences were observed ($p = 0.199$, effect size = 0.103) (Figure 8).

Serum Free Fatty Acids Hypothesis Conclusion

- H_3: There will be a significantly higher serum FFA concentration in the fasting condition. There was a significantly higher serum FFA concentration in the fasting condition; therefore, the hypothesis is accepted.

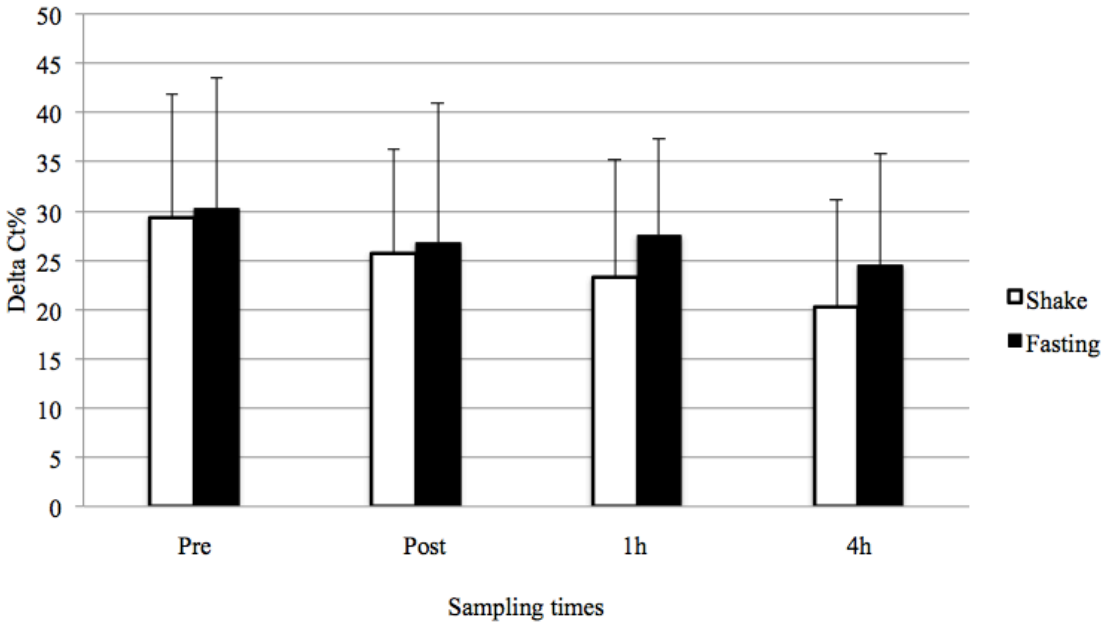


Pre	0.4 ± 0.5	0.7 ± 0.9
Post	0.9 ± 0.7	1.5 ± 1.2
1h	0.7 ± 0.6	1.3 ± 1.1
4h	1.5 ± 1.1	1.6 ± 0.8

Figure 8. Serum Free Fatty Acids Concentration in Response to Fasting and Shake Conditions. The asterisk (*) indicates a significant main effect for time ($p < 0.05$). Within the shake condition a significant increase in FFA concentration was observed at 4h compared with pre-exercise ($p = 0.005$), while no significant differences were noted for the fasting condition ($p = 0.199$, effect size = 0.103). Data expressed as means ± SD.

PPARα mRNA Expression

There was no statistically significant condition by time interaction in skeletal muscle PPARα mRNA expression ($p = 0.929$, effect size = 0.005). The main effect for condition revealed no significant differences in PPARα mRNA expression between experimental conditions ($p = 0.291$, effect size = 0.013). The main effect for time also showed no significant differences in PPARα mRNA expression ($p = 0.197$, effect size = 0.053) (Figure 9).



Sampling time	Shake	Fasting
Pre	29.39 ± 12.52	30.25 ± 13.26
Post	25.71 ± 10.51	26.77 ± 14.16
1h	23.26 ± 11.99	27.53 ± 9.88
4h	20.19 ± 20.19	24.47 ± 24.47

Figure 9. ΔCt % of PPARα mRNA Expression in Response to Fasting and Shake Conditions. No significant diferences were observed (p> 0.05). Data expressed as means ± SD

PPARα Hypotheses Conclusion

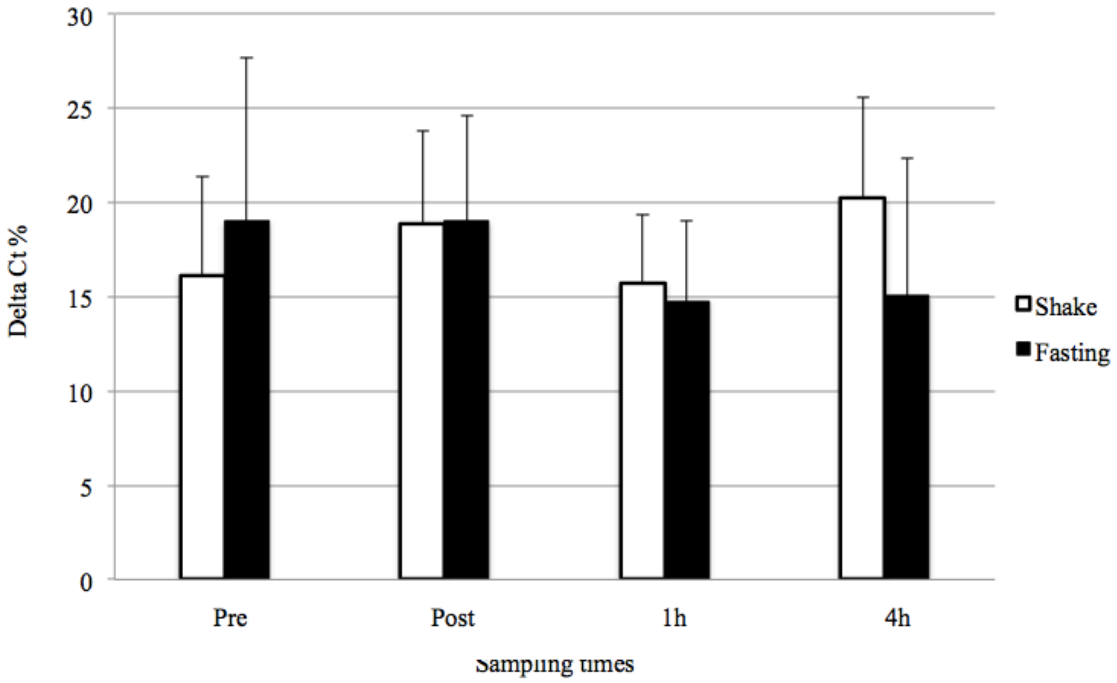
- H_4: There will be a significantly higher PPARα mRNA expression in the fasting condition. Since there was no significant increase in PPARα mRNA expression, the hypothesis is rejected.

- H_5: There will be a significant increase in PPARα mRNA expression after exercise in both conditions. There was no significant effect of exercise in PPARα mRNA expression; therefore, the hypothesis is rejected.

PGC-1α mRNA Expression

There was no significant condition by time interaction for skeletal muscle PGC-1α mRNA expression ($p = 0.132$, effect size = 0.063). The main effect for condition revealed no significant differences in PGC-1α mRNA expression between experimental conditions ($p = 0.523$, effect size = 0.005). The main effect for time also demonstrated no significant

differences in PGC-1α mRNA expression between time points (p = 0.205, effect size = 0.0.052) (Figure 10).



Sampling time	Shake	Fasting
Pre	16.14 ± 5.25	19.00 ± 8.66
Post	18.90 ± 4.94	19.01 ± 5.61
1h	15.72 ± 3.65	14.75 ± 4.32
4h	20.23 ± 5.40	15.08 ± 7.31

Figure 10. ΔCt % of PGC-1α mRNA Expression in Response to Fasting and Shake Conditions. No significant diferences were observed (p> 0.05). Data expressed as means ± SD

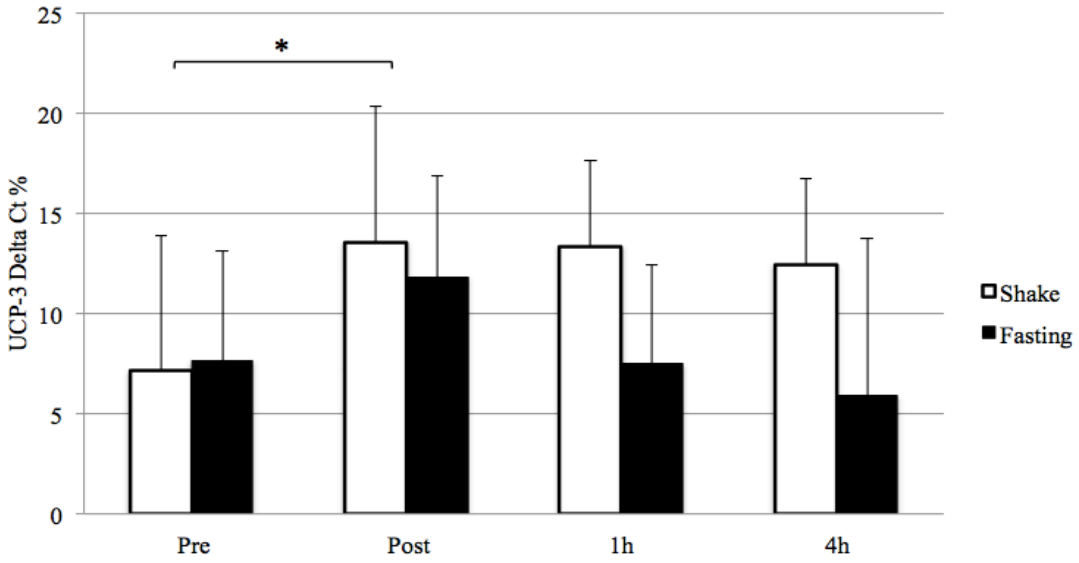
PGC-1α Hypotheses Conclusion

- H_6: There will be a significantly higher PGC-1α mRNA expression in the fasting condition. There was no significant increase in PGC-1α RNA expression. As a result, the hypothesis is rejected.

- H_7: There will be a significant increase in PGC-1α mRNA expression after exercise in both conditions. Since there was no significant difference in PGC-1α mRNA expression in response to exercise, the hypothesis is rejected.

UCP-3 mRNA Expression

There was no significant condition by time interaction for skeletal muscle UCP-3 mRNA expression ($p = 0.127$, effect size = 0.064). The main effect for condition showed a significant increase in UCP-3 mRNA expression in the shake condition ($p = 0.006$, effect size = 0.085). The time main effect also demonstrated a significant difference in UCP-3 mRNA expression ($p = 0.020$, effect size = 0.108). Post-hoc analyses revealed a significant increase in UCP-3 mRNA expression at the post-exercise sampling point compared with pre-exercise ($p = 0.042$) in the shake condition; however, no significant differences ($p = 0.108$, effect size = 0.130) in UCP-3 mRNA expression were observed in the fasting condition (Figure 11).



Sampling time	Shake	Fasting
Pre	7.14 ± 6.77	7.65 ± 5.49
Post	13.55 ± 6.81	11.83 ± 5.07
1h	13.35 ± 4.32	7.50 ± 4.92
4h	12.44 ± 4.28	5.88 ± 7.85

Figure 11. ΔCt % of UCP-3 mRNA Expression in Response to Fasting and Shake Conditions. The asterisk (*) indicates a significant main effect for time ($p < 0.05$). Within the shake condition a significant increase in UCP-3 mRNA expression occurred at the post-exercise sampling point compared with pre-exercise ($p = 0.042$), while no significant differences were noted for the fasting condition ($p = 0.108$, effect size= 0.130). Data expressed as means ± SD.

UCP-3 mRNA Expression Hypotheses Conclusion

- H_8: There will be a significantly higher UCP-3 mRNA expression in the fasting condition. There was a statistically significant increase in UCP-3 mRNA expression; however, the increase occurred in the shake condition. As a result, the hypothesis is rejected.

- H_9: There will be a significant increase in UCP-3 mRNA expression after exercise in both conditions. There was a significant increase in UCP-3 mRNA expression at the post-exercise sampling time compared with pre-exercise only in the shake condition; therefore, the hypothesis is rejected.

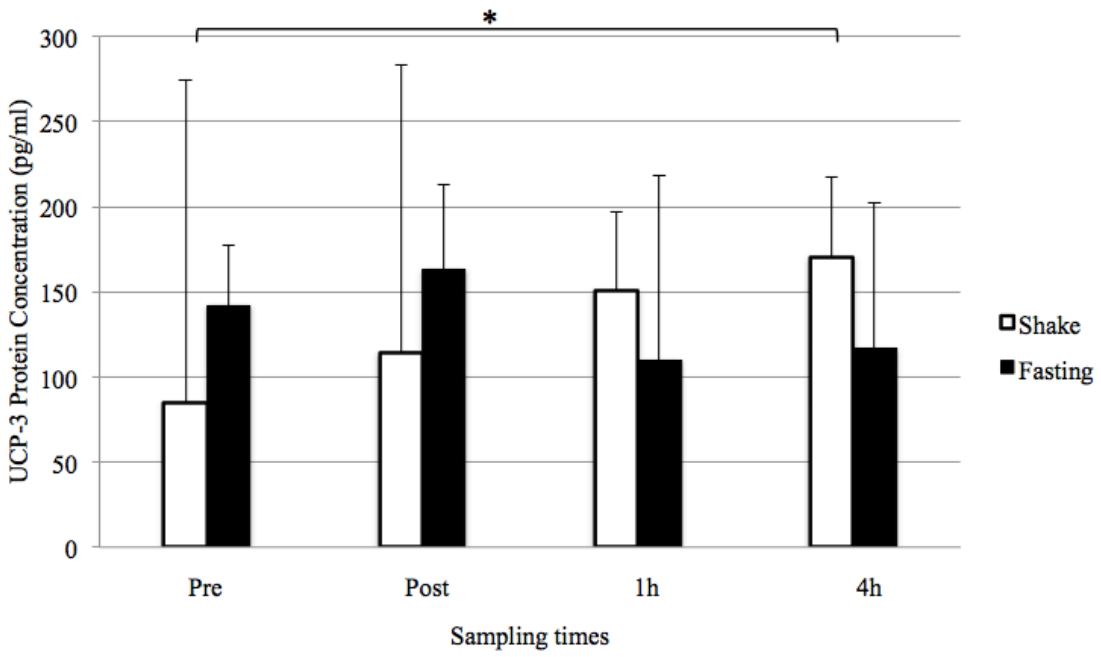
UCP-3 Protein Levels

There was no significant condition by time interaction for skeletal muscle UCP-3 protein concentration ($p = 0.165$, effect size = 0.057). The main effect for condition revealed no significant differences in UCP-3 protein between conditions ($p = 0.889$, effect size = 0.000). Time main effect also demonstrated no significant changes in UCP-3 protein levels ($p = 0.782$, effect size = 0.12); however, upon separate analysis a significant increase in UCP-3 protein levels was presented at 4 h compared with pre-exercise ($p = 0.036$) in the shake condition. In the fasting condition, no significant ($p = 0.753$, effect size = 0.027) differences in UCP-3 protein levels were observed (Figure 12).

UCP-3 Protein Hypotheses Conclusion

- H_{10}: There will be a significantly higher UCP-3 protein concentration in the fasting condition. Since, there were no statistically significant increases in UCP-3 protein in either condition the hypothesis is rejected.

- H_{11}: There will be a significant increase in UCP-3 protein concentration after exercise in both conditions. There was a significant increase in UCP-3 protein concentration only in the shake condition; therefore, the hypothesis is rejected.



Sampling time	Shake	Fasting
Pre	84.97 ± 35.42	142.19 ± 189.61
Post	113.95 ± 49.34	163.52 ± 169.60
1h	150.74 ± 108.13	110.02 ± 46.25
4h	170.38 ± 85.49	116.73 ± 46.67

Figure 12. UCP-3 Protein Concentration in Response to Fasting and Shake Conditions. The asterisk (*) indicates a significant main effect for time ($p < 0.05$). Within the shake condition a significant increase in UCP-3 protein levels was presented at 4h compared with pre-exercise ($p = 0.036$), while in the fasting condition no significant differences were observed ($p = 0.753$, effect size= 0.027). Data expressed as means ± SD.

CHAPTER FIVE

Discussion

Uncoupling proteins have been proposed as potential therapeutic targets to treat obesity and other related diseases (Busiello et al., 2015). These mitochondrial proteins dissipate the mitochondrial electrochemical gradient by transporting protons back into the matrix. Five isoforms, UCP-1, UCP-2, UCP-3, UCP-4 and UCP-5 have been identified (Adams, 2000). Of the three, the isoform that has been found to be mainly expressed in skeletal muscle is UCP-3. FFA and reactive oxygen species (ROS) have been proposed as possible UCP-3 activators; however, the existing evidence is still under debate. Some of the health benefits that have been attributed to UCP-3 are prevention of oxidative damage and lipid peroxidation, as well as promotion of fatty acid oxidation and mitochondrial uncoupling (Azzu et al., 2010; Busiello et al., 2015).

Exercising while fasted can increase UCP-3 expression; however, this response seems to be blunted by glucose consumption before and during exercise (Azzu et al., 2010; Cluberton et al., 2005; Hildebrandt & Neufer, 2000; Millet et al., 1997; Schrauwen et al., 2002). The exact mechanism by which UCP-3 expression in skeletal muscle is regulated is still unclear. Although glucose consumption decreases UCP-3 mRNA expression in skeletal muscle, insulin produces the opposite response, which is an unexpected effect due to the insulinemic effect of glucose and their close positive correlation (Pedersen, Lund, Buhl, & Richelsen, 2001). A high-carbohydrate diet by itself or in combination with exercise causes down-regulation of UCP-3 expression in skeletal muscle (Brun et al., 1999b; Cluberton et al., 2005; Schrauwen et al., 2002). Conversely,

both high-fat and high-protein diets seem to induce the opposite effect, increasing UCP-3 expression (Brun et al., 1999b; Frier et al., 2011; Petzke et al., 2007). Even though UCP-3 shows a macronutrient dependent response in skeletal muscle, until now, most of the research has only focused on the response to exercise between fasting and glucose consumption. In an effort to clarify the regulation in the mRNA and protein expression of the mitochondrial protein UCP-3 in skeletal muscle, the primary purpose of the present study was to determine the effect of a multi-macronutrient meal replacement shake containing carbohydrates, protein, and fat in the pre-exercise period. A secondary purpose of the study was to assess the possible involvement of the transcription factors, PGC1-α and PPARα, on the regulation of the expression of this mitochondrial protein (Azzu et al., 2010; Brun et al., 1999a; Cluberton et al., 2005).

Serum Glucose Concentration

Contrary to our hypotheses, no significant difference in serum glucose concentration was observed between fasting and shake conditions. Although, an increase in glucose concentration was observed after the shake ingestion, it was not considered statistically significant. These outcomes could have been caused due to the lower amount of carbohydrates and subsequent glycemic load contained in the provided shake in comparison with previous studies (Schrauwen et al., 2002). The glucose solution consumed by the participants from Cluberton et al. (2005) doubled the amount of carbohydrates used in the present study; however, they were also not able to observe significant alterations in serum glucose. Conversely, using a concentration of carbohydrates 6 times higher than the one used in the present study, Schrauwen et al. (2002) were able to observe a significant increase in serum glucose concentration. The

meal replacement shake used in the current study contained 11g of fat and 13g of protein, in addition to carbohydrates, providing a total of 350 kcal. The caloric content provided by the shake was also lower than the caloric intake provided to the participants in other studies, nonetheless, it was still enough to overcome the 350 kcal expended in the exercise session (Cluberton et al., 2005; Schrauwen et al., 2002). The aforementioned studies provided their participants with a range of 470 to 1,200 kcal, respectively, of glucose. In the present study, the rationale for the selected multi-macronutrient shake in the pre-exercise period was to try to resemble the consumption of a typical meal in the pre-exercise period, which is more practically realistic as it is very unlikely that an individual would consume 1,200 kcal of glucose or any form of pure carbohydrate approximately prior to exercise. However, individuals often consume multi-macronutrient meals throughout the day, including the pre-exercise time period. Overall, the lack of a significant increase in serum glucose in the shake condition was most likely caused by the lower carbohydrate content from the multi-macronutrient shake.

Free Fatty Acids

Previous studies have consistently showed a significant increase in serum FFA during fasting conditions, which represents an increased lipolysis in order to satisfy the metabolic demands when glucose is scarce (Noland et al., 2003; Pilegaard, Saltin, & Neufer, 2003; Schrauwen et al., 2002). In the present study, a significantly higher FFA concentration was found in the fasting condition, which is in agreement with previous research (Cluberton et al., 2005; Schrauwen et al., 2002). This outcome is a critical factor for the present study since FFA are considered to be triggers of UCP-3 mRNA expression (Brun et al., 1999a; Schrauwen et al., 2002; Yoshitomi et al., 1998). Therefore, the fact

that FFA was significantly increased in the fasting condition could have potentially created a suitable environment for increased UCP-3 mRNA and protein expression, assuming that FFA triggers its expression.

PPARa and PGC-1α

PPARα and PGC-1α are two crucial transcription factors involved in the response to metabolic and physiologic stressors and regulate proteins which control lipolysis, beta-oxidation, and mitochondrial uncoupling (Azzu et al., 2010; Brun et al., 1999a; Leone et al., 1999; Minnich et al., 2001; Norheim et al., 2014; Yessoufou & Wahli, 2010). PPARα and PGC-1α are considered to be involved in UCP-3 expression activation and have shown to be increased by fasting (Cluberton et al., 2005). In the present study, no significant treatment or time main effects were observed for PPARα or PGC-1α mRNA expression, which is in disagreement with our hypotheses and with the general consensus for these transcription factors (Azzu et al., 2010; Cluberton et al., 2005). Furthermore, even though exercise caused an increase in PGC-1α mRNA expression in the shake condition at 4 h post-exercise, the increment was not considered statistically significant. Due to the fact that we were unable to see changes in PPARα or PGC-1α mRNA expression, besides observing significant alterations in both UCP-3 mRNA and protein expression, we conclude that the involvement of these transcription factors on the exercise induced UCP-3 expression needs to be further evaluated.

Uncoupling Protein-3 mRNA Expression

UCP-3 mRNA expression has shown to be increased by exercise in fasting conditions; however, it decreases when glucose is provided before and during the exercise session (Azzu et al., 2010; Cluberton et al., 2005; Hildebrandt & Neufer, 2000;

Millet et al., 1997; Schrauwen et al., 2002). In the present study, a significantly higher degree of UCP-3 mRNA expression was observed in the shake condition, which is contrary to previous results showing a significantly higher UCP-3 expression only in the fasting condition (Cluberton et al., 2005; Schrauwen et al., 2002). Furthermore, an increase in UCP-3 mRNA expression in both conditions was expected as a result of exercise; however, this effect took place only in the shake condition (Pedersen et al., 2001; Pilegaard et al., 2000). In the current study, the fact that exercise induced UCP-3 expression only in the shake condition suggests that exercise by itself is not a potent activator of UCP-3 mRNA expression (Schrauwen et al., 2002). Hildebrandt et al. (2000) demonstrated that fasting itself was able to increase UCP-3 expression in skeletal muscle while, in accordance with our results, increasing the metabolic demand by exercising attenuated UCP-3 expression. The decrease in UCP-3 expression in skeletal muscle was accompanied by decreases in the expression of enzymes involved in lipolysis such as lipoprotein lipase, CPT-1 and long chain acyl-CoA dehydrogenase. It seems that exercising while fasting creates an additional metabolic stressor, which changes the effects of fasting on mRNA and subsequent protein expression in skeletal muscle. Nonetheless, is interesting to note that a fasting period of 72 h did not cause a significant difference in the expression of the aforementioned genes which suggests an different regulatory mechanism for fasting and exercise regulating UCP-3 mRNA expression in skeletal muscle (Hildebrandt & Neufer, 2000). FFA have been proposed as a possible trigger for UCP-3 expression (Schrauwen et al., 2002; Yoshitomi et al., 1998); however, even though we observed a significantly higher FFA concentration in the fasting condition, the increase in UCP-3 mRNA expression was found only in the shake

condition (Schrauwen et al., 2002; Yoshitomi et al., 1998). These results are contrary to the results obtained by Schrauwen et al. (2002) and Cluberton et al (2005), who observed a significant increase in FFA and UCP-3 expression in the fasting condition. The different outcome between the present study and the studies performed by Schrauwen et al. (2002) and by Cluberton et al. (2005) could have been caused by three main variations between the protocols: the training status of the participants, the different nutritional approaches, and the intensity and duration of the exercise sessions. In the studies conducted by Schrauwen et al. (2002) and Cluberton et al. (2005), they recruited participants who were moderately active ($VO_{2\ peak}$ = 50.5 ± 2.4 mL/kg/min and 47 ± 6 mL/kg/min respectively), while in the present study untrained participants ($VO_{2\ peak}$ = 41.8 ± 7.4 mL/kg/min) were selected. The different training status of the participants could have accounted for the observed differential outcome, since it has been repeatedly demonstrated that training modifies the molecular response to exercise (Noland et al., 2003; Pilegaard et al., 2000; Tsuboyama-Kasaoka et al., 1998). With this in mind, the rationale for the selection of untrained participants in our study was with the intention of facilitating the identification of an acute response to exercise, unaffected by the training status. Another factor that could potentially have influenced the different outcome between the studies was the significantly lower caloric content and the different macronutrient composition of our selected nutritional approach. Our study involved the consumption of a multi-macronutrient shake containing carbohydrates, protein and fat in comparison with glucose solutions from previous studies that had higher caloric content (Cluberton et al., 2005; Schrauwen et al., 2002). Lastly, the duration as well as the intensity of the exercise sessions differ among the studies. These factors could explain, at

least in part, the different results obtained between the current study and previous studies. However, our results support the contention that factors other than FFA might regulate in a stronger way UCP-3 gene expression during moderate exercise. Kusuhara et al. (2005) demonstrated that UCP-3 expression was increased as soon as 5 min after the beginning of the exercise session, even though plasma FFA did not peak until the end of the session, suggesting that FFA did not play a key role in inducing UCP-3 mRNA expression (Kusuhara et al., 2005). Likewise, Noland et al. (2003) found no significant correlation between exercise-induced FFA and UCP-3 expression in skeletal muscle. Moreover, in lactation, over-nutrition has shown to increase UCP-3 expression in skeletal muscle, while under-nutrition has been shown to decrease it (Brun et al., 1999b). Another potential explanation of the results obtained in the present study could be a stimulatory effect by insulin on UCP-3 expression in skeletal muscle, an effect that has been shown to appear even in total absence of glucose (Pedersen et al., 2001). The shake consumption could have increased serum insulin, which in turn, increased UCP-3 expression only in the shake condition. This hypothesis explains the results of the present study; however, it does not explain the results obtained by Schrauwen et al. (2002), in which the increase in UCP-3 mRNA expression was observed only in the fasting condition. An alternative explanation of our results is that the multi-macronutrient composition of the shake changed the molecular effects that glucose itself has exhibited in previous studies. In this regard, high-fat and high-protein diets have independently been shown to induce UCP-3 expression in skeletal muscle, which differs from the effect observed with a high-carbohydrate diet (Brun et al., 1999b). Unlike most of the previous studies that have compared the effect of fasting and a post-prandial condition with glucose as a sole source

of energy, our study used a shake containing carbohydrates (50g), fats (11g) and protein (13g). The consumption of a multi-macronutrient shake at just one time point is not equivalent and cannot be compared with the acute diets performed by (Brun et al., 1999b). However, the fact that a high-protein and a high-fat diet regulate UCP-3 expression in an opposite fashion than a high-carbohydrate diet suggests that macronutrients could produce different metabolic effects on UCP-3 expression (Frier et al., 2011; Petzke et al., 2007). This hypothesis could explain why the increase in UCP-3 expression was observed only in the shake condition. Moreover, this hypothesis also explain the results presented by Schrauwen et al. (2002) in which glucose consumption totally blunted the normal increase in UCP-3 expression caused by exercise.

Uncoupling Protein-3 Protein Concentration

Although UCP-3 protein levels showed no significant differences between conditions, a significant increase in protein levels was observed at 4 h post-exercise compared with pre-exercise in the shake condition. Schrauwen et al. (2002) found no significant treatment or time effect on UCP-3 protein concentration. The fact that Schrauwen and his team found no significant alterations in protein concentration, even when they observed significant increments in UCP-3 mRNA expression, could be explained by a delay in translation. Since the peak of UCP-3 mRNA expression was observed at the very last sampling point (4 h), they were not able to detect significant changes in protein concentration due to translation delay. The same effect was observed in the current study in which UCP-3 mRNA expression increased at 1h post-exercise. This result allowed enough time for mRNA translation to occur, and conceivably resulted in our observed increase in UCP-3 protein concentration at 4 h post-exercise. Lastly, it

has also been shown that food intake is required for UCP-3 expression in newborn mice (Brun et al., 1999a). This evidence cannot be directly translated to adult humans; however, it does generate the question as to whether feeding could play a key role in UCP-3 activation in humans. Taking together from the UCP-3 mRNA and protein results, we can conclude that even though a significant increase in FFA concentration was observed in the fasting group, a consequent increase in UCP-3 mRNA expression was not observed in the same group. More importantly, a significant increase in UCP-3 mRNA expression and protein content was observed in the shake condition. This could be the result of an agonist effect of insulin, an inducing effect of protein and fat from the multi-macronutrient shake, or just from differences between the methodologies used among the studies.

Conclusion

Exercising while fasting and exercising in the post-prandial period cannot be treated as similar scenarios to fasting and the post-prandial period during resting conditions. According to Hildebrandt et al. (2000), and in accordance with the current study, fasting while exercising represents different and possibly opposite triggers for UCP-3 expression in skeletal muscle than fasting itself. This is the first study evaluating the effect of a multi-macronutrient meal consumed in the pre-exercise period on UCP-3 expression in skeletal muscle. Our results show that in addition to a significant increase in FFA in the fasting condition, a significant increase in both UCP-3 mRNA and protein expression was present only in the shake condition. Whether the increase in UCP-3 mRNA and protein expression was an effect of insulin or the multi-macronutrient composition of the shake, remains unclear. Our data suggest that macronutrients might

control in a different way UCP-3 expression in skeletal muscle in a unique way. In addition, our results are in disagreement with a potential effect of FFA on exercise-induced UCP-3 expression in skeletal muscle. In sum, the outcome of the present study shows that the multi-macronutrient shake was not able to down-regulate UCP-3 gene expression, moreover, the shake consumption surprisingly caused an increase in the expression of this mitochondrial protein. The before, could be interpreted as a favorable gene expression activation caused by the shake consumption in the pre-exercise period, which could potentially increase the metabolic rate.

Future perspectives

Researchers should seek to evaluate the effect of the following variables on UCP-mRNA and protein expression in skeletal muscle: 1) the various transcription factors involved in UCP-3 expression, 2) the individual effect of protein, fat and carbohydrate intake in the pre-exercise period, 3) the possible involvement of hormones and novel molecules (e.g. insulin and BAIBA) which may regulate UCP-3 expression, 4) the comparison between the response to single bouts and exercise training and, 5) the response of untrained versus trained participants.

APPENDICES

APPENDIX A

Informed Consent

BAYLOR UNIVERSITY

Department of Health, Human Performance, & Recreation
Informed Consent Form

Title of Investigation: Effect of Pre-Exercise Nutrition on Irisin Signaling and Metabolic Pathway Regulation in Participants Who Are of Normal Body Weight and Overweight

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Sponsor:
Exercise and Biochemical Nutrition Lab, Baylor University

Rationale:

The obesity epidemic has increased to the extent that the incidence of chronic hypokinetic diseases (e.g. ischemic heart disease, stroke and diabetes mellitus) are now the main cause of death in Western Society. A healthy diet along with an exercise program are the first line treatment for obesity; however, there is not enough scientific research on how to combine both treatments to get maximize the effectiveness of diet and exercise.

Uncoupling proteins, also known as UCP, increase energy dissipation as heat and increase energy expenditure in the long term. In this regard, it has been proven that UCP expression increases after exercise, an effect that is reverted when the exercise is preceded by glucose ingestion. Moreover, it seems that the elevation in free fatty acids results in increased UCP expression, and not the exercise by itself. In other words, glucose ingestion in the pre-exercise period prevents the normal increase in free fatty acids caused by exercise, thereby blunting exercise-induced UCP expression. Finally, Irisin signaling has shown to increase UCP expression after exercise. The absence in UCP activation by exercise when enough glucose has been provided could be caused by a lack of Irisin signaling. Having a blunted response in Irisin signaling can be a factor involved in decreased UCP expression, which can lead to decreased energy expenditure in the post-exercise period.

Based on the above mentioned, the purpose of this study is to assess the response of Irisin's metabolic pathway to exercise, when food has been provided in the pre-exercise period. Furthermore, an additional purpose is to assess the difference between participants who are considered to be of normal body weight and overweight.

Description of the Study

You will be one of 22 apparently healthy, physically-inactive males between the ages 18 to 30 who will participate in this study. You will be required to visit the laboratory on 3 separate occasions in the following manner: visit 1 = entry/familiarization session, visit 2 = exercise testing session with or without a pre-exercise meal, and visit 3 = exercise testing session with or without a pre-exercise meal. Relative to the testing sessions (visits 2 & 3), you will perform an exercise session in the bike in order to collect measurements of caloric expenditure. Each session will involve the gathering of data for the analysis of markers of metabolic function in blood and muscle samples (which you will have obtained at visits 2 & 3).

During an initial familiarization session (visit 1), you will be informed of the requirements of the study and sign an informed consent statement approved by the Institutional Review Board for the Protection of Humans in Research Baylor University. You will then be examined by trained study personnel and will complete health history questionnaires to determine if you are qualified to participate in this study. If you are cleared to participate, you will be familiarized to the methods and procedures involved in the study. Once the familiarization session is completed, you will be scheduled for the first testing session (visit 2) and instructed to refrain from any type of physical activity (other than routine walking) for 48 hours prior to each testing session (visits 2 & 3). You will also be informed to undergo a fasting period of 8 hours before each testing session at visits 2 and 3. You will be provided with a dietary analysis form to complete 24 hours prior to each testing session.

Once you report to the lab for each of the two testing sessions (visits 2 & 3), you will turn in the completed dietary analysis form. Body composition (body fat and muscle mass) will be determined using dual energy x ray absorptiometry (DEXA). The DEXA body

composition test will involve you lying down on your back in a comfortable position in a pair of shorts/t-shirt. A low dose of radiation will then scan your entire body for approximately 6 minutes. Radiation exposure from the DEXA is approximately 1.5 mR per scan. This is similar to the amount of natural background radiation you would receive in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose will be less than 5 mR for the entire study. Even know the DEXA scan only provides minimal radiation, there is an accumulative effect over a lifetime. Thus, if you have had had numerous x-rays over the course of the last year, or years, etc. you should take this into account when deciding whether or not to participate in the study.

At this point, you will undergo an exercise test on the bike. After baseline measurements at rest are completed for heart rate and blood pressure, Therefore, during the entry session, participants will perform the maximal cycling test. In brief, each exercise session will start with 1 min collection of resting data, 3 min warm up cycling at 25 W. Thereafter, the cycle ergometer work rate will be increased 25-W every minute, until you reach your maximum level of tolerance. The test will be terminated once you achieve volitional fatigue. The pedal rate will be maintained at 50 rpm throughout the test as confirmed by a metronome. You will be verbally encouraged by study personnel to provide a true maximal effort. The $VO_{2\ peak}$ test will be considered valid if you achieved a plateau in their $VO_{2\ peak}$ with an increased workload, if your respiratory exchange ratio is above 1.1 or if you attained the age predicted HR_{max} within 10 beats/min. Once you have reached a point in which your oxygen uptake does not continue to increase, you have reached your peak oxygen uptake and this test will be terminated. You will be encouraged to exercise maximally unless you begin to experience clinical signs to terminate the exercise test (i.e., angina, breathlessness, dizziness, a decline in blood pressure, lightheadedness, confusion, paleness, nausea, excessive increase in blood pressure, or excessive increase in heart rate. After exercise you will have undergo a 5-minute period of very low resistance in the bike for an active recovery. During exercise and both recovery periods, study personnel will be checking for abnormal responses (signs and symptoms) to exercise and will also be asking how you feel.

You will be required to fast for 8 hours prior each testing session at visits 2 & 3. Once you report to the laboratory, you will turn in the 24 hour dietary analysis form. You will rest for 5 minutes and then have your heart rate and blood pressure determined. You will then have the venous catheter inserted and the first blood sample obtained. For one exercise session you will then ingest the pre-exercise meal which will involve a commercially-available meal replacement drink (Ensure) which contains 350 kilocalories containing 53 g of carbohydrates (29 g sugar), 10 g of fat and 13 g of protein. After ingesting the milk-shake, you will wait for 30 minutes and then have another blood sample obtained at 1 hour and 4 hours post workout. For the remaining exercise session, the only difference is the absence of the milk-shake.

The workout will consist on working out in the bike at 70% of your $VO_{2\ peak}$ until you burn out 350 kcal (typically takes approximately 35 minutes). Your heart rate, oxygen

consumption and carbon dioxide production will be measured during the duration of the study.

At each of the two testing sessions (visits 2 & 3) you will donate a total of 50 milliliters (10 milliliters each time) of venous blood from an in-dwelling catheter inserted in a forearm vein in the arm using sterile techniques by an experienced phlebotomist involving standard procedures. Catheter insertion might take around 5 minutes and it may cause some pain as well as some bleeding and bruising, and it will securely remain in place by tape until the end of the testing process. However, proper pressure will be applied when removal to reduce bruising and experienced and qualified in phlebotomy personnel will be inserting the intravenous catheter. Prior to each blood sampling, the catheter will be flushed with sodium heparin to avoid coagulation (clotting within the catheter). You may also experience some dizziness, nausea, and/or faint if unaccustomed to having blood drawn. The personnel who will be performing the blood draws are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services.

In addition to the blood draws at each testing session (visits 2 & 3), you will have four muscle samples obtained by biopsy. The biopsy location will be identified on the thigh and the area will be shaved clean of leg hair, washed with antiseptic soap, and cleaned with rubbing alcohol. In addition, the biopsy site will be further cleansed by swabbing the area with Betadine (fluid antiseptic). A small area of the cleaned skin approximately 2 cm in diameter will then be anesthetized with a 1.0 mL subcutaneous (under the skin) injection of the topical anesthetic Lidocaine. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will only take 15-20 seconds. Complications resulting from the muscle biopsy are rare, especially in this case with the fine-needle biopsy technique, where the biopsy is similar to receiving a routine intramuscular injection as there is no open incision. However, there is a risk of infection if you do not adequately cleanse the area for approximately 24 hours post-biopsy. You will be instructed to remove the band-aid and cleanse the biopsy puncture site with soap and water every 4-6 hours, pat the area dry and reapply a fresh adhesive band-aid. You will be instructed to leave the band-aid on for 24 hours (unless unexpected bleeding). You will also be further advised to refrain from vigorous physical activity with the affected leg for 24 hours after the biopsy. There is a potential risk of an allergic reaction to the Lidocaine. You will be asked by study personnel about any such known allergies to local anesthetics (e.g. Lidocaine, Xylocaine, etc.) that they may have been previously given during dental or hospital visits. If you do have a known allergy to anesthesia medications you will not be allowed to participate in the study. The biopsy procedure may cause a small amount of pain when the needle is inserted to subcutaneously inject the Lidocaine and you may also experience some dizziness, nausea, and/or faint if unaccustomed to needles. However, due to the localized effects of the anesthetic, you should feel no pain during this process. However, the biopsy procedure may cause some slight bleeding and bruising. If needed, you may take non-prescription analgesic medication such as acetaminophen to relieve pain if needed. However, medications such as aspirin, Advil, Nuprin, Bufferin, or Ibuprophen are discouraged as these medications may lead to bruising at the biopsy site. Soreness of the area may occur for about 24 hours

post-biopsy. Darryn Willoughby, Ph.D., Sarah McKinley, M.S.Ed., and Tom Andre, M.S. are trained in blood draws and muscle biopsies and will be the only study personnel to perform these two procedures.

You agree to: 1) follow the instructions outlined by the investigators; 2) show up to all scheduled testing times; 3) ingest the pre-exercise meal as indicated; 4) not to take any nutritional supplement during the course of the study; 5) to fast for a period of 8 hours prior to the exercise session; and 6) not to take any non-medical prescribed medication. Failure to adhere to these guidelines may result in your removal from the study.

Exclusionary Criteria

You understand that in order to participate in the study, trained research personnel will examine you to determine whether you are qualified to participate. You also understand that you will not be allowed to participate in this study if you: 1.) have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; 2.) have a history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 3.) are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (ie, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications; 4.) have any bleeding disorders; 5.) have any chronic infections (e.g., HIV); 6) have a known allergic reaction to topical anesthetics; 7) have a known food allergy to dairy/milk products and/or soy products; and 8) have been involved in a weight loss regimen within the last 6 months.

You will be asked on the medical history questionnaire to report all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that you may be presently taking. You will also be asked to report if you have any known allergies to local anesthetics and if you have had any prior allergic reactions to topical anesthetics. You will also be asked if you have any known food allergies to milk and soy. The most common allergy-related symptoms after consuming milk protein are vomiting, hives and wheezing. Other symptoms that may develop alongside these primary ones are diarrhea, abdominal cramping, runny nose, watery eyes, loose stools, itchy skin, tingling around the mouth, chest tightness and coughing. The meal replacement supplement to be used in the study also contains soy. Symptoms of a soy allergy may be limited to one area of the body or may involve many areas. They can include flushing and/or itching skin, swelling lips and/or tongue, wheezing, shortness of breath, hoarseness or tightness in the throat, nausea and vomiting, colic, abdominal cramps and diarrhea.

By a medical history questionnaire, you will be asked to report whether you have any additional medical problems that would prevent participating in this study. You must agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that you take during the course of the investigation to Darryn Willoughby, Ph.D. (254-710-3504). If you experience any unexpected problems or adverse events from participating in this study you may be referred to discuss the problem with your primary

care physician, or if you are a Baylor student, the student health center to determine whether any medical treatment is needed and/or whether you can continue in the study.

You agree to report all changes in medical status, nutritional and/or pharmacological agents that you take during the study to the principal investigator Darryn Willoughby, Ph.D. or study personnel. If clinically significant side effects are reported from participation in the study, you should report them to study personnel and you will be referred to discuss the problem with Darryn Willoughby, Ph.D. Upon his discretion, you may be referred to discuss the matter with the Baylor Health Center and/or your primary care physician to determine whether any medical treatment is needed and/or whether you can continue in the study. Failure report your progress and health status to study personnel may result in you being removed from the study.

Risks and Benefits

There are minor risks of muscular pain and soreness associated with the endurance exercise protocol required in this study which are not uncommon to any exercise program especially for individuals who do not exercise on a regular basis. During each of the two testing sessions (visits 2 & 3) during this study, you will have approximately 10 milliliters of blood drawn from a forearm vein using standard procedures at five different time points. All blood sampling will be performed by an experienced phlebotomist. This procedure may cause a small amount of pain when the needle is inserted into as well as some bleeding and bruising. You may also experience some dizziness, nausea, and/or faint if unaccustomed to having blood drawn. Also, at each of the two testing sessions (visits 2 & 3), you will undergo a muscle biopsy in which a small sample of muscle will be obtained from the thigh at three different time points. Darryn Willoughby, Ph.D., Sarah McKinley, M.S.Ed., or Tom Andre, M.S. will perform all blood draws and muscle biopsies and that a local anesthetic (Lidocaine) will be injected into the skin of my thigh prior to the biopsy, which will help prevent any pain and discomfort during the procedure. A small puncture will be made in your skin and a biopsy needle introduced 1 cm into the muscle. The puncture is so small that it will be simply covered with an adhesive bandage (band-aid). After the anesthetic wears off within 2-3 hours, the sensation at the biopsy site is comparable to that of a bruise and may persist for up to 24 hours after the procedure. You are required to inform study personnel if you have had any prior allergic reactions to anesthesia (e.g. while in the hospital or during a dental visit).

The exercise test and the exercise sessions that will be performed may cause symptoms of fatigue, shortness of breath and/or muscular fatigue. However, they will be similar to the risks of participating in my normal training program. Trained, non-physician, exercise physiologists, certified in CPR, will supervise the exercise sessions and that a telephone and an automated electronic defibrillator will be available in the laboratory in case of any possible emergency. Emergency instructions are also posted in the laboratory in case of an unlikely event occurs.

What should you do if injured as a result of being in this study? If you become ill or injured as a result of your participation in the study, you should seek medical treatment

through your doctor or treatment center of choice. You should promptly tell the researcher about any illness or injury. There are no plans for Baylor University to pay you or give you other compensation for your injury or illness. You do not give up any of your legal rights to seek compensation by signing this form.

The primary benefit obtained from participating in this study is to gain insight about your health and fitness status from the body composition, exercise, and nutritional assessments to be performed. In addition, you may also gain insight into how your body responds to endurance exercise on the bike.

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, you must obtain treatment for any medical problem that may arise from your personal physician.

Costs and Payments

If a Baylor University student, you will not receive any academic credit for participating in this study. If an intercollegiate scholarship athlete, you will not be eligible to receive payment to participate in this study. If you successfully complete all requirements of the study will be paid $150. However, if you withdraw after the first testing session you will receive $75. You will be given free nutrition, body composition, and exercise assessments during the course of the study as described above and may receive information regarding results of these tests if you desire. Please check the appropriate space indicating whether or not you would like a copy of your results for being a participant in the study.

_____ I would like to receive a copy of my study results.

_____ I would NOT like to receive a copy of my study results.

New Information

Any new information obtained during the course of this research that may affect your willingness to continue participation in this study will be provided to you. In addition, you will be informed of any unusual/abnormal clinical findings in which medical referral to your personal physician may be warranted. If desired, you may request that this information be provided to your physician.

Confidentiality

Any information obtained about you in this research, including medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review your records from this study, which may include medical history, laboratory findings/reports, statistical data,

and/or notes taken about your participation in this study. In addition, records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. The data derived from your participation in this study may be used in reports, presentations, and publications. However, you will not be individually identified unless your consent is granted in writing. Additionally, confidentiality will be maintained by assigning code numbers to your files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. Unused blood and muscle samples will be kept in a locked freezer for no longer than one year. If any subsequent analysis occurs with the samples, they will be re-coded to further instill confidentiality.

Right to Withdrawal

You are not required to participate in this study and are free to refuse to participate or to withdraw from the study at any time. Further, your decision to withdraw from the study will not affect your care at this institution or cause a loss of benefits to which you might be otherwise entitled. If there is concern about your medical safety, you may be referred to seek medical attention.

Compensation for Illness or Injury

If you are injured as a direct result of taking part in this study, you should consult with your personal physician to obtain treatment. The cost associated with the care and treatment of such injury will be the responsibility of you and or your insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate you for injury or illness that may result from your participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, you may be referred to your personal physician if any clinically significant medical/psychological findings are observed during the course of this study. You agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss you suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

This study is funded by the Exercise and Biochemical Nutrition Laboratory at Baylor University, and that the researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

You certify that you have read this consent form or it has been read to you and understand the contents, and that any questions that you have pertaining to the research have been, or will be, answered by Darryn Willoughby, Ph.D. (principal investigator,

Department of Health, Human Performance & Recreation, 123 Marrs McLean Gymnasium, Baylor University, phone: 254-710-3504) or one of the research associates. Your signature below means that you are at least 18 years of age and that you freely agree to participate in this investigation. You will be given a copy of this consent form for your records. If you have any questions regarding your rights as a participant, or any other aspect of the research as it relates to you as a participant, please contact the Baylor University Committee for Protection of Human Subjects in Research, Dr. David W. Schlueter, Ph.D., Chair Baylor IRB, Baylor University, One Bear Place #97368 Waco, TX 76798-7368. Dr. Schlueter may also be reached at (254) 710-6920 or (254) 710-3708.

Date _____ Participant's Signature_____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

APPENDIX B

Proposal

Application to the Baylor IRB
For Review of Research/Activity Proposal

Part 1: Signature Page

1. Name Darryn S. Willoughby, Ph.D., FACSM, FISSN

2. Email Address (optional) Darryn_Willoughby@baylor.edu

3. Complete Mailing Address P.O. Box 97313

4. Position Associate Professor

5. Faculty Advisor (if researcher is Graduate Student) _____

6. Department/School HHPR/SOE & Biomedical Science Institute

7. Telephone # x3504 FAX # x3527

8. Are you using participants in research (***Y*** or N) or in teaching exercises (Y or ***N***)?

9. Title of the research project/teaching exercise: "Effect of Pre-Exercise Nutrition on Irisin's Metabolic Pathway on Participants with Different BMI"

10. Please return this signed form along with all the other parts of the application and other documentation to the University Committee for Protection of Human Subjects in Research; The chairman is Dr. Michael Sherr, School of Social Work, P.O. Box 97320, Waco, Texas 76798, phone number (254) 710-4483. If you have questions, or if you would like to see a copy of the OHRP Report on protection of human subjects in research, contact Dr. Sherr at extension 4483.

05/20/2014

_____ _____
Signature of Principal Investigator Date

_____
Signature of Faculty Advisor (required if researcher is a Graduate Student)
Departmental Review: _____
Department Chair or the Chair's Designate

Part 2. Introduction and Rationale

Obesity increases the prevalence of comorbidities, causing around 2.8 millions of deaths per year (WHO, 2013; Hall et al. 2010; Kenchaiah, 2002). Among other important factors, it is believed to be mainly generated by energy unbalances, either increased energy consumption, decreased energy expenditure or both (Pereira-Lancha et al. 2012; Wirfält et al. 2001; Denova-gutie et al. 2010).

Nutrition and physical activity are environmental factors that play a vital role in health status maintenance. Both are located in the first line of obesity treatment. From both, the most likely factor that has changed during human development is the levels of physical activity. The decrease of domestic duties along with the increase in sedentary activities, have considerably lowered the actual energy expenditure. According to Speakman and Selman, in order to achieve the energy expenditure of our primitive ancestors it would be necessary for us, to engage in 90 min of intense daily physical activity (Speakman & Selman, 2003).

Moreover, it has been shown that high levels of physical activity decrease mortality rates, effect that is further marked when the exercise is performed at high intensity (Samitz, Egger, & Zwahlen, 2011).

These physiological effects of physical activity have been known for decades, however, it was not until some years ago that the biochemical and molecular effects of exercise started to receive special attention from scientists (Moreno-Navarrete et al., 2013). One of the most recently discovered effects of exercise is the ability to convert white into beige adipocytes.

Beige adipocytes are similar than white adipocytes at basal conditions but, under stimulation (like cAMP activation), they change their protein expression towards brown like adipocytes. This configuration involves a higher production of uncoupling proteins - 1 (UCP-1), affecting by this way mitochondrial respiration. The increase in mitochondrial energy expenditure by beige adipocytes, seems to be equal or even higher than the observed in brown adipose tissue. It is endorsed by increases in UCP-1, Prdm16 and Cox8b expression, all of them markers of brown adipose tissue. The metabolic pathway activated to accomplish this configuration seems to be triggered by Irisin (Wu et al., 2012).

Irisin is a polypeptide of 112 amino acids produced my muscle that has shown to increase thermogenesis by augmenting in a dose-dependent manner the UCP-1 content in adipose tissue, which is able to rise energy expenditure by respiration uncoupling, preventing obesity and some of its metabolic comorbidities in the long term (Feldmann, Golozoubova, Cannon, & Nedergaard, 2009; Seale et al., 2011).

Human adipose tissue is also able to synthetize Irisin, however, its expression is 1/100 of the Irisin expressed by skeletal muscle. The before implies a possible endocrine effect from skeletal muscle to adipose tissue (Huh et al., 2012).

Irisin is the result of the C-terminal cleavage and posterior glycosylation of the Fibronectin Domain Containing Protein 5 (FNCD5) receptor, which is activated by the transcription factor Peroxisome Proliferator Activated Receptor γ Coactivator-1α (PGC-1α) (Boström et al., 2012; Schumacher, Chinnam, Ohashi, Shah, & Erickson, 2013) and it

is 100% identical in humans and mice, being at a concentration of 50.7-166.5 ng/ml in humans and around 40 nM in rodents (Boström et al., 2012).

PGC-1α is involved in a considerable number of cellular responses to external energy demanding factors. It controls mitochondrial biogenesis, expression of electron transport chain and oxidative phosphorylation proteins, expression of lipogenic genes, thermogenic response to cold and body fat regulation (Leone et al., 2005).

PGC1α is required for the increased UCP-1 expression in white adipose tissue after both a single bout and a chronic period of exercise training. Moreover, a single bout of aerobic exercise increases UCP-1 and UCP-3 expression in white adipose tissue and skeletal muscle respectively. However, the increased UCP expression in adipose tissue is the only one maintained after a training period of aerobic exercise (Noland et al., 2003; Ringholm et al., 2013).

Recently, it has been shown that the nutrition in the pre-exercise period is able of changing the gene expression normally affected by exercise. In this regard, it has been demonstrated that when enough glucose is provided before and during the exercise session, the normal increase in UCP expression observed after the exercise session is totally blunted, which could be the result of a null increase on serum fatty acid levels (Schrauwen et al., 2002).

Since Irisin has demonstrated to be a UCP expression activator and this one has shown to be blunted after exercise when glucose has been ingested before and through the exercise session, it is interesting to find out if the metabolic pathway leading to a blunted UCP response is changed by Irisin's metabolic pathway, however, no studies have been done in this area.

The goal of the present study is to assess from the molecular standpoint, the effect of pre-exercise nutrition on Irisin's metabolic pathway activation in the post exercise period. Furthermore, we will asses if differences exist in the activation of this metabolic pathway between a normal and an increased BMI (BMI >30 kg/m^2). The before will help to understand the metabolic post-exercise response when energy supply has been provided, and will also provide valuable information regarding possible differences among BMI, which could be helpful in the development of effective regimens aimed at magnifying energy expenditure caused by exercise.

Part 3: Methods

Experimental Approach

In a randomized, uniform-balanced, cross-over clinical trial, participants will perform two endurance exercise sessions, after a fasting period of 8 hours and after ingesting a provided breakfast (in one of the exercise sessions). Each exercise session will be separated by at least 7 days.

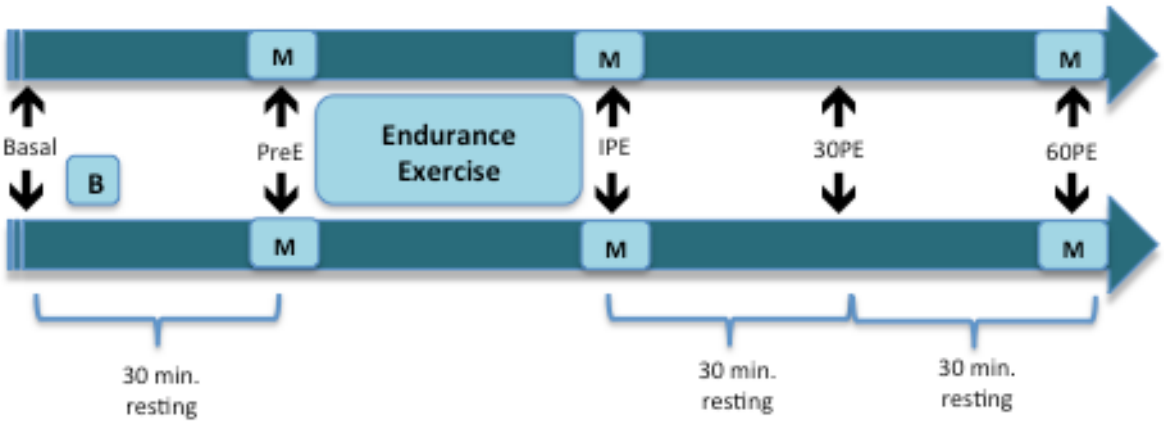

Figure 1. Experimental Design (B: breakfast, M: muscle biopsy, PreE: Pre-exercise, IPE: Immediately Post Exercise, 30PE: 30 minutes Post Exercise, 60PE: 60 minutes Post Exercise.

Participants

Thirty apparently healthy, young male (18-30 years) participants with an inactive lifestyle (less than three hours per week of doing low-intensity activities) will be recruited to perform two exercise sessions. Volunteers will be divided in one of two groups (15 participants each) according to their BMI (normal BMI or BMI > 30 kg/m^2). Enrollment will be open to men of all ethnicities.

Participants will be required to fill a medical screening before enrollment to ensure clearance to do exercise, according to the American College of Sports Medicine (ACSM) criteria. Volunteers will be excluded from participating in this study if they have consumed nutritional supplement a month prior the study.

Finally, all eligible participants will sign an informed consent approved by Baylor University. Approval will be granted by the Institutional Review Board for Human Subjects and all experimental procedures involved in this study will conform the ethical considerations of Helsinki Code.

Study Site

All exercise testing and exercise sessions will be conducted in the Baylor Laboratories for Exercise Science and Technologies (BLEST) and sample analyses will be performed on the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Independent and Dependent Variables

The independent variables will be the BMI of the participants and the provided breakfast (milk-shake). The dependent variables will include variables collected at the following times, at rest, immediately after exercise and during recovery:

- Irisin, FNDC5, PGC1α and UCP protein levels on skeletal muscle.

- mRNA expression of FNDC5, PGC1α and UCP on skeletal muscle.
- Irisin, glucose, fatty acids and insulin levels on serum.

Table 1. Overview of Research Design.

Familiarization	Testing Session 1	Testing Session 2
Familiarization session Informed Consent Form Health History Form General Exam to Determine Qualifications to Participate in Study Determination of Height and Body Weight Assessment of Body Composition Assessment of Peak Oxygen Consumption	24h Dietary Intake Form Muscle Biopsy (3) Blood Sampling (3) Provided Breakfast (randomly) Treadmill Exercise	24h Dietary Intake Form Muscle Biopsy (3) Blood Sampling (3) Treadmill Exercise

Entry and Familiarization Session (Visit 1)

Participants expressing interest in the study will be interviewed on the phone to determine whether they fulfill the inclusion criteria to participate in this study. Volunteers who meet eligibility criteria will then be invited to attend an entry/familiarization session.

Once in the lab, participants will complete a medical history questionnaire and undergo a general physical examination to determine whether they meet eligibility criteria.

Participants satisfying these criteria will be familiarized with the study design via a verbal and written explanation and will perform an assessment of their body composition and peak oxygen uptake ($VO_{2\ peak}$). At the end of the familiarization session, participants will be given an appointment in which to attend their next exercise session.

Finally, each participant will refrain from any type of exercise 24 hours before every exercise session, will come after 8 hours fasting (preferably in the morning) and will record their dietary intake the day before the exercise session.

Provided Breakfast

The nutritional information of a breakfast normally considered as healthy by american population (1 cup of non-sugary cereal with 1 cup of low fat milk) was calculated to assist in the proper milk-shake selection, to ensure a similar but reliable energy intake, that will be easy to digest and absorb. Ensure ® Original milk-shake was the shake chosen for the purpose of this study.

Assessment of Body Composition (Visit 1)

Lean and fat mass, along with body fat percentage, will be assed by Dual X Ray Absorptiometry (DEXA) during the familiarization session.

Assessment of Peak Oxygen Uptake ($VO_{2\ peak}$) (Visit 1)

During the entry session, participants will perform a $VO_{2\ peak}$ test on a treadmill using the Bruce protocol (ACSM, 2014). Oxygen uptake will be measured every 30 seconds via an open-circuit sampling system (Parvo Medics, Provo, UT), and the highest VO_2 will be defined as the $VO_{2\ peak}$.

Muscle Biopsies (Visits 2 & 3)

Percutaneus muscle biopsies (50-70 mg) will be obtained from the middle portion of the *vastus lateralis* of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. After the initial biopsy, for the remaining biopsies attempts will be made to extract tissue from 0.5 cm to the former from medial to lateral. Three muscle samples will be taken every exercise session, with a total of six muscle samples per participant during the course of the study.

Blood Sampling (Visits 2 & 3)

Venous blood samples will be obtained into 2 (10ml) vacutainer tubes from a 20 gauge intravenous catheter inserted into the antecubital vein. Immediately prior each blood sample is taken, the catheter will be flushed with 10 UI/ml of sodium heparin. Blood samples will stay at room temperature for 10 min and then centrifugated. The serum will be collected in micro-tubes and stored at -80°C for their later analysis. Five blood samples will be obtained at each exercise session, with a total of ten blood samples being obtained during the course of the study. At each testing session, blood samples will be obtained: 30 min before exercise, immediately before exercise, immediately after exercise, 30 min and 1 hour after exercise.

Dietary Analysis (Visits 2 & 3)

Participants will record their dietary intake 24 h before every exercise session. With exemption of the breakfast provided before one of the exercise sessions, diets won´t be standardized and participants will be asked to keep their normal dietary habits throughout the duration of the study. The 24 h recall will be evaluated using Food Processor dietary assessment software to determine the consumption of macronutrients throughout the duration of the study.

Testing Protocol (Visits 2 & 3)

In a randomized, cross-over fashion, participants will participate in two exercise sessions; each exercise session will be separated from at least 7 days. An illustration of the testing protocol can be seen in Figure 1 and Table 1. At each exercise sessions participants will report to the laboratory after an 8 hours fasting period. They will turn in their dietary logs, have their provided breakfast in the lab (just before one of the exercise sessions), have a muscle biopsy obtained, and then have an indwelling venous catheter inserted and the first blood sample obtained. Participants will then undergo a 20 min resting period (to give enough time for nutrients, when breakfast is provided). At the end of this period participants will then perform a standardized 3 min warm-up on the treadmill at 2 mph and 2% grade, then speed and grade will be adjusted to elicit the appropriate intensity for each condition (determined from the maximal oxygen consumption test performed in their first visit). Heart rates and respiratory analysis values will be checked every 5 min intervals and if necessary, the treadmill grade and speed will be adjusted to ensure a proper intensity throughout the duration of the study. At the end of the exercise session, participants will have their second muscle biopsy and blood draw, after which they'll rest for an hour and have their third muscle biopsy and blood sample.

Assessment of Serum Fatty Acids

Fatty acid content will be determined spectophotometrically using a free fatty acid quantification kit (Bio Vision, Mountain View, CA) at a wavelength of 540 nm. The assay will be read using the microplate reader Wallac Victor 1420, Perkin Elmer, Boston MA. Data analysis will be analyzed with MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany). All assays will be performed by duplicate.

Assessment of Serum Glucose, Insulin, Irisin and FNDC5 levels

Serum glucose levels will be measured enzymatically (GAHK20 Sigma-Aldrich), serum insulin levels will be determined using ELISA kits (RAB0327 Sigma-Aldrich), Irisin and FNDC5 will be analyzed by EIA (EK-067-29 and EK-067-19, respectively, from Phoenix Pharmaceuticals). Assays will be read with the Walac Victor 1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA), will be performed according to the manufacturer instructions and compared against a standard curve.

Assessment of Skeletal Muscle Protein Levels

Protein levels of Irisin (EIA kit from Phoenix Pharmaceuticals, EK-067-29), FNDC5 (EIA kit from Phoenix Pharmaceuticals, EK-067-19), PGC1-α and UCP-3 (using the ELISA kits No. ABIN422955 and ABIN836878, respectively, from antibodies online) will be measured by duplicate according to manufacturer instructions. Data analysis will be performed using MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany), and the final concentration will be expressed relative to muscle weight.

Assessment of Skeletal Muscle Protein Expression

Gene expression of FNDC5, PGC1-α and UCP-3 will be analyzed using the kits Hs00401006_m1, Hs01016719_m1 and Hs01106052_m1 from TaqMan Assays, Applied Biosystems. Samples will be run by duplicate using RT-PCR.

Statistical Analyses

Analysis of Variance (ANOVA) with repeated measures for each criterion variable will be used. Significance between groups will de determined using SPSS 11.0 software and a probability level of 0.05 will be adopted throughout the analysis.

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Research Team

Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN. Dr. Willoughby will serve as the principal investigator. He is an Associate Professor of Exercise and Muscle Physiology and Biochemistry in the Department of Health, Human Performance, & Recreation at Baylor University. He is also an Associate Professor of Baylor's Biomedical Science Institute. Dr. Willoughby is an internationally recognized exercise biochemist and molecular physiologist. He has conducted a vast amount of research focusing on the biochemical and molecular regulatory mechanisms regarding exercise performance and nutrition. Dr. Willoughby will be the principal supervisor of the project. He will oversee all aspects of the study and perform the majority of the biochemical and clinical chemistry assays involved in the project.

Brian Leutholtz, Ph.D., FACSM. Dr. Leutholtz is a Professor of Exercise Physiology in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Leutholtz will assist in providing oversight, in data collection, strength and body composition testing, and performing clinical laboratory assessments.

Elisa Morales, B.S. is a dietitian pursuing her M.S. in Exercise Physiology and serves as a research assistant in the EBNL. She will perform blood draws, muscle biopsies and bench work.

Sarah McKinley, M.S.Ed. Ms. McKinley is an exercise physiologist pursuing her Ph.D. in Kinesiology, Exercise Nutrition, and Preventative Health and serves as a research assistant in the EBNL. She will assist in all areas involved in the project.

Tom Andre, M.S. is an exercise physiologist pursuing his Ph.D. in Kinesiology, Exercise Nutrition, and Preventative Health and serves as a research assistant in the EBNL. He will assist in all areas involved in the project.

Procedures

Medical Monitoring. Interested participants will be invited to a familiarization session in which participants will sign a consent letter and complete a medical history questionnaire. Participants will then undergo a general exam, including a training history

and a cardiovascular risk assessment to determine whether the subject meets entry criteria to participate in the study. Trained exercise physiologist certified in CPR will supervise participants undergoing testing and assessments. A telephone is located inside the laboratory in case of any emergency, and there will be no more than two researchers working with each participant during testing sessions. In the event of any unlikely emergency, one of the researchers will check for signs and begin any necessary intervention while the other contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone to ask for assistance. Staff is required to inform any unexpected problem or adverse event throughout the study to the principal investigator, Darryn S. Willoughby Ph.D. If clinically significant signs or symptoms are reported, the participants will be referred to their personal physician for medical follow up. Recurrent findings or medical referrals of adverse events will be documented, placed in the participants research file and reported to the Baylor IRB committee.

Screening for Cardiopulmonary Disease Risk and Exercise Contraindications.

All participants will have their risk of cardiopulmonary disease and their possible contraindications to exercise assessed by Certified Exercise Physiologists in accordance to the standard procedures described by the American College of Sports Medicine (ACSM's Guidelines for Exercise Testing and Prescription, 9th ed. Williams & Wilkins Publishers, 2013). Only those participants considered as low to moderate risk for cardiovascular disease with no contraindications to exercise will be considered eligible:

ACSM Risk Stratification Criteria for Cardiovascular Disease (page 28)

Low Risk
Younger individuals (men < 45 years of age; women < 55 years of age) who are asymptomatic for cardiovascular disease and possess no more than two positive cardiovascular disease risk factor.

Moderate Risk
Older individuals and/or those who are asymptomatic for cardiovascular disease and possess two or more cardiovascular disease risk factors.

High Risk
Symptomatic patients or with known cardiovascular, pulmonary, renal or metabolic disease.

ACSM Criteria for Signs and Symptoms Suggestive of Cardiovascular Disease

1. Pain, discomfort in the chest, neck, jaw, arms, or other areas that may be due to myocardial ischemia.
2. Shortness of breath at rest or with mild exertion.
3. Dizziness or syncope.
4. Orthopnea or paroxysmal nocturnal dyspnea.

5. Ankle edema.
6. Palpitations or tachycardia.
7. Intermittent claudication.
8. Known heart murmur.
9. Unusual fatigue or shortness of breath with usual activities.

ACSM Absolute and Relative Contraindications to Exercise

Absolute Contraindications
1. Unstable angina.
2. Uncontrolled dysrhythmias.
3. Recent ECG changes and cardiac events.
4. Symptomatic severe aortic stenosis
5. Uncontrolled symptomatic heart failure.
6. Acute myocarditis or pericarditis.
7. Acute pulmonary embolism or acute myocardial infarction.
8. Dissecting aneurysm.
9. Acute infections.

Relative Contraindications
1. Left main coronary stenosis.
2. Moderate stenotic valvular heart disease
3. Electrolyte abnormalities
4. Severe arterial hypertension (> 200/115).
5. Tachycardia or bradycardia.
6. Neuromotor or rheumatoid disorders that are exacerbated by exercise.
7. Uncontrolled metabolic disease.
8. High-degree AV block.
9. Chronic infectious disease.
10. Hypertrophic cardiomyopahty and outflow obstructions.
11. Ventricular aneurysm.
12. Mental or physical impairment leading to inability to exercise adequately.

Assessment of Hemodynamic Safety Markers (Heart Rate & Blood Pressure)

Heart rate will be determined using a heart rate monitor (POLAR) placed in the chest. Blood pressure will be assessed in the supine position after resting for 5 min using a standardized procedure with a mercurial sphygmomanometer.

Estimated Energy Intake/Dietary Inventories

Participants will record their food intake 24 hours before every exercise session, using a provided food log form in order to standardize their nutritional intake. Participants will bring those forms at the beginning of every exercise session. Dietary intake will be assessed using the Food Processor IV Nutrition Software.

Body Composition Assessment

Body composition will be determined using a calibrated Hologic 4500 W Dual Energy X-Ray Absorptiometry (DEXA). This test divides the body in three components: fat, free mass and bone mass. It involves having the participant lie down on their back in a standardized position. A low dose of radiation will then scan the body for approximately 6 min. Radiation exposure from DEXA for the whole body scan is approximately 1.5 mR per scan, which is similar to the amount of natural background radiation a person would receive in one month while living in Waco, TX. The maximal permissible X-Ray dose for non-occupational exposure is 500 mR per year.

Aerobic Exercise Capacity Test

A sterile mask will be secured on the subject. Resting expired gases will be collected using the Parvo Medics 2400 TrueMax Metabolic Measurement System (Sandy, Utah). Once the participant is ready to begin the test protocol, the subject will straddle the treadmill with both legs while the treadmill is turned on at a speed of 2 mph at a 2% grade to help the participant feel comfortable while using the treadmill. Then, the participant will perform a standard symptom-limited Bruce Treadmill maximal exercise test using the following speeds and grades:

Stage	Speed	Grade (%)	Duration (min.)
1	1.7	10	3
2	2.5	12	3
3	3.4	14	3
4	4.2	16	3
5	5.0	18	3
6	5.5	20	3
7	6.0	22	3

The participant will be encouraged to exercise to their maximum unless the subject experiences clinical signs or symptoms to terminate the exercise test as stated by the ACSM's Guidelines for Exercise Testing and Prescription (i.e., angina, dyspnea, dizziness, a decline in systolic blood pressure, lightheadedness, confusion, ataxia, cyanosis, nausea, excessive rise in systolic blood pressure over 250 mmHg or diastolic over 120 mmHg, chronotropic impairment, failure of the monitoring system, or other signs or symptoms for terminating the test). The test can also be terminated by participant's petition. Once the exercise test is complete, the participant will stay in active recovery for 3-6 min followed by a 3-6 min seated recovery period. Expired gases and heart rate will be monitored continuously throughout the exercise test. Participants will

be asked to report any unusual signs or symptoms to the exercise specialists during the exercise test. This test will determine maximal aerobic capacity and ventilator anaerobic threshold.

Blood Samples

Participants will donate approximately 20 mililiters of venous blood at each blood draw. Using standard phlebotomy, blood samples will be obtained from an intravenous catheter placed into the antecubital vein by Darryn Willoughby, Ph.D., Elisa Morales, B.S. or Sarah McKinley M.S.Ed. and Tom Andre, M.S.Ed. who are trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. While drawing blood, study personnel will wear personal protective clothing (gloves, lab coats, etc.) when handling blood samples. Subjects will be seated in a phlebotomy chair, a tourniquet will be applied high on the brachium and will be tight enough without causing discomfort (Figure 2). The site will be cleaned first with an alcohol pad and then with a betadine swab (Figure 3). The participant will be instructed to make a fist several times in order to maximize venous engorgement. The appropriate vein will be selected (Figure 4), however, if a suitable vein is difficult to find, the pads of the first and second fingers will be used to slap the veins gently to help dilate them. Alternately, the arm may be covered with a warm, moist compress to help with vasodilation. If no suitable veins are found, then the tourniquet will be released from above the elbow and placed around the forearm to search in the distal forearm, wrist and hand. If still no suitable veins are found, the other arm will be checked.

To puncture the vein, the 20 gauge will be held in the dominant hand, entering the skin at a 30 degree angle and in the direction of the vein. After entering the vein the angle will be decreased until it is nearly parallel to the skin (Figure 5). If the vein appears to “roll” (move around freely under the skin), the venipuncture will begin by applying counter tension against the skin just below the entry site using the thumb of the non-dominant hand and pulling distally toward the wrist carefully. The catheter will be advanced into the vein until blood is seen in the “flash chamber” of the catheter. After entering the vein, the plastic catheter (located over the needle) will be advanced into the vein while leaving the needle stationary (Figure 6). The hub of the catheter will be all the way to the skin puncture site. The tourniquet will be released. The needle will be removed from within the plastic catheter and disposed in an appropriately, labeled sharps container (Figure 7). The catheter will be taped in place using the strips of tape and a sterile dressing (Figure 8). Immediately prior to blood sampling, the catheter will be flushed with 10 IU/ml of sodium heparin. Once samples are obtained, the catheter will be removed and discarded as hazardous waste in an appropriately labeled biohazard waste container. The site of the blood draw will then be cleaned with a sterile alcohol pad and a sterile band-aid will be placed on the site. The alcohol wipe and gauze then will be discarded in an appropriately-labeled biohazard waste receptacle. The blood collection tubes will be labeled and placed in a test tube rack for 10 min, then it will be centrifugated and transferred into serum labeled containers, and finally stored at -80 C for later analysis.

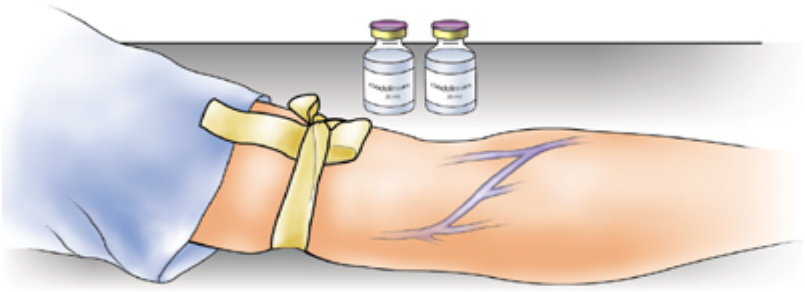


Figure 1.

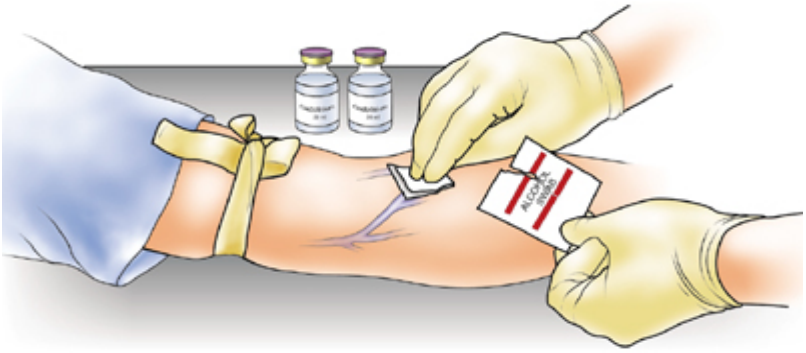


Figure 2.

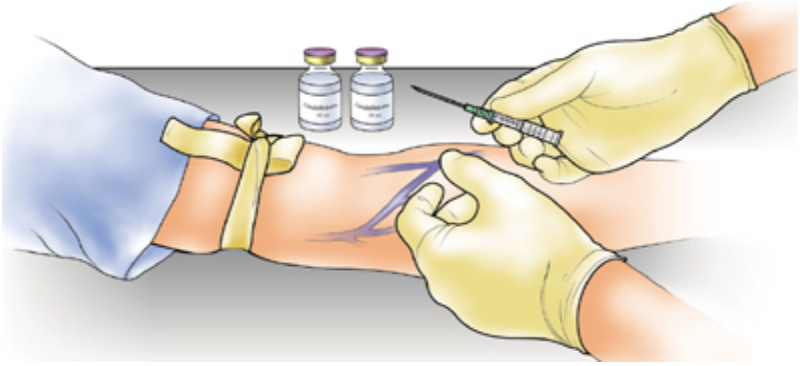


Figure 3.

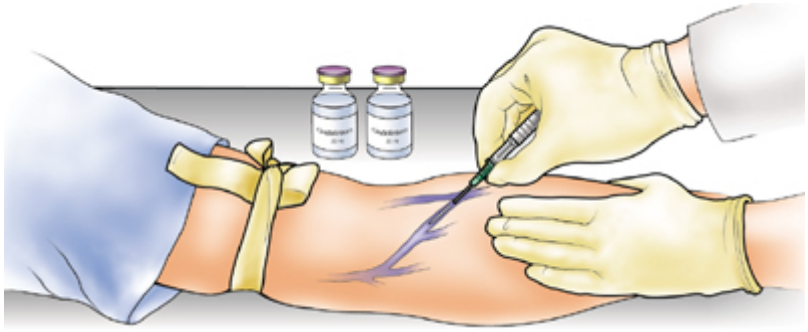


Figure 4.

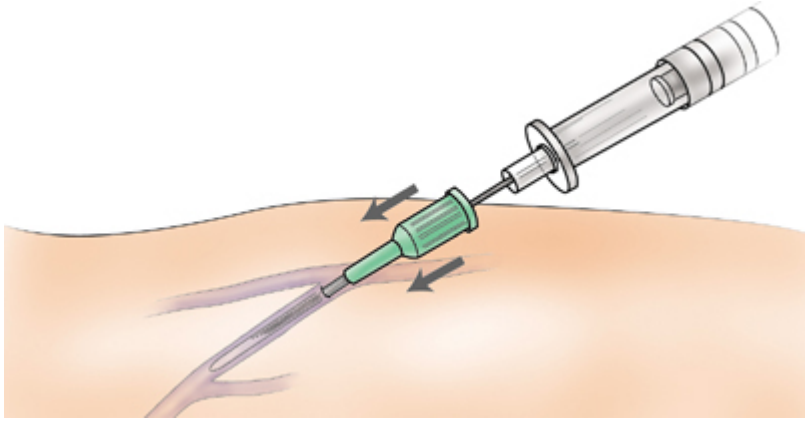


Figure 5.

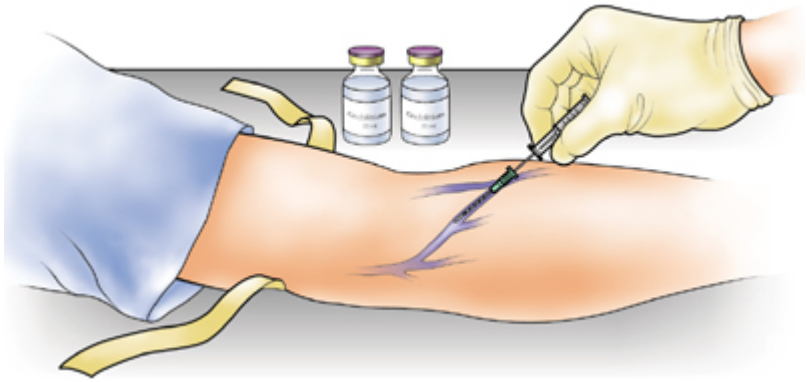


Figure 6.

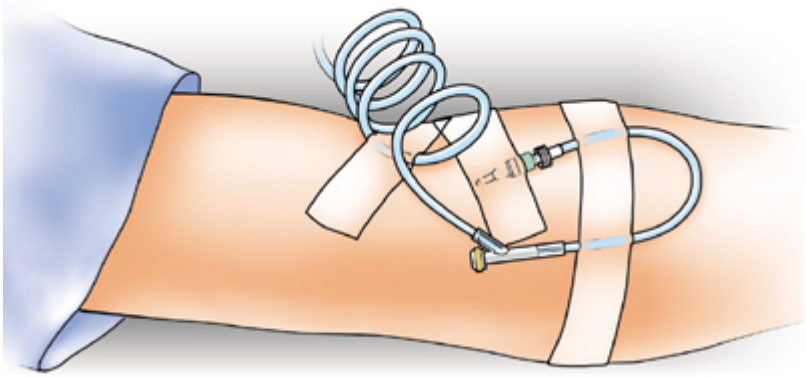


Figure 7.

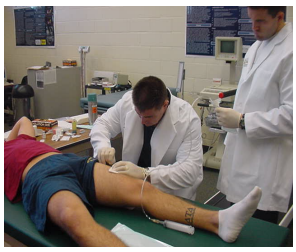
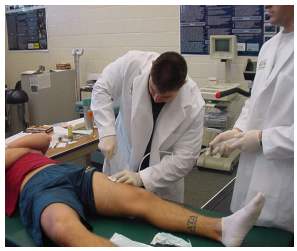
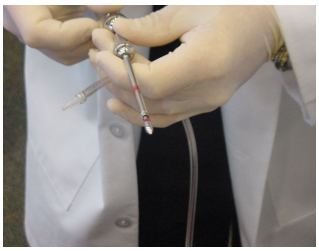
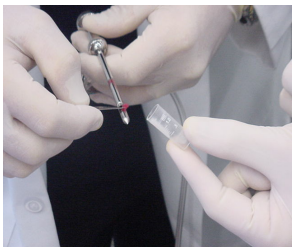
Muscle Biopsies

Percutaneous muscle biopsies (approximately 50-70 mg) will be obtained from the vastus lateralis of each participant's thigh. Samples will be extracted after 1% Lidocaine at the midway between the patella and the greater trochanter of the femur by Darryn Willoughby. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will take approximately 15-20 seconds. For each biopsy, muscle tissue will be extracted from the same location by using the previous markings. First, the participant will be in supine position on a sterilized table. Once the extraction point is identified, the area will be shaved and cleaned with rubbing alcohol and a Betadine swab. A small area of the skin will be anesthetized with a 1.0 mL of Lidocaine. Once anesthetized, a needle point will be used to produce the initial biopsy site by making an incision through the skin, the participant shouldn't feel pain during this process due to the localized effects of the anesthetic. Then, the biopsy needle will be advanced into the incision approximately 1 cm and during the process, the subject may feel pressure to the thigh area. Once the muscle sample has been obtained, pressure will be immediately applied and the wound will immediately be bandaged.

Due to the small incision, bleeding is so slight; therefore, only a butterfly bandage is needed to close the incision, which is then covered with a pressure bandage. The needles will be discarded as hazardous waste in an appropriately labeled sharps container. The site of the biopsy will be cleaned with a sterile alcohol wipe and gauze, which will be then discarded in an appropriately labeled biohazard waste receptacle. The tissue sample will be stored at -80 C for future analyses.

Written instructions for post-biopsy care will be given to the subjects. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report back to the lab within 24 hours to have the old bandages removed, the incision inspected and bandages applied. The participant will be further advised to refrain from vigorous physical activity during the following 48 hours post-biopsy. These suggestions will minimize pain and possible bleeding of the affected area. If needed, the subject may take non-prescription analgesic medication such as

acetaminophen to relieve pain if needed. However, medications such as aspirin, Advil, Nuprin, Bufferin or Ibuprophen will be discouraged as these medications may lead to ecchymosis at the biopsy site. Soreness of the area may occur for about 24 hours post-biopsy.

Equipment

Digital Scale. Body weight will be measured using a digital scale ± 0.02 kg.

Mercurial Sphygmomanometer. Blood pressure will be assessed by auscultation of the brachial artery with a mercurial sphygmomanometer using standard clinical procedures.

Dual-Energy X-Ray Absorptiometer (DEXA). Body composition measurements will be determined by qualified personnel (in compliance with State Regulations) using a Dual Energy X-ray Absorptiometer (Watlham, MA). This system segments the body into three compartments (i.e., bone mass, fat mass, and fat-free mass). Calibration will be performed on a spine phantom (Hologic X-CALIBER Model DPA/QDR-1 anthropometric spine phantom) prior to each testing session.

Aerobic Exercise Capacity Testing. Maximal cardiopulmonary measurements will be obtained using Parvo Medics 2400 TrueMax metabolic measurement system (Sandy, Utah) and a treadmill ergometer (Quinton, Seattle, WA).

Serum Analyses. Enzyme-Linked Immunosorbent Assay (ELISA) and Enzyme Immunoassay (EIA) will be read spectrophotometrically with a Wallac Victor-1420 micoplate reader (Perkin-Elmer Life Sciences, Boston, MA). All assays will be performed against a standard curve.

Participants

Recruitment

Thirty participants with either normal BMI or BMI $\geq$ 30 kg/m^2 , between the ages of 18 - 30 years, with non physically active lifestyles (< thrice weekly of doing low to moderate physical activity) will be assigned in one of two groups according to their BMI (15 participants per group). Enrollment will be open for all ethnicities. Recruitment will be achieved by postings on campus and at near fitness centers.

Selection Criteria

Non-inclusion criteria:

1. Have been involved in an habitual training program (more than 3 hours/week) of low to moderate intensity exercise.
2. Use tobacco.
3. Have allergy to anesthetics.
4. Have an orthopedic limitation that would limit aerobic exercise.
5. Have any of the following diseases or disorders: heart disease, arrhythmias, diabetes, thyroid disease, bleeding disorder, history of pulmonary disease, hypertension, hepatorenal disease, musculoskeletal disorder, neuromuscular/neurological disease, autoimmune disease, cancer, peptic ulcers, anemia or chronic infection (HIV).
6. Are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic, psychotropic, neuromuscular or androgenic medications.
7. Have taken ergogenic levels of nutritional supplements that may affect muscle mass (e.g., creatine) or anabolic/catabolic hormone levels (e.g., androstenedione, DHEA, etc.) within three months prior to the start of the study.
8. Have any absolute or relative contraindication for exercise as outlined by the American College of Sports Medicine.
9. Report any unusual adverse events associated with this study that in consultation with the principal investigator recommends no acceptance or removal from the study.
10. Are bodyweight stable during the previous six months and are not currently undergoing any type of weight loss intervention.

Compensation or Incentives

Participants completing familiarization and testing sessions as well as turning in all required materials (food logs) in the study will be paid $150. Subjects may receive information regarding their results if they desire. In case the subject is a Baylor student, they will not receive extra credits for participating in the study.

Potential Risks

Participants who meet eligibility criteria will be subjected to a treadmill exercise session to determine peak oxygen consumption involving repetitive muscle contractions. Even though the participants are physically active, they may also experience short-term muscle soreness, moderate fatigue and muscle strains. During the familiarization session participants will be informed of the treadmill protocol and the possible soreness associated with it.

Participants will donate about 20 milliliters of venous blood a total of ten times during the study by using an intravenous catheter and the standard procedure already described. This procedure could cause a small amount of pain, as well as some bleeding and

bruising. However, proper pressure will be applied upon removal to reduce bruising. The subject may also experience some dizziness, nausea and or faint if they are unaccustomed to have blood drawn.

Complications resulting from the muscle biopsy are rare, because of the type of biopsy (similar to routine intramuscular injection). However, there is a risk of infection if the subject does not adequately clean the area for approximately 48-72 hours post biopsy. participants will be instructed to clean the biopsy area with soap and water every 4-6 hours, dry the area and apply a fresh adhesive bandage. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report back to the lab within 24 hours to have the old bandage removed, the incision inspected and new bandages applied. Volunteers will be advised to refrain from vigorous physical activit the following 24 hours after the biopsy. There is a potential risk for an allergic reaction to Lidocaine. All subjects will be asked if they have known allergies to local anesthetics that they may have been previously given during dental or hospital visits. Participants with known allergies to anesthesia medications will not be allowed to participate in the study. Darryn Willoughby Ph.D. will perform all muscle biopsies. Researchers involved in collecting data are trained, non-physician exercise physiologists. All personnel involved in collecting data will be certified in CPR. A telephone and automated electronic defibrillator (AED) are located in the laboratory in case of any emergency and there will be no less than two researchers working with each subject during the testing. In the event of any unlikely emergency, one researcher will check for vital signs and the other will call Baylor's campus police (# 2222). Instructions for emergencies are posted above the phone.

Potential Benefits

The direct benefit the participants will obtain from this study is to gain insight about their health and fitness status from the assessments to be performed. Moreover, their participation in this study will help to elucidate whether or not nutrition in the pre-exercise period does affect energy expenditure throughout mainly Irisin pathway activation blockage.

Assessment of Risk

This study will help to elucidate the role of nutrition in the pre-exercise period in order to achieve the maximum benefit in energy expenditure, through activation of Irisin and UCP related metabolic pathways. The biggest risk associated with participating in this study will be performing the single bouts of aerobic exercise. However, since the participants to be recruited will be physically fit and all will undergo medical screening, the risk will not differ from doing exercise by tier own. Therefore, the potential benefits of participating in this study outweigh the potential risks.

Compensation for Illness or Injury

Each participant will agree to indemnify and hold harmless Baylor Unviersity, its officers, directors, employees and students for any injury, damage, loss or claim related as a result of participation in this study regardless of the cause.

Confidentiality

Information obtained from this research (including questionnaires, medical history, laboratory findings, physical examinations, statistical data or notes taken through this study) to the extent required by law. However, as sustained by FDA regulations, records will be open to FDA representatives if necessary. Records of the research can also be open by court order or federal regulatory authorities. Participants will have a code to identify their results and data derived from this study will not be individually identify unless participants give their written consent. Data will be stored and locked in the Exercise and Biochemistry Laboratory and only the principal investigator Darryn Willoughby, Ph.D. will have access to the key. Personnel involved in the research can have access to the data through Darryn Willughby Ph.D. permission. All evidence of primary data will be stored for exactly three years after the completion of the study. At this time data will be destroyed.

Data Presentation and Publication

Data will possibly be presented at an appropriate scientific conference (International Society of Sports Nutrition, American College of Sports medicine, Experimental Biology, etc.) and published in peer review scientific journals (International Journal of Sports Nutrition and Exercise Metabolism, Medicine & Science in Sport and Exercise, Journal of Sport and Medicine, etc.).

Statement of Conflict of Interest

Funding for this study will be provided by the Exercise and Biochemical Nutrition Laboratory. Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Informed Consent Form Checklist

When using humans as subjects in research you must obtain their informed consent. Check each of the following items as they appear on your Informed Consent Form and include this checklist with your protocol:

__x_(a) A statement explaining the purpose of the research.

__x_(b) A statement of the expected duration of the subject's participation.

__x_(c) A description of the procedures to be followed.

__x_(d) A description of any reasonable foreseeable risks or discomforts to the subject, including invasion of privacy.

__x_(e) A description of any benefits resulting from the research, either to the subject or to others.

__x_(f) A statement that informs subject of his/her right not to be a subject in a research project that is also a teaching exercise.

__x_(g) A statement informing subject about how his/her anonymity will be guarded; i.e., that their confidentiality will be protected by assigned code numbers, by limitations of who has access to data, by data storage in locked cabinets, by locked computer files, etc.

__x_(h) A statement that the subject's participation is voluntary, and that his/her refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

_na_(i) A disclaimer, if applicable, regarding the use of the Internet to collect data.

__x_(j) For research involving more than minimal risk, an explanation regarding the availability of any compensation or any medical treatments if injury occurs (if applicable, see OHRP Reports).

__x_(k) If written informed consent is required, a place for the subject to sign and date the form and a statement that a copy of the signed consent form will be given to the subject for his/her records.

_na_(l) If the subject is a minor, a statement of parental responsibility in consenting to the child's participation in the study with a place for the parent to sign and date the form in addition to the participant's signature.

__x_(m) Include a short summary of your expertise related to this research proposal.

__x_(n) The name, address, and telephone number of the principal investigator of the research project, and his/her affiliation with Baylor University. If the principal investigator is a graduate student, the name and telephone number of the faculty advisor is also required.

__x_(o) A statement informing subject that inquiries regarding his/her rights as a subject, or any other aspect of the research as it relates to his/her participation as a subject, can be directed to Baylor's University Committee for Protection of Human Subjects in Research. The chairman is Dr. Matt Stanford, Professor Psychology and Neuroscience, PO Box 97334, Waco, Texas 76798-7334, phone number 254-710-2236.

APPENDIX C

Recruitment Flyer

GUYS, WE NEED YOU!

Men Needed for an Exercise Study

Researchers in the Department of Health, Human Performance, and Recreation at Baylor University are recruiting 22 healthy men who normally perform low to moderate intensity physical activity no more than three hours weekly. Participants will be required to be located between the ages of 18-30 years and have either normal (18.5-24.99 kg/m^2) or increased body mass index (BMI) ($\geq$30 kg/m^2). The purpose of the study is to evaluate the effect that nutrition in the pre-exercise period has on skeletal muscle hormones related with energy expenditure on people with different body weights/BMI. Participants will be required to engage in 2 separate testing/exercise sessions and undergo aerobic exercise, body composition testing, blood sampling, and muscle biopsies (fine needle technique). Eligible participants will receive $150 for completing the study. Participants will also receive free body composition analysis (DEXA), free aerobic fitness exercise testing ($VO_{2\ peak}$) and nutritional counseling.

For more information contact:
Elisa Morales
E_Morales@Baylor.edu or
Darryn Willoughby, Ph.D.
Exercise & Biochemical Nutrition Lab
Marrs McLean Gym, Room 123
darryn_willoughby@baylor.edu
254-710-3504

APPENDIX D

Wound Care for the Muscle Biopsy Procedure

Complications resulting from the muscle biopsy procedure are rare. Furthermore, after the procedure, you can reduce your risk of chance of infection by adhering to the following course of action for wound care:

For approximately 24 hours post biopsy,

- Leave the bandage(s) on for 24 hours (unless unexpected bleeding or pain occurs, which should be immediately reported to the lab)
- lightly clean around the bandage(s) if necessary
- report back to the lab within 24 hours to have the old bandage(s) removed, the incision inspected and new bandages applied
- refrain from vigorous physical activity with the leg during the first 24 hours post-biopsy

After the 24-hour follow-up, (for approximately 72 hours post biopsy)

- leave the butterfly bandage in place • adequately cleanse the area surrounding the bandage with soap and water every 4- 6 hours, and pat the area dry • reapply a fresh adhesive bandage

At approximately 72 hours post biopsy

- return to the lab, allow the incision sight to be inspected, butterfly removed, and new bandages applied
- leave these bandages on for 24 hours (unless unexpected bleeding or pain occurs)
- return to normal hygiene practices unless complications arise

Possible pain & side effects

Soreness of the area comparable to that of a bruise will likely persist for 24 hours, and possibly even 36 hours, after the biopsy procedure. Following the procedures outlined above should significantly minimize pain and possible bleeding of the area. However, some subjects experience no significant pain post biopsy.

- If needed, the subject may take non-prescription analgesic medication such as Acetominophen to relieve pain if needed.
- Medications such as aspirin, Advil, Bufferin, Nuprin, and Ibuprofen are discouraged as they may lead to excess bruising at the biopsy site.

If any questions or complications arise please contact: Darryn Willoughby, Ph.D.
Room # 120 Marrs McLean Gym (254) 710-3504 Darryn_Willoughby@baylor.edu

APPENDIX E

Medical History Inventory

BAYLOR UNIVERSITY
EBNL

Directions. The purpose of this questionnaire is to enable the staff of the Exercise and Sport Sciences Laboratory to evaluate your health and fitness status. Please answer the following questions to the best of your knowledge. All information given is CONFIDENTIAL as described in the Informed Consent Statement.

Name:_____ Age: _____Date of Birth: _____

Name and Address of Your Physician:

_____

MEDICAL HISTORY

Do you have or have you ever had any of the following conditions? (Please write the date when you had the condition in blank).

___ Heart murmur, clicks, or other cardiac findings?
___ Frequent extra, skipped, or rapid heartbeats?
___ Chest Pain (with or without exertion)?
___ High cholesterol?
___ Diagnosed high blood pressure?
___ Heart attack or any cardiac surgery?
___ Leg cramps (during exercise)?
___ Chronic swollen ankles?
___ Varicose veins?
___ Frequent dizziness/fainting?
___ Muscle or joint problems?
___ High blood sugar/diabetes?
___ Thyroid Disease?
___ Low testosterone/hypogonadism?
___ Glaucoma?
___ Asthma/breathing difficulty?
___ Bronchitis/Chest Cold?
___ Melanoma/Skin Lesions?
___ Stroke or Blood Clots?
___ Emphysema/lung disease?
___ Epilepsy/seizures?
___ Rheumatic fever?
___ Scarlet fever?
___ Ulcers?
___ Pneumonia?
___ Anemias?
___ Liver or kidney disease?
___ Autoimmune disease?
___ Nerve disease?
___ Psychological Disorders?

Do you have or have you been diagnosed with any other medical condition not listed?

_____

Please provide any additional comments/explanations of your current or past medical history.

_____

Please list any recent surgery (i.e., type, dates etc.).

_____

List all prescribed/non-prescription medications and nutritional supplements you have taken in the last 3 months.

_____

What was the date of your last complete medical exam?

_____

Do you know of any medical problem that might make it dangerous or unwise for you to participate in this study (including strength and maximal exercise tests) ____ If yes, please explain:

_____

Recommendation for Participation

____ No exclusion criteria presented. Subject is cleared to participate in the study.

____ Exclusion criteria is/are present. Subject is not cleared to participate in the study.

Signed: _____ Date: _____

APPENDIX F

Pre-Screening Form

Pre-Screening Form of the study "Effect of Pre-Exercise Nutrition on Irisin's Metabolic Pathway on Participants with Different BMI"

Name:_____Date:_____
Address:_____City:_____State:_____ Zip Code:_____
Phone:_____E-mail:_____ Birth date: ___/___/____ Age:___

Inclusion Criteria	Satisfy?	Comments
Are you a male with an age between 18-30 years?		
Do you perform exercise less than three hours per week of low to moderate intensity activities?		
Are you currently taking supplements?		
Are you willing to stop using supplements during the duration of the study?		
Have you been involved in a weight lost program in the last month?		
Have you decreased your body weight in the last month?		
Do you consume any medication?		
Do you have a problem with blood draws or muscle biopsies?		
Would you be able to attend for a period of 6 hours to the lab, starting from 7:00-10:00 am any day of the week in two sessions separated for a week?		
Is your BMI located in the normal range or $\geq$ 30 kg/m^2?		
Are you lactose tolerant?		

Researcher	Check
Explanation of experimental design to participant	
Fill out Pre-Screening Form (above table)	
Sign Consent Form	
Complete Health-History Questionnaire	
Take anthropometric measurements	
Take blood pressure and heart rate	
Perform $VO_{2\ peak}$	
Arrange the hour and dates for next appointments	

Anthropometric measurements	Measurement
Height (m)	
Weight (kg)	
BMI (kg/m^2) IMPORTANT! Participant most have a Normal BMI or $\geq$ 30 kg/m^2 in order to be included in the study	
Waist Circumference (cm) (umbilicus)	
Waist Circumference (cm) (greatest protuberance of the butt)	
Waist to Hip Ratio	
DXA saved and printed?	
Blood pressure 1st visit (right before test)	
Heart rate 1st visit (right before test)	
$VO_{2\ peak}$ test saved and printed?	

APPENDIX G

24h Food Log

Name:_____Date:_____

Day: _____Phone:_____E-mail:_____

Record right after your meals everything you eat and drink (except for water) 24 hours before each exercise session. The amount of the food or beverage can be specified in grams or household measure. **If you consumed your meal in a dining hall, write down its name in the Brand/Restaurant column so we can request the nutritional information directly from them**.

Meal & Time	Food or Beverage	Amount	Cooking method	Brand/Restaurant

APPENDIX H

$VO_{2\ peak}$ Form

ID:_____ Age:_____ Gender:_____ Date:_____
Weight:_____lbs_____Kg Height:_____ft/in_____inches_____m
Waist circumference (umbilicus):____cm Hip circumference (max protuberance):__ cm

Time (min)	**Intensity (Watts)**	**Cadence**	**HR**	**RPE scale**	**Annotations**
0-1	Start metronome				Resting Data
1-4	25 W	50 rpm			Warm up
4	50 W	50 rpm			
5	75 W	50 rpm			
6	100 W	50 rpm			
7	125 W	50 rpm			
8	150 W	50 rpm			
9	175 W	50 rpm			
10	200 W	50 rpm			
11	225 W	50 rpm			
12	250 W	50 rpm			

*Notes: HR is taken at the last seconds of every stage.
RPE is taken right before HR.

Maximum calculated HR(beats/minute) (Tanaka) = 208 - (0.7 x Age) = _____
Seat height: _____
Face mask:_____
Maximum HR:_____
Maximum $VO_{2\ peak}$:_____
70% of $VO_{2\ peak}$:_____
70% Intensity:_____

Mark the criteria that the participant achieved to consider the test as a true $VO_{2\ peak}$:

- Plateau in their $VO_{2\ peak}$ with an increased workload_____
- Respiratory exchange ratio above 1.1 _____
- Attained the age predicted HR_{max} within 15 beats/min_____
- Achieved volitional fatigue with any of the above criteria_____
- Another:_____

APPENDIX I

Exercise Testing Forms

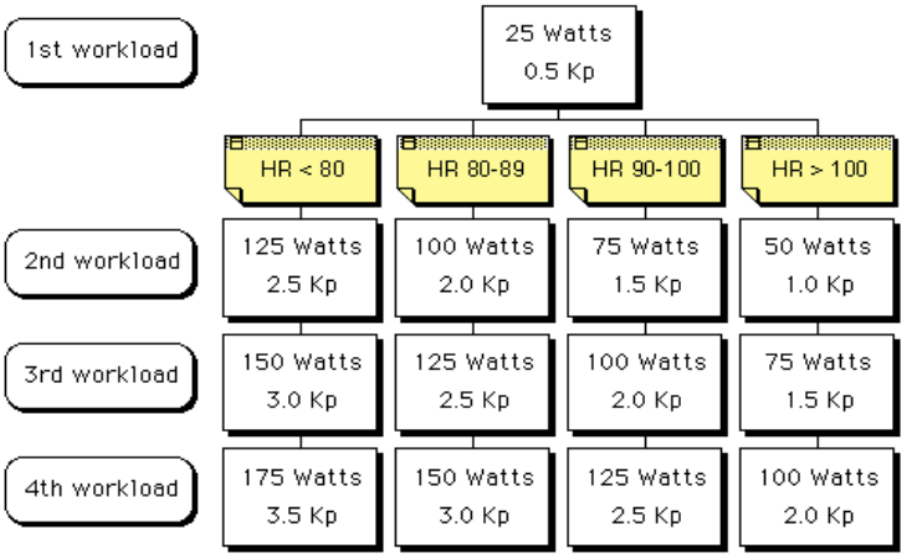
ID:_____ Age:_____ Gender:_____ Date:_____ Fasting time:_____

Time (min)	Intensity (Watts)	Cadence	HR	Annotations
0-1	Start metronome			Resting Data
1-4	25 W	50 rpm		Warm up
4-7		50 rpm		
7-10		50 rpm		
		50 rpm		
		50 rpm		
		50 rpm		
		50 rpm		
		50 rpm		
		50 rpm		
		50 rpm		
		50 rpm		

*Notes: HR is taken at the last seconds of every stage.

GOAL ➔ 70% of $VO_{2\ peak}$:_____

Follow YMCA protocol until achieving 70% of $VO_{2\ peak}$



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