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The Immediate and Extended Effect of Diet and Exercise on Metabolic Flexibility

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THE IMMEDIATE AND EXTENDED EFFECT OF DIET AND EXERCISE ON METABOLIC FLEXIBILTY

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ABSTRACT

Metabolic inflexibility is an emerging physiological marker indicative of metabolic dysfunction and associated with type 2 diabetes (T2D) and obesity. Exercise is a potent stimulus to improve metabolic health, however, not much is known about the acute effects of exercise on metabolic flexibility (METFLEX). The purpose of this dissertation was study the time-course of diet and exercise and its effects on METFLEX. Three studies were conducted to investigate molecular and clinical aspects of diet and exercise and how these stimuli may alter the metabolic response to fuel.

The first study was conducted on obesity-prone, Osborne-Mendel (OM) and obesity-resistant S5B/Pl (S5B) rats. This experiment investigated inherent differences in EE, metabolic rate, METFLEX and skeletal muscle markers of metabolism, lipid storage and lipid oxidation between OM and S5B, as well as HFD-induced strain differences in these parameters. The consumption of a HFD in S5B rats increased metabolic rate and EE and decreased metabolic rate and EE in OM rats. These strain differences were not due to the differences in activity or food intake. These results suggest that HFD-induced differences in metabolic rate and EE may be mediated by HFD-induced differences in pAMPK and PPARγ expression and lipid accumulation in the gastrocnemius muscle of OM and S5B rats.

The second study was designed to investigate the effect of acute aerobic exercise at an intensity that maximizes the rate of fat oxidation (FATMAX) on glucose tolerance, insulin action, and METFLEX compared to acute aerobic exercise at lactate threshold (OBLA) resulting in greater carbohydrate (CHO) oxidation. OBLA exercise appears to have deleterious effects on oral glucose tolerance and metabolic flexibility acutely, however, FM exercise does not confer improved METFLEX. These results suggest that predominate substrate utilization does not promote improved glucose tolerance and metabolic flexibility in young overweight men. The third and final study of this dissertation was designed to

investigate the immediate and extended of a single bout of high intensity interval exercise (HIIE) on METFLEX and mitochondrial function. Participants were recruited with a family history (FmHx) of T2D (n=8) and without a family history of T2D (n=8). The extended effects (48 hours) work via the reduction fat oxidation by increasing resting fat oxidation rate and improving the suppression of fat oxidation in response to a mixed meal.

CHAPTER 1. INTRODUCTION

Metabolic flexibility (METFLEX) and metabolic inflexibility (MI) are biomarkers for metabolic health and dysfunction respectively. Kelley and colleagues (1999) first noted a profound lack of plasticity in obese skeletal muscle to reduce fat oxidation and increase carbohydrate oxidation in the presence of insulin [1]. In this seminal work, the authors define METFLEX as "a high capacity to modulate rates of energy production, blood flow, and substrate utilization". Since that time, METFLEX is more often described as the body's ability to match fuel oxidation to fuel availability. Interestingly, MI is not only a characteristic of T2D and obese patients, but also those with a family history (FmHx) of T2D and is linked to mitochondrial dysfunction [2-6].

Exercise interventions (6-12 weeks) have been shown to improve insulin sensitivity and metabolic flexibility in obese and type 2 diabetic patients, with greater improvements demonstrated with higher intensities [7,8]. Recent research suggests that 2-6 weeks of high intensity interval training (HIIT) improves insulin sensitivity and skeletal muscle oxidative capacity [9,10]. Interestingly, the time course of glucose tolerance and insulin action in response to a single bout of exercise reveals periods of increased insulin sensitivity (immediately post exercise to 3 days) and resistance (5 to 7 days' post exercise) [11,12]. Moreover, improvements found in insulin sensitivity due to exercise training often do not coincide with improvements in METFLEX, suggesting MI does not simply occur secondarily to insulin resistance or obesity [13,14].

The purpose of this dissertation was study the time-course of diet and exercise and its effects on METFLEX. Three studies were conducted to investigate molecular and clinical aspects of diet and exercise and how these stimuli may alter the metabolic response to fuel. These include work using both animal models and human participants. The first study was conducted on obesity-prone, Osborne-Mendel (OM) and obesity-resistant S5B/Pl (S5B) rats. This experiment investigated inherent differences in energy expenditure (EE),

metabolic rate, METFLEX and skeletal muscle markers of metabolism, lipid storage and lipid oxidation between OM and S5B, as well as HFD-induced strain differences in these parameters. The consumption of a HFD in S5B rats increased metabolic rate and EE and decreased metabolic rate and EE in OM rats. These strain differences were not due to the differences in activity or food intake. These results suggest that HFD-induced differences in metabolic rate and EE may be mediated by HFD-induced differences in pAMPK and PPARγ expression and lipid accumulation in the gastrocnemius muscle of OM and S5B rats.

The second study was designed to investigate the effect of acute aerobic exercise at an intensity that maximizes the rate of fat oxidation (FM) on glucose tolerance, insulin action, and metabolic flexibility (METFLEX) compared to acute aerobic exercise at lactate threshold (OBLA) resulting in greater carbohydrate (CHO) oxidation. OBLA exercise appears to have deleterious effects on oral glucose tolerance and metabolic flexibility acutely, however, FM exercise does not confer improved METFLEX. These results suggest that predominate substrate utilization does not promote improved glucose tolerance and metabolic flexibility in young overweight men.

The third and final study of this dissertation was designed to investigate the immediate and extended of a single bout of high intensity interval exercise (HIIE) on METFLEX and mitochondrial function. In study 3, participants were recruited with a family history (FmHx) of T2D (n=8) and without a family history of T2D (n=8). Participants completed baseline (no previous exercise) mixed tolerance tests (MMTT) to assess METFLEX. On a separate week participants completed a single bout of HIIE follow by an immediate (1 hour later) MMTT and follow-up MMTT (48 hours later) MMTT. The immediate effects (1 hour) of HIIE on METFLEX are primarily directed on the increase in carbohydrate oxidation and blood flow in response to a mixed meal. The extended effects (48 hours) work via the reduction fat oxidation by increasing resting fat oxidation rate and improving the suppression of fat oxidation in response to a mixed meal.

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CHAPTER 2. HIGH FAT DIET DIFFERENTIALLY REGULATES METABOLIC PARAMETERS IN OBESITY-RESISTANT S5B/Pl RATS AND OBESITY-PRONE OSBORNE-MENDEL RATS

2.1 Introduction

The current prevalence of obesity has been linked to a number of health consequences. [1,2]. Differences in the susceptibility to becoming obese exists and a subset of individuals gain weight and become obese on high-fat/energy dense diets, while others resist weight gain on the same diet [3-7]. Studies indicate that the susceptibility to develop obesity is due to several central and peripheral factors. These factors may include an inability to adapt to the consumption of an energy dense diet by the obesity-prone individuals [8-10]. Inherent differences in metabolic parameters and a differential response to the consumption of an energy dense diet on energy expenditure (EE) may influence the susceptibility to develop obesity.

Energy expenditure and carbohydrate and fat metabolism is regulated by skeletal muscle and alterations in the regulation of EE, metabolic rate and skeletal markers of glucose and fatty acid metabolism may affect the susceptibility to developing obesity [11-18]. Studies have demonstrated that respiratory exchange ratios (RER) measured in isolated muscle tissue during fasting in obese people and those with type 2 diabetes are elevated and do not respond to insulin stimulation [19-21]. An increased susceptibility to obesity has been linked to metabolic inflexibility, which may be due to alterations in the transport and oxidation of fatty acids and glucose in skeletal muscle [18,20,22]. The fatty acid receptor, CD36, and the glucose transporter, GLUT4, are important regulators of fatty acid and glucose uptake in skeletal muscle and are regulated by the phosphorylated state of AMP-activated protein kinase (AMPK) [23]. Defects in peroxisome proliferator-activated receptor-gamma (PPARγ), a regulator of fatty acid storage and glucose metabolism, are also associated with increased susceptibility to developing obesity [24]. Obesity-

prone and obesity-resistant rodent models and obese and lean humans demonstrate differential expression of skeletal muscle proteins induced by a high fat diet (HFD) [14] suggesting that dysregulation of one or more of these skeletal muscle markers may affect the susceptibility to becoming obese.

Obesity-prone, Osborne-Mendel (OM) and obesity-resistant S5B/Pl (S5B) rats are animal models used to assess the mechanisms underlying HFD intake and obesity [3-5,8,25-31]. OM rats are susceptible to diet-induced obesity and gain more weight and adiposity when eating a HFD, than S5B rats, which are resistant to obesity, when consuming the same diet. The current experiment tested the hypothesis that consumption of a HFD would differentially affect metabolic parameters and skeletal muscle markers of metabolism in obesity-prone, OM and obesity-resistant, S5B rats. This experiment investigated inherent differences in EE, metabolic rate and skeletal muscle markers of metabolism, lipid storage and lipid oxidation between OM and S5B, as well as HFD-induced strain differences in these parameters. Assessment of metabolic rate, EE, glucose and carbohydrate metabolism, and lipid oxidation and storage in skeletal muscle will provide insight into mechanisms which alter the susceptibility to developing obesity.

2.2 Methods

2.2.1 Animals:

Male obesity-prone Osborne-Mendel (OM; n=8/diet) and obesity-resistant S5B/Pl (S5B; *n*=8/diet) rats (8-9 weeks old) used in these studies were bred in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved Pennington Biomedical Research Center vivarium. Rats were individually housed on a 12/12 h light/dark cycle (lights on at 0700) with food and water available *ad libitum*. Animals were given access to a pelleted high fat diet (HFD, 60% kcal from fat, Research Diets, New Brunwick, NJ) or a pelleted low fat diet

(LFD, 10% from fat, Research Diets). All procedures were approved by the Pennington Biomedical Research Center Institutional Animal care and Use Committee.

2.2.2 Fasting Glucose:

Following 2 weeks access to HFD and LFD, OM and S5B rats underwent an overnight fast (16 hours) and glucose levels were measured. For the determination of circulating glucose level, blood was taken from the tail and glucose was measured using a glucometer (Contour blood glucose monitoring system, Bayer Health Care, Mishawaka, IN).

2.2.3 Body Composition:

OM and S5B rat were fed the HFD or the LFD for 2 weeks prior to assessment of metabolic parameters. Immediately prior to placement in the metabolic chambers, rats were weighed and body composition was determined using nuclear magnetic resonance imaging (NMR, MiniSpec Plus, Bruker Corp.). Percent body fat was determined for each rat ((fat mass (g)/body weight (g))*100) and percent lean mass for each rat was calculated ((lean mass (LBM)(g)/body weight (g))*100).

2.2.4 Effect of HFD consumption on metabolic parameters in OM and S5B:

For metabolic testing, OM-LFD (*n*=6), OM-HFD (*n*=6), S5B-LFD (*n*=6) and S5B-HFD (*n*=6) rats were individually housed in metabolic chambers (PhenoMaster System,TSE Lab Master System, Homburg, Germany), which performed continuous metabolic measurements for 5 days (1 day of habituation and 4 days of data collection). The PhenoMaster System at PBRC contains 24 metabolic cages. All rats were placed in the metabolic chambers at the same time and measurements for all rats were collected concurrently. Rats were weighed immediately prior to being placed in the metabolic chambers. Throughout metabolic assessment, rats continued to

receive *ad libitum* access to HFD or LFD. Data from the metabolic chambers were assessed, calculated and analyzed using the TSE Systems LabMaster software (v.1.5). Respiratory exchange ratio (RER), EE (kcal/h/LBM), metabolic rate (VO2/kg LBM), food intake (g), and activity (beam breaks) were assessed every 40 min throughout the experiment by the TSE system. EE and VO2 were calculated based on lean body mass (kg) as determined by NMR. Food intake was converted to kilocalories. For each day, the data point for an individual rat was averaged across the light and the dark cycle. Total daily food intake and activity values were obtained by combining the average data from the light cycle and the dark cycle for each rat. The values for each day were combined and used to provide an average value for the light cycle, the dark cycle, and the total daily value. To determine the shift in substrate utilization (RER) between the light and dark phase, the RER value during the light phase was subtracted from the RER value from the dark phase.

2.2.5 Protein Expression in Skeletal Muscle:

OM and S5B rats fed HFD or LFD diet (n=6/strain/diet) were used to assess protein expression of skeletal muscle markers of glucose and lipid metabolism. At the time of sacrifice (0900-1100), the gastrocnemius muscle was dissected and a 100mg sample was collected, frozen in liquid nitrogen and stored at -80°C until further processing. Rats were not fasted prior to sacrifice. Protein was isolated and Western Blot analyses were conducted as previously described [8]. For protein isolation, samples were incubated on ice in RIPA buffer (Sigma-Aldrich, St. Louis, MO) containing 1:100 protease inhibitor (Sigma-Aldrich), and 1:100 phosphatase inhibitor (Sigma-Aldrich) and then homogenized for 30 seconds or until a smooth suspension resulted. Homogenized samples were centrifuged for 10min at 16,874 *x g* at 4^{o} C. Supernatant was collected and measured for protein concentrations using a BCA protein assay kit

(Pierce/Thermo Fisher Scientific, Rockford, IL). For Western Blot, equal amounts of protein (30 μg) were separated on a 10% Tris-Hepes-SDS premade gel (Pierce/Thermo Fisher Scientific) and transferred to a PVDF membrane (Amersham, Amersham, UK) as indicated by the manufacturer. The membrane was then blocked in TBS (20 mM Tris-Base, 150 mM NaCl, pH 7.6) containing 5% nonfat dry milk overnight at 4º C. On Day 2, the membrane was incubated for 1h with primary antibody for CD36 (1:500; Novus Biologicals, Littleton, CO), GLUT4 (1:1000; Abcam, Cambridge, MA), PPARγ (1:1000; Abcam), pAMPK (1:2000; Cell Signaling, Danvers, MA), total AMPK (AMPK-α; 1:1000; Cell Signaling) or alpha-tubulin (1:500; Abcam) against rabbit in TBS containing 5% nonfat dry milk. Following several washes the membrane was incubated for 45min with horseradish peroxidase-conjugated anti-rabbit antiserum (1:10,000, Abcam). Immunoreactivity was visualized using ECL Western blotting detection reagents (Amersham). Images were obtained using exposure to chemiluminescence film (Amersham). Bands were quantified using ImageJ for densitometry and values were normalized to alpha-tubulin. pAMPK was also normalized to total AMPK. For quantification in Image J, films were scanned and saved as jpeg files. Images were cropped to contain the appropriately sized band. Using Image J, a box was drawn around the band for each sample. This same box was used for each sample on each image. Image J provided graphical information on the relative density of each band, which reflected the portion of the drawn box that contained the band. This allowed for a more precise measurement of densitometry of the actual band and not the surrounding image.

2.2.6 Histological Analysis of gastrocnemius muscle in OM and S5B (Succinate Dehydrogenase and Oil Red O):

Fat and connective tissue were dissected from gastrocnemius muscle, frozen in isopentane, and stored at -80°C until histological processing. At the time of processing, sections (10μm) were cut using a cryostat (Shandon Cryotome, Thermo Scientific) at -20°C and mounted on slides. Slides with cross sections of the gastrocnemius muscle were incubated with succinate dehydrogenase (SDH) to evaluate the presence of oxidative enzymes or Oil Red O to detect the presence of intramuscular lipids. Images were captured via light microscope (Nikon Eclipse 80i) with a connected digital camera under a 10x objective. Within the cross section, quantitative image analysis was conducted using the Image J Software on a minimum of 75 fibers. Image J provided a numerical value for the density of staining for each fiber. The oxidative capacity of the muscle was determined by the intensity of SDH staining according to validated methods [32]. With this method, there is a positive linear correlation between color and oxidative potential. Type I fibers (slow twitch, oxidative) stain darkest and Type II fibers (fast twitch, glycolytic) stain lighter. Oil Red O staining was quantified according to the area occupied by the lipid staining within individual muscle fibers [33].

2.2.7 Statistical Analysis:

Data is presented as mean $\pm$ SEM. Two way ANOVAs were used to determine if significant differences between groups (strain vs. diet). If an overall ANOVA demonstrated significant differences, post hoc comparisons were performed using a Bonferroni t-test. Differences were considered significant if $p < 0.05$. Statistical analyses were performed using SPSS.

2.3 Results

2.3.1 Fasting Glucose:

Fasting glucose levels were significantly affected by diet and strain (F=6.03, p<0.05; Figure 2.1A). Consumption of HFD increased fasting glucose levels in the OM, but not the S5B rats.

2.3.2 Body Composition:

OM rats weighed more than S5B rats fed either the HFD or the LFD (F=10.6, p<.01; Figure 2.1B). Body weight was increased by the consumption of a HFD in OM and S5B rats, with a greater increase in body weight in the obesity-prone OM rats, compared to the obesity-resistant S5B rats (58.3 *g* vs. 15.3 *g*, respectively). Body composition was assessed by NMR and a higher percentage of body fat was detected in OM rats, compared to S5B rats (F=5.9, p<.05; Figure 2.1C). HFD intake increased the percentage of body fat in OM rats only. The percentage of LBM was not altered by strain or diet (Figure 2.1D).

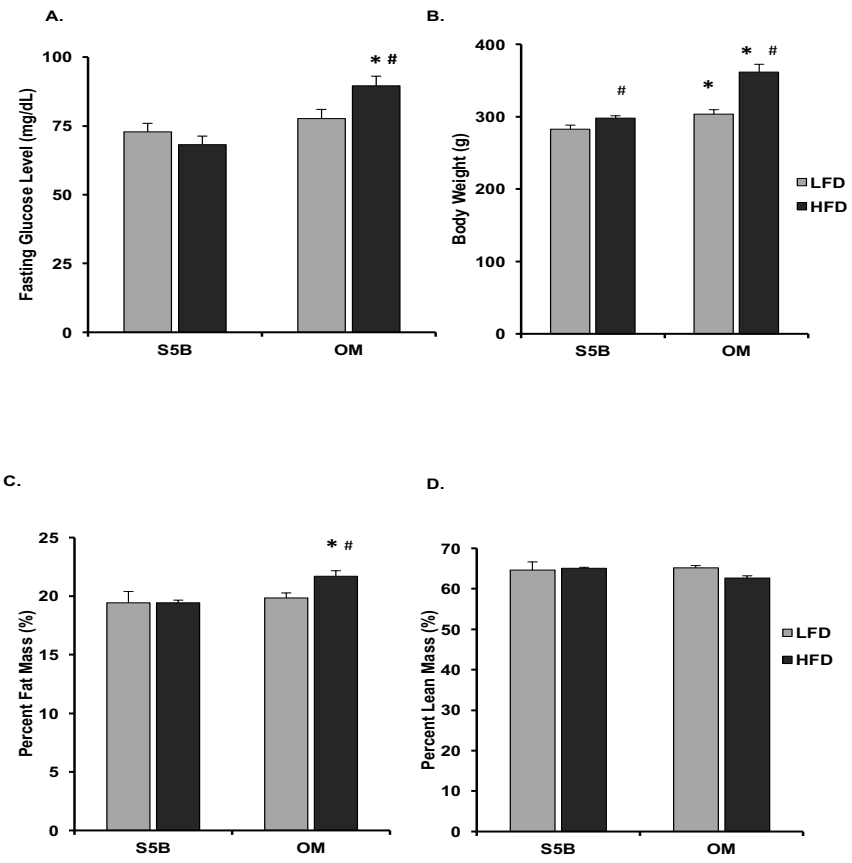

Figure 2. 1: A. Fasting glucose levels were elevated in OM rats consuming the HFD. B. OM rats weighed more than S5B rats. HFD intake increased body weight in both strains. OM rats consuming a HFD weighed more than S5B rats consuming a HFD. C. Consumption of a HFD increased the percentage of body fat in OM rats. D. The percentage of LBM was not altered by HFD consumption. Data is shown as mean $\pm$ SEM, $p<.05$, # diet differences, *strain differences.

2.3.3 Effect of HFD consumption on metabolic parameters in OM and S5B:

Rats were placed in the metabolic chambers for 5 days (1d habituation, 4d data collection), during which food intake, RER, activity, metabolic rate (VO2) and EE were measured. Data collected during the dark phase and the light phase was analyzed, as well as over the total 24h period (for food intake and activity). Rats consuming the HFD exhibited a significantly lower RER value than rats consuming the LFD during the light phase (F=4552.8, p<.001; Figure 2A, 2B) and an interaction between strain and diet was detected during the dark phase (F=22.0,

p<.001). During the light phase, S5B rats fed the LFD were able to shift their substrate utilization toward fat oxidation, compared to the OM rats (difference in light to dark: OM-LFD 0.020 $\pm$ .007 vs. S5B-LFD 0.074 $\pm$ .008, p<.001).

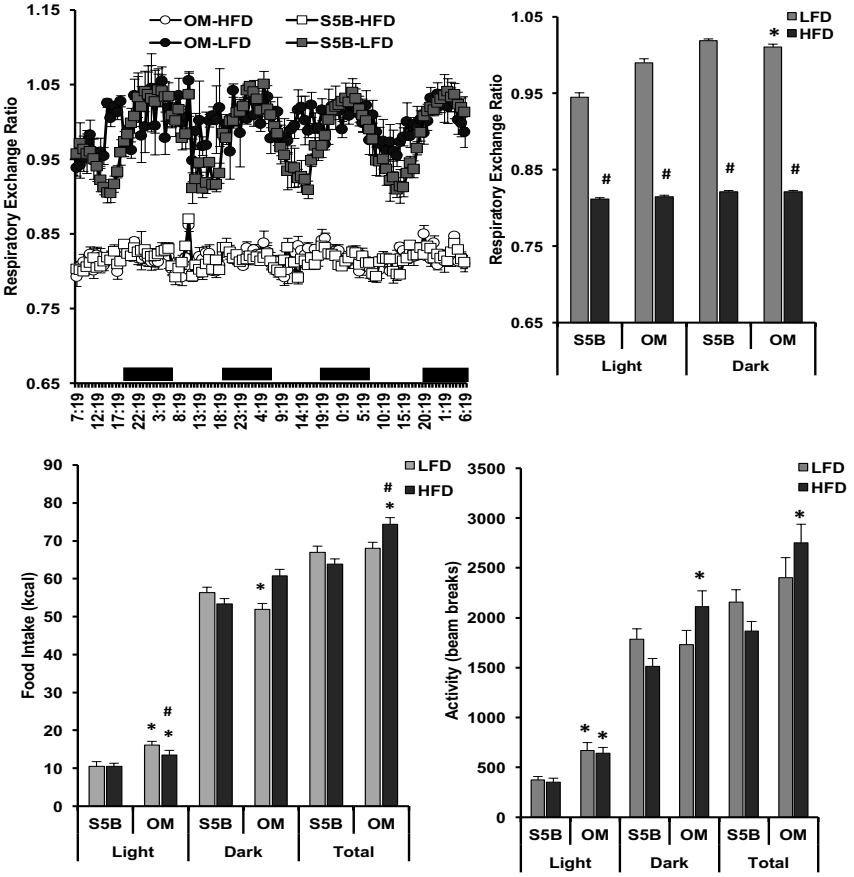

Figure 2.2: A. RER time course over 4 days of measurements in metabolic chambers. B. RER was lower in rats consuming HFD, reflecting the predominance of fat utilization. During the dark cycle, OM rats consuming LFD exhibited a higher RER than S5B rats consuming a LFD. C. OM rats consumed more LFD and HFD during the light cycle. During the dark cycle, OM rats reduced their consumption of LFD. OM rats consumed more HFD over the 24h period than S5B rats. Black bars indicate dark period. Data is shown as mean $\pm$ SEM, p<.05, # diet differences, *strain differences.

Differences in HFD and LFD intake were seen across the light and dark cycles. During the light cycle, OM rats consumed more HFD and LFD than S5B rats and more LFD, than HFD (F=15.2, p<.001; See Figure 2C). During the dark cycle, OM rats consumed less LFD than the S5B rats and similar amounts of HFD (F=15.7, p<.001). Total daily HFD intake was higher in the OM rats (F=7.9, p<.001). OM rats were more active in the metabolic cages than S5B rats during the light cycle (F=6.9, p<.01; Figure 2D) and HFD fed OM rats were more active than S5B rats during the dark cycle (F=27.5, p<.001). Total daily activity was highest in OM rats consuming the HFD (F=4.2, p<.05).

Metabolic rate (VO2 based on kg LBM) was differentially affected by diet in the OM and S5B rats during the light (F=20.6, p<.001) and dark phase (F=27.8, p<.001; Figures 2.3A, 2.3B). In both the light and the dark phase, HFD consumption increased metabolic rate in the obesity-resistant S5B rats and decreased metabolic rate in the obesity-prone OM rats. Estimated hourly EE was calculated by the TSE software and based on kg LBM. Estimated EE was differentially affected by diet in the OM and S5B rats during the light (F=20.5, p<001) and dark (F=27.1, p<.001) cycles (Figures 2.3C, 2.3D). Estimated hourly EE was increased by HFD consumption in the S5B rats and decreased by HFD intake in the OM rats.

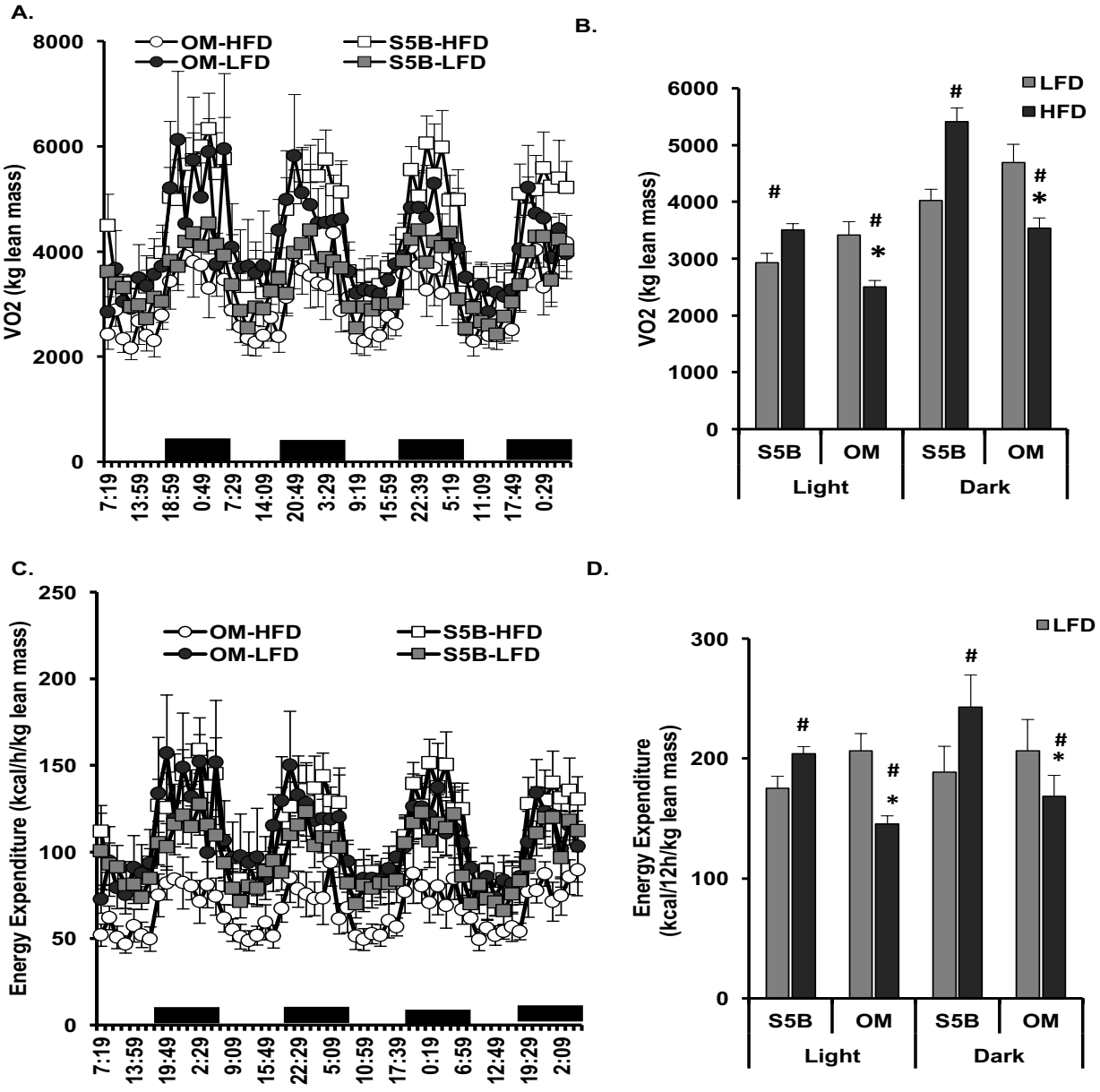

Figure 2.3: A. Time course of metabolic rate (VO2 (kg LBM). B. HFD intake differentially affected VO2 level in OM and S5B rats. HFD intake increased metabolic rate in obesity-resistant S5B rats and decreased metabolic rate in obesity-prone OM rats. C. Time course of estimated EE over 4 days of measurements in the metabolic chambers. D. HFD consumption increased EE in S5B rats and decreased EE in OM rats. Black bars indicate dark period. Data is shown as mean $\pm$ SEM, $p<.05$, # diet differences, *strain differences.

2.3.4 Skeletal Muscle CD36, GLUT4, pAMPK and PPARγ protein expression:

Protein expression of skeletal muscle markers of fat and carbohydrate metabolism were assessed by Western blot. There were no inherent strain differences or HFD-induced alterations in the expression of CD36 (Figure 2.4A). GLUT 4 expression was elevated in OM rats (F=5.6, p<.05; Figure 2.4B). A differential response to HFD consumption was detected for pAMPK/total AMPK (F=12.2, p<.01, Figure 2.4C) and PPARγ (F=4.8, p<.05; Figure 2.4D) between OM and S5B rats. Consumption of HFD decreased pAMPK and PPARγ in the obesity-prone OM rats. In LFD fed rats, PPARγ expression was higher in OM rats, compared to S5B rats.

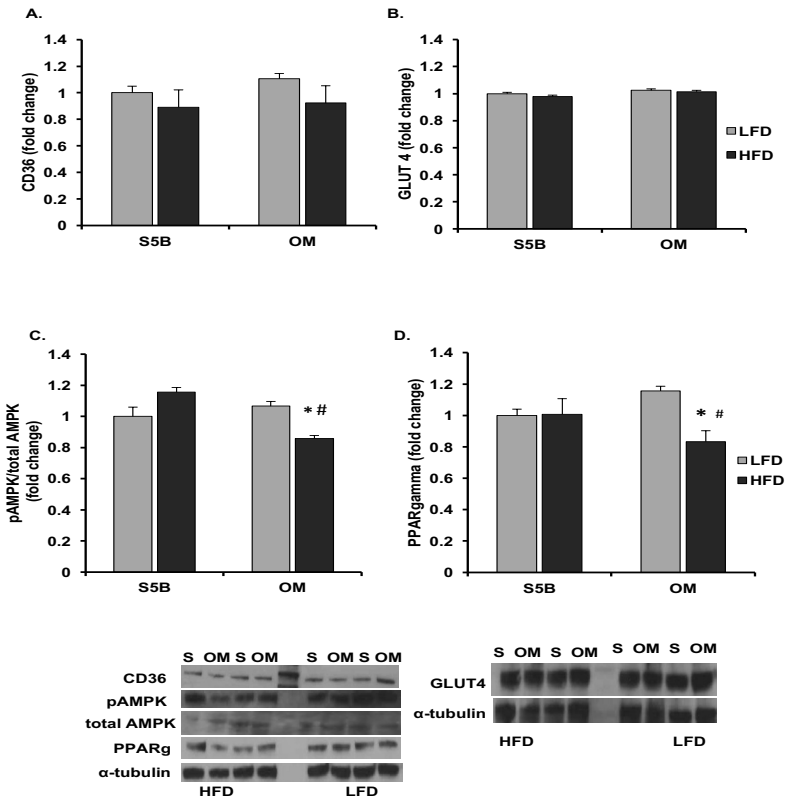

Figure 2.4: Western Blots were used to assess protein expression of skeletal muscle markers of metabolism in OM and S5B rats fed LFD or HFD. A. CD36 protein levels were not altered by strain or diet. B. GLUT4 protein expression in the gastrocnemius muscle was increased in OMrats OM rats. C. pAMPK levels in the gastrocnemius muscle were decreased by HFD in OM rats. D. PPARɣ protein expression was decreased in OM rats consuming a HFD. Data is shown as mean ± SEM, p<.05, # diet differences, *strain differences.

2.3.5 Histological Analysis of gastrocnemius muscle in OM and S5B (Succinate Dehydrogenase (SDH) and Oil Red O:

Assessment of inherent and HFD-induced differences in oxidative activity of the gastrocnemius muscle, as measured by SDH activity, indicated that HFD consumption increased the oxidative capacity of the skeletal muscle in both strains (F=172.9, p<.01, Figure 2.5). SDH activity indicates a greater number of Type I, slow twitch fibers following HFD consumption. HFD consumption increased intra-muscular lipid droplets, as measured by Oil Red O, in both OM and S5B rats (F=178.4, p<.001, Figure 2.6). The intensity of Oil Red O staining was higher in the OM rats fed the HFD, than the S5B rats fed the HFD.

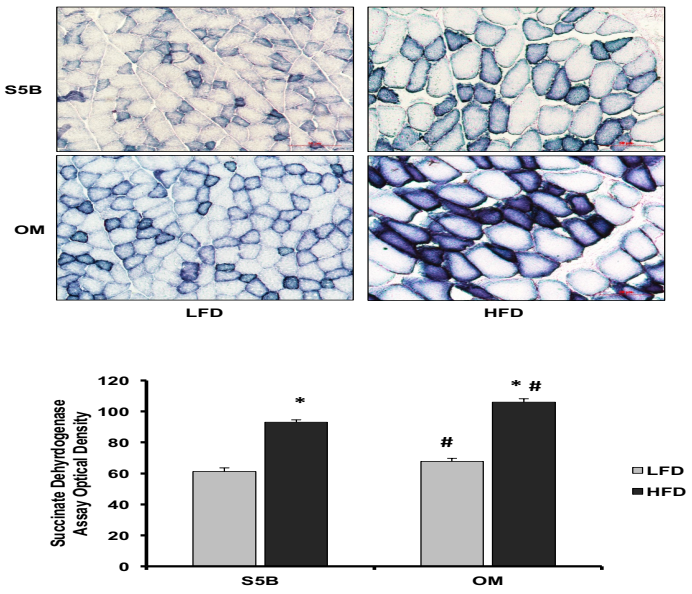

Figure 2.5: SDH assay was used to determine oxidative capacity of the gastrocnemius muscle in OM and S5B rats fed LFD or HFD. The consumption of a HFD increased SDH staining in OM and S5B rats, suggesting an enhanced oxidative capacity. OM rats fed LFD had greater SDH staining than S5B rats fed LFD.

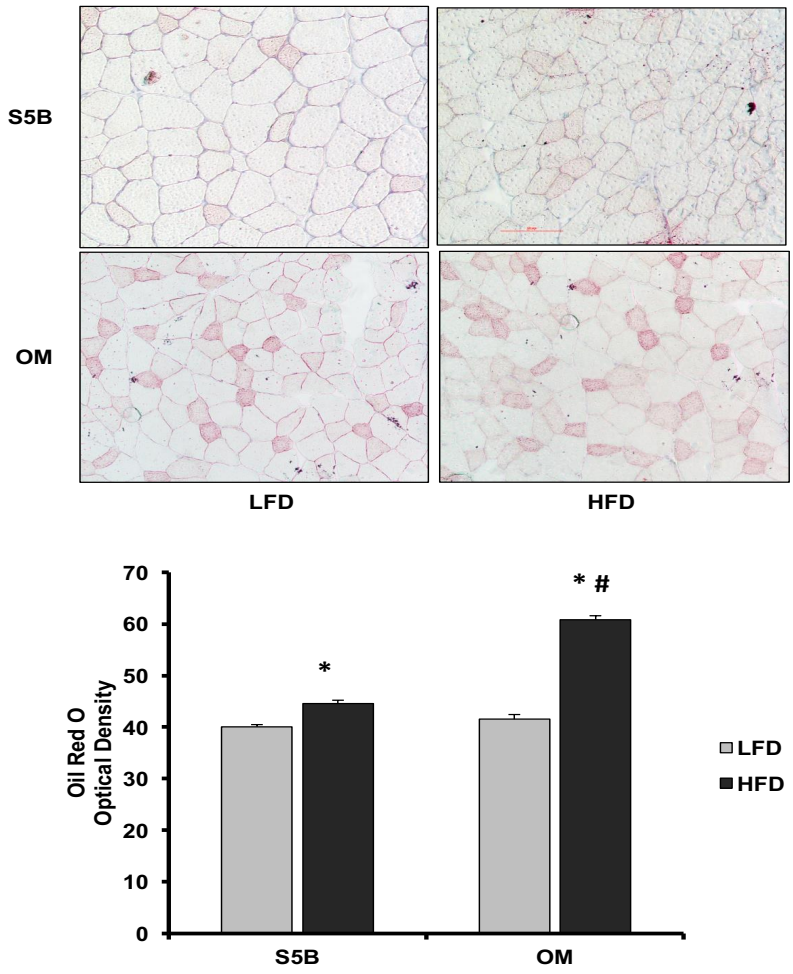

Figure 2.6: Oil Red O was used to determine lipid deposition in the gastrocnemius muscle S5B and OM rats fed LFD or HFD. An increase in density of Oil Red O stain is reflective of an increase in lipid deposition. The consumption of a HFD increased Oil Red O staining in OM and S5B rats. Lipid deposition in HFD fed was markedly enhanced in OM rats, compare to S5B rats. Data is shown as mean $\pm$ SEM, $p<.05$, # diet differences, *strain differences.

2.4 Discussion

OM rats are susceptible to diet-induced obesity and gain more weight and body fat when eating a HFD, than obesity-resistant S5B rats. The current experiment investigated inherent strain differences and HFD-induced differences in metabolic parameters (e.g. metabolic rate) and skeletal muscle markers of metabolism, lipid storage, lipid oxidation and muscle fiber type in these animal models. It was hypothesized that the consumption of HFD would differentially affect metabolic parameters and skeletal muscle markers of metabolism in the obesity-prone, OM and the obesity-resistant, S5B rats. The results from this experiment elucidate HFD-induced changes in the metabolic phenotype of these two strains, which may contribute to the susceptibility to develop diet-induced obesity.

The animals models used in the current study have been studied for a number of years to assess physiological and pharmacological differences that would alter the susceptibility to developing diet-induced obesity. Multiple studies have demonstrated innate strain differences as well as strain differences in response to HFD consumption [3-5,8,25-31]. However, metabolic parameters have not been assessed in these models and these strains may differ from other models of diet-induced obesity on these parameters. Previous studies using other models of obesity have examined differences in metabolism and activity as potential mechanisms to account for the susceptibility to obesity. A recent study by Azzout-Marniche and colleagues [34] failed to demonstrate differences in EE, or substrate utilization in fat-resistant and fat-sensitive Wistar rats fed a HFD, though fat-sensitive rats were more active. Jackman and colleagues [9] reported a sex difference in the response to dietary fat in obesity-prone and obesity-resistant rats, indicating that their male rats demonstrated minor increases in EE, compared to female rats. Their study concentrated on the switch from a LFD to a HFD, and reported a lack of difference

in activity levels. Reported differences in activity have also been suggested as a method by which obesity-resistant rats are able to resist obesity. Specifically, high levels of spontaneous physical activity (SPA, very low-intensity physical activity) were associated with obesity resistance in selectively-bred obesity resistant Sprague-Dawley rats. [6,35-37]. In these studies, obesity-resistant rats spent more time being ambulatory than Sprague-Dawley rats, and exhibited decreased body weight, fat mass and food intake, suggesting that EE generated from SPA accounts for the difference between obesity resistance and propensity in this model.

In the current experiment, HFD consumption increased fasting glucose levels in the obesity-prone OM rats, but not the obesity-resistant, S5B rats. These data are in congruence with previous reports suggesting that OM rats have higher circulating insulin and leptin levels [26,28], in response to a HFD. OM and S5B rats gained weight on the HFD and OM rats consuming the HFD weighed considerably more than the OM rats consuming the LFD (approximately 58g). A HFD-induced increase in the percentage of body fat was only seen in the OM rats and there were no differences in percentage of LBM between the strains, regardless of diet. Similar percentages of LBM in these strains suggest that differences in metabolic parameters and skeletal muscle markers are not due to HFD-induced muscle degradation. As expected, daily food intake was highest in the OM rats consuming the HFD. This increase in total HFD intake by the OM rats appears to be driven by an increase in HFD consumption during the light phase. An increase in activity levels during the light phase was also seen in the OM rats, suggesting that these rats were awake, active and eating. This increase in LFD consumption and increase in activity, compared to the S5B rats, may account for the lack of "flexibility" in RER of the OM rats during the light phase. Multiple studies have suggested that obese individuals are metabolically inflexible, suggesting that these individuals are not able to adequately shift from carbohydrate to fat

oxidation during times of fasting (e.g. sleep) [18,20,22]. In the current study, the rats fed the HFD exhibited a RER that reflected fat oxidation. Since this diet contains 60% of its kilocalories from fat (20% from carbohydrates), this was expected. Metabolic inflexibility was not able to be assessed in the HFD groups, since the RER did not fluctuate between the light and dark cycle.

Energy expenditure and metabolic rate were assessed in the OM and S5B rats fed the HFD and LFD. The consumption of the HFD increased metabolic rate (VO2) and estimated EE in the obesity-resistant S5B rats during the light cycle and the dark cycle. This was not due to an increase in activity level, since S5B rats fed the HFD were not more active than those fed the LFD. This was also not due to an increase in HFD intake, since the S5B consumed the same number of kilocalories regardless of diet. Conversely, consumption of HFD decreased metabolic rate and estimated hourly EE in the obesity-prone OM rats. These decreases were not due to a decrease in HFD intake, since OM rats consumed more HFD than LFD and they were not due to a decrease in activity level, since OM rats consuming the HFD were more active than those consuming the LFD. These data suggest that there is an alternate mechanism regulating the HFD-induced alterations in metabolic rate and EE in the OM and S5B rats. Previous studies have investigated altered sympathetic output, as measured by peripheral norepinephrine, in these strains and have shown higher concentration of norepinephrine in brown adipose tissue following HFD intake in S5B, compared to OM rats, supporting decreased EE in OM rats consuming HFD [38].

Skeletal muscle is a major component of glucose utilization and metabolic rate, and is predicted to be a major factor in the resistance to developing obesity. Therefore, the current study assessed skeletal muscle markers of carbohydrate and lipid metabolism, as well as lipid oxidation and storage. Expression of the fatty acid receptor, CD36, did not differ between OM

and S5B rats and was not affected by HFD consumption. GLUT 4 expression was higher in OM rats. Inherent strain differences in pAMPK and PPARγ expression were not detected, however, HFD consumption decreased pAMPK (pAMPK/total AMPK) and PPARγ expression in obesity-prone OM rats, but not in obesity-resistant S5B rats. AMPK serves as a key protein kinase that integrates nutrient and hormonal signals to regulate whole-body metabolism by stimulating glucose uptake and partitioning fatty acids toward oxidation [23,39-42]. Investigations linking obesity with impaired skeletal muscle AMPK have provided largely inconsistent findings. In several genetic models of obesity, AMPK levels in skeletal muscle were altered [16,43,44], however, other studies assessing AMPK levels in obese and insulin resistant models did not indicate differences [45-50]. In the current experiment, pAMPK expression was decreased by HFD consumption in OM rats. Due to the role of AMPK as an energy sensor and a regulator of fat oxidation, these data suggest that HFD-induced decreases in AMPK phosphorylation in the skeletal muscle of OM rats may be a mechanism by which OM rats decrease their metabolic rate and EE in response to a HFD. Previous research has implicated defects in PPARγ , a regulator of fatty acid storage and glucose metabolism, as a mediator of the susceptibility to developing obesity [24], which supports data from the current study.

Inherent and HFD-induced alterations in lipid accumulation in the skeletal muscle of OM and S5B rats were measured by Oil Red O. HFD intake increased lipid accumulation in both strains, but to a greater extent in the OM rats. Oxidative capacity of the skeletal muscle was assessed by an SDH assay and these data suggest that the gastrocnemius muscle of OM rats have a greater oxidative capacity (more Type I fibers). HFD consumption increased the oxidative capacity of the muscle in both strains. Lipid accumulation in skeletal muscle, in most cases, is indicative of the cellular energy status, which favors storage rather than oxidation, and is

associated with obesity and type 2 diabetes [51]. Previous studies have demonstrated excessive fat transport and β-oxidation in skeletal muscle following HFD intake [52], which rendered rats incapable of switching to carbohydrate oxidation in the postprandial state, and therefore inhibited metabolic flexibility. The accumulation of intramuscular triglycerides and the intermediaries of incomplete fat oxidation (DAG, ceramides) have been shown to alter the insertion of GLUT4 in the plasma membrane [53-55]. Excessive, but incomplete fat oxidation leading to an accumulation of lipids in skeletal muscle has been seen in rats fed a HFD [52] and is similar to the pattern seen in the current study, in which HFD intake increased fat oxidation and lipid accumulation in OM rats. Incomplete fat oxidation and excessive lipid accumulation may explain, in part, the increased susceptibility to developing obesity in the OM rats.

The current experiment tested the hypothesis that there were inherent differences and HFD-induced alterations in metabolic parameters, skeletal muscle markers of carbohydrate and fat metabolism, and oxidation and lipid accumulation in skeletal muscle of obesity-prone and obesity-resistant rats. Obesity-prone OM rats gained more weight and body fat on a HFD and consume more HFD, than obesity-resistant S5B rats. HFD consumption also increased fasting glucose levels in the OM rats, suggesting glucose intolerance. No inherent strain differences were detected in metabolic rate and estimated EE. However, the consumption of a HFD in S5B rats increased metabolic rate and EE and decreased metabolic rate and EE in OM rats. These strain differences were not due to the differences in activity or food intake. HFD-induced differences in metabolic rate and EE may be mediated by HFD-induced differences in pAMPK and PPARγ expression and lipid accumulation in the gastrocnemius muscle of OM and S5B rats. HFD intake decreases in pAMPK and PPARγ expression in OM rats predict a decrease in EE and an increase in lipid accumulation. Taken together these data suggest that the susceptibility to

developing HFD-induced obesity, in these two strains, may be mediated by differential rates of EE, which are mediated by AMPK and PPARγ and by lipid accumulation in skeletal muscle.

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CHAPTER 3. DIFFERENTIAL EFFECTS OF FATMAX VS. HIGH CARBOHDRATE OXIDATION (OBLA) EXERCISE ON ORAL GLUCOSE TOLERANCE AND METABOLIC FLEXIBILITY

3.1 Introduction

Exercise is an important lifestyle intervention used to prevent, delay, or treat the development of metabolic disorders such as obesity and type 2 diabetes [1]. However, there is a need to develop more effective exercise strategies to account for the large variability in the response to exercise in people with metabolic disorders [2]. For example, an acute bout of exercise has been shown to improve insulin sensitivity for up to 24 hours, in some, [3-5],but not all studies [6,7]. This discrepancy is due to numerous factors including exercise intensity [8-10] , glycemic status of subjects [11], dietary behavior [12,13], or timing of the post-exercise measurement of insulin sensitivity [14]. The total dose of exercise at differing intensity is also a consideration that might explain the variability in post exercise insulin sensitivity.

The improvements in glucose tolerance with aerobic exercise may be the result of 1) increased glucose uptake to support glycogen synthesis [3] or 2) more complete oxidation of intramuscular triglycerides (IMTG), and lipid species [15]. Previous research suggests that a single bout of aerobic exercise at a higher intensity (as a percentage of maximal cardiorespiratory fitness; VO_{2max}) improves glucose tolerance to a greater extent [16]. However, this result may be partially due to a lower variability in individual responses regarding fuel utilization as high rates of carbohydrate oxidation are common at higher exercise intensities. At lower exercise intensities, physiologic, nutritional, and genetic factors lead to an increase in the variability in fuel selection potentially obscuring the benefits of increased fat oxidation during acute exercise [17,18]. Aerobic exercise can also be prescribed at an intensity (FATMAX) eliciting a maximal fat oxidation rate (MFO; g/min). The FATMAX varies between individuals, but is easily

determined by a standard graded exercise test to exhaustion (VO_{2max}) [19]. To our knowledge, only one study has examined the effect of aerobic training at FATMAX [20]. In this study, insulin sensitivity improved after only 4 weeks of aerobic training at FATMAX compared to 4 weeks of aerobic interval training of equal caloric dose. The effect of acute exercise at FATMAX on glucose tolerance is unknown. Additionally, no research exists comparing a single bout of aerobic exercise at FATMAX to a higher intensity exercise in which the primary fuel source is carbohydrate (i.e onset of blood lactate accumulation). Determining the intensity of aerobic exercise that has the greatest impact on glucose tolerance has practical applications and could potentially offer options for individuals looking to start exercise programs, especially sedentary individuals at risk for diabetes. Likewise, a lower intensity exercise may also promote greater adherence through improved affective (feeling and arousal) responses during exercise.

The purpose of this study is to investigate the effect of acute aerobic exercise at an intensity that maximizes the rate of fat oxidation (FATMAX) on glucose tolerance compared to acute aerobic exercise at a higher intensity resulting in greater carbohydrate oxidation (OBLA). We hypothesize that glucose tolerance will improve acutely post FATMAX and OBLA exercise. Additionally, we expect that affective responses will be stable and more positive during the FATMAX trial compared with the OBLA trial.

3.2 Methods

3.2.1 Participants

Eight apparently healthy, sedentary, overweight/obese college-aged men gave written informed consent to participate in this study. Individuals who presented with any known cardiovascular disease, type 2 diabetes, resting blood pressure ≥140/90mmHg, and/or body mass index ≥35kg/m^2 were excluded. Baseline participant characteristics are provided in table 1. The

participants enrolled were all college aged, sedendtary, and overweight. All participants had fasting plasma glucose levels below the threshold criteria (≥126 mg/dl) and 120-minute threshold of ≥200 mg/dl. All participants were physically inactive, nonsmokers, presently not taking any medications, and had physician approval to participate. This study was approved by the Institutional Review Board at Louisiana State University.

Table 3.1. Baseline Characteristics

Age (y)	20.5 ± 1.5
Height (m)	1.81 ± 0.04
Weight (kg)	95.9 ± 13.8
BMI (kg/m^2)	29.5 ± 4.7
VO_{2peak} ($ml \bullet kg^{-1} \bullet min^{-1}$)	33.2 ± 6.8
Fasting plasma glucose (mg/dl)	93.9 ± 10.6
120 min plasma glucose (mg/dl)	93.2 ± 12.2
Fasting RER	0.80 ±0.05

VO_{2peak} maximal aerobic capacity; BMI body mass index;
Respirator Exchange Ratio (VCO2/VO2) ±SD

3.2.2. Experimental Protocol

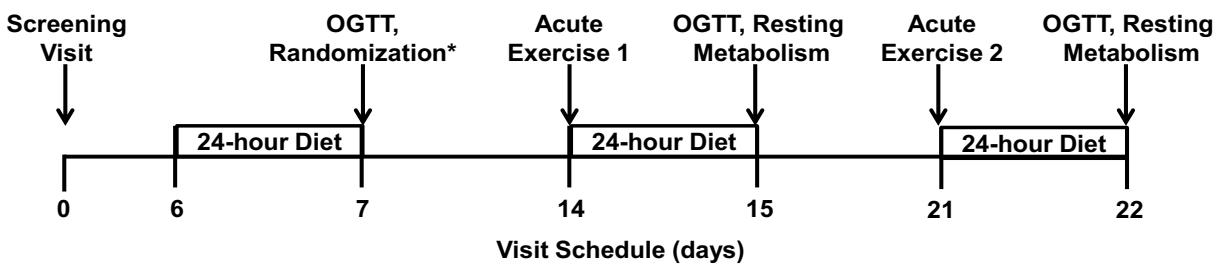


Figure 1. Visit Schedule. *After confirmation of non-diabetic status, participants undergo 2 acute aerobic exercise sessions in random order. The intensity of the acute exercise sessions will be 1) FatMax or 2) high carbohydrate oxidation. 24-hour diet records will be kept prior to the baseline OGTT and replicated between the acute exercise sessions and the OGTTs.

Participants underwent a maximal cardiorespiratory fitness test (VO_{2max}) with electrocardiogram (ECG) on a cycle ergometer (Velotron, Racermate, Inc., Seattle, WA). Participants started at an initial workload of 25 Watts for 4 minutes. The workload was increased by 25 Watts every 4 minutes until respiratory exchange ratio reached 1.05 after which the workload increased by 25 Watts every 2 minutes until volitional exhaustion. Throughout the test, respiratory gases were analyzed using a metabolic cart (ParvoMedics Inc., Sandy, UT) and fat oxidation rates were determined using stoichiometric equations[21]. FATMAX was determined using a 3-parameter logistic line of best fit calculated from the fat oxidation vs. exercise intensity (%VO_{2max}) graph (figure 3.2) [19]. During the VO_{2max} test, we also obtained finger stick blood lactate concentrations (Lactate Plus,Waltham,MA), at the end of each interval for the determination of onset of blood lactate accumulation (OBLA; 4 mM lactate). The exercise ECG was reviewed by a cardiologist for final study approval.

The baseline OGTTs occurred ~1 week after the screening visit and VO_{2max} test. Prior to the baseline visit participants recorded all food and beverages ingested for the 24-hour period prior to the baseline OGTT. Participants were required to abstain from exercise 48 hours prior to the visit. Participants then completed, in random order separated by ~1 week, two bouts of

isocaloric (400 kcal) exercise at either FATMAX or OBLA. Additional OGTTs were conducted 24 hours after the 2 acute exercise sessions separated by ~1 week. Prior to the post-exercise OGTTs, participants replicated the 24-hour food and beverage diary recorded prior to the baseline OGTT. All OGTTs and exercise sessions were conducted in the morning hours after a 10-hour fast.

The acute aerobic exercise sessions occurred ~1 week after the screening visit and VO_{2max} test and were performed on a cycle ergometer at intensities eliciting 1) FATMAX and 2) high carbohydrate oxidation (lactate threshold). All participants underwent the acute aerobic exercise sessions in a random, counter-balanced order. The targeted total dose of exercise was 400 kcal for both exercise sessions. Caloric expenditure rates were monitored periodically throughout exercise. The time to finish the 400 kcal exercise dose was accurately determined by extrapolating the kcal expenditure rate (from the metabolic cart) at each time point to the final caloric dose. We estimated that a 100 kg male (VO_{2peak}=35 $ml \cdot kg^{-1} \cdot min^{-1}$) will finish the FATMAX session in ~50 minutes and the OBLA session in ~30 minutes [22].

3.2.3 Experimental Measures

3.2.3.1 Exercise Prescription

FATMAX was determined using a 3-parameter logistic line of best fit calculated from the fat oxidation vs. exercise intensity (%VO_{2max}) graph [23] (figure 3.2). According to this method, we required $\geq$3 valid fat oxidation points with 2 valid inflection points. Once MFO was identified FATMAX intensity could be determined by using the corresponding power generation (watts) performed during the GXT (Figure 2). FATMAX calculated for each individual participant. Lactate was measured at the end of each stage during the GXT. OBLA was considered the onset

of blood lactate (4mM). The corresponding intensity was determined by the inspection method [24].

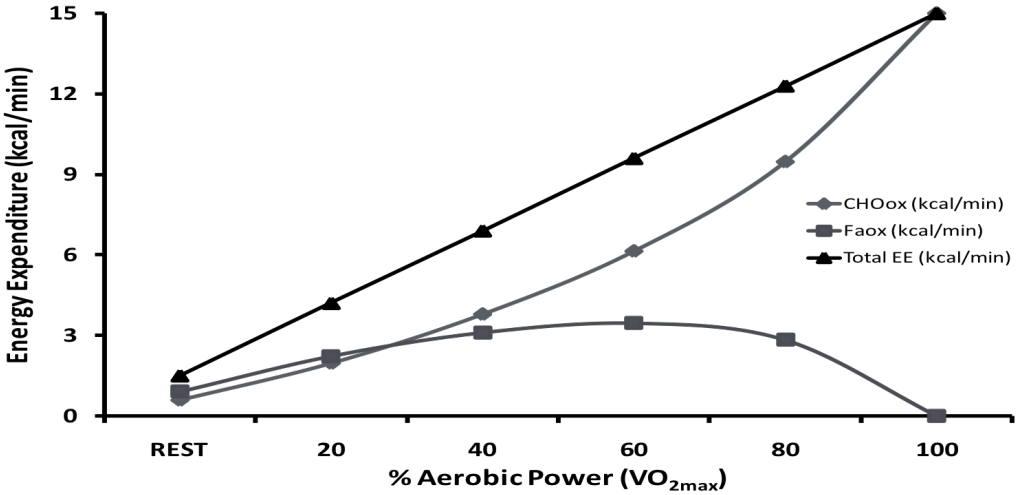


Figure 3.2 Example of FATMAX analysis. FatMax and MFO calculated by MatLab (.exe) from VO_{2max} test. Fat oxidation (FAOx) by Jeukendrup and Walis equation (2005). Required 3 PL regression with ≥ 3 valid FAOx points with 2 valid inflection points. Lower Bound = 0%; forced equation through origin (0,0). Upper Bound (UB) ≤ 100% and LB < FatMax < UB.

3.2.3.2 Exercise Sessions

During both exercise sessions, respiratory gases were measured at 5-min intervals prior to 25%, 50%, 75% and 100% of the exercise bout, equivalent to 100, 200, 300, and 400kcal, respectively, and the time to complete the exercise bout was adjusted accordingly. Any excessive energy expenditure achieved during the first of the randomized sessions was accounted for in the subsequent session to match energy expenditure.

Affective responses were measured from the perspective of the circumflex model of affect. Affect is defined by the orthogonal and bipolar dimensions of affective valence (pleasure-displeasure) and perceived activation (low-high). To capture these dimensions, two single-item measures were used. The Feeling Scale is an 11-point measure of affective valence. The scale ranges from -5 to +5. Anchors are provided at zero ("Neutral") and at all odd integers, ranging

from “Very Bad” (-5) to “Very Good” (+5). The Felt Arousal Scale [25] is a 6-point measure of perceived activation. The scale ranges from 1 to 6, with anchors at 1 (“Low Arousal”) and 6 (“High Arousal”). Affective responses were measured pre- and post-exercise in all 3 trials as well as at each minute during the initial VO_{2max} test and at regular intervals (e.g., ~25%, 50%, 75%, and 100% of the 400 kcal exercise dose) during the experimental conditions. Additionally, rating of perceived exertion (RPE) was recorded at the same intervals as other affective responses.

3.3.3.3 *Oral Glucose Tolerance Test (OGTT)*

The baseline OGTT occurred ~1 week after the screening visit and VO_{2max} test. . The baseline OGTT completed the exclusion criteria for high fasting glucose (> 125 mg/dL) and abnormal 2-hour glucose (> 200 mg/dL). Additional OGTTs was conducted 24 hours after the 2 acute exercise sessions and was separated by ~1 week. Prior to the post-exercise OGTTs, participants were asked to replicate the 24-hour food and beverage diary recorded prior to the baseline OGTT. Glucose area under the curve was measured by the trapezoidal method [26].

Indirect calorimetry was performed prior to and during each OGTT. Resting energy expenditure and substrate utilization were assessed using the ParvoMedics, TruOne 2400 metabolic cart (Sandy, UT) calibrated with standard gas mixtures . A transparent plastic hood connected to the metabolic cart was placed over the participant’s head and calculations of energy expenditure and carbohydrate and fat oxidation rates were made from expiratory gases diluted in a constant air flow (~40 L/min). Resting metabolic measurements were assessed before the OGTT (0 min) and at 60 and 120 minutes after ingestion of the glucose drink. The average of the last 20 minutes of the 30 minute measurement was used to calculate resting metabolic rate and substrate oxidation using the equations of Jequier et al [27]. Participants remained motionless

and awake during these periods. Metabolic flexibility was measured at 60 minutes (RER 60-0 min) and at 120 minutes (120-0 minutes).

3.2.4 Statistical Analysis

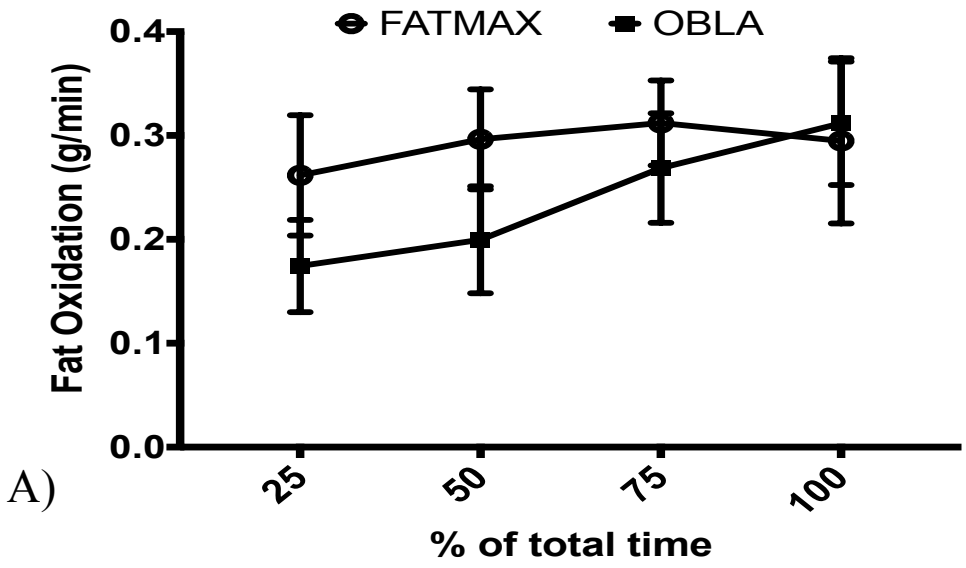
All statistics were performed using JMP statistical software (SAS Institute Inc., Cary, NC). Data analysis generally followed CONSORT recommendations using General Linear Models and repeated measures analysis of variance (RM-ANOVA) co-varied as needed depending on normality distributions at baseline and order of intervention trials. In addition, two-way RM-ANOVA (exercise session x time point) were used to determine differences in responses in both the primary and secondary outcome variables. Primary outcomes included AUC plasma glucose and change in metabolic flexibility (shift in RER pre to post oral glucose). Baseline (non-exercise) and exercise (FATMAX and OBLA combined) comparisons were evaluated using contrast statements with the one-way RM-ANOVAs. Significant effects were further evaluated using student's t-test post-hoc analyses were appropriate. Data will be reported as mean±SD and differences declared at $P<0.05$.

3.3 Results

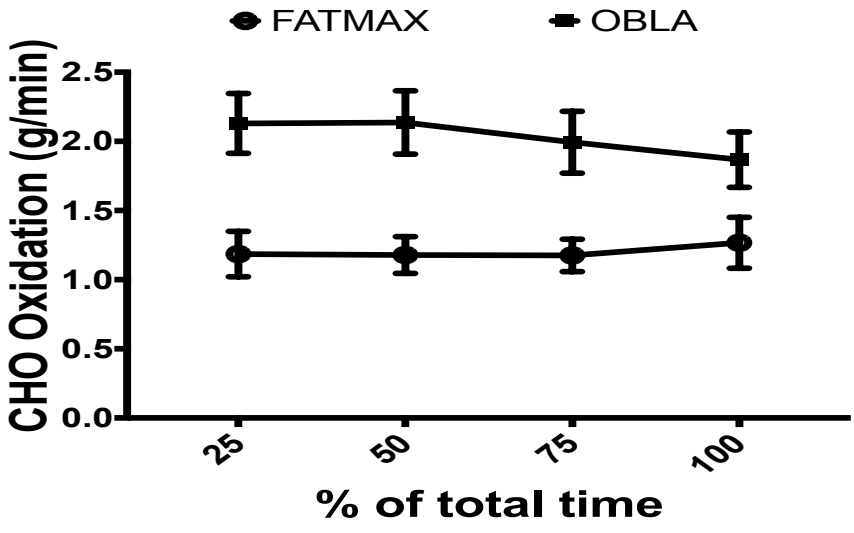
3.3.1 Substrate Oxidation During Fat Max and OBLA Exercise

The percent VO_{2max} at FATMAX (41±12%) and OBLA (68±10%) differed significantly ($p<0.001$). Substrate oxidation for fat (figure 3.3A) and carbohydrate (figure 3.3B) was measured during FATMAX and OBLA exercise. Total energy expenditure was 416.1 ± 11.4kcal for FATMAX and 422.1 ± 10.4kcal for OBLA exercise. FATMAX exercise elicited greater average fat utilization (18.6 ± 12.1g) than OBLA (10.1 ± 20.3g; $p=0.03$) exercise (figure 3.4). Accordingly, OBLA (82.8 ± 12.1g) exercise elicited greater carbohydrate utilization than FATMAX (62.4 ± 20.3g; $p<0.05$) exercise. As expected, the average time to complete the OBLA

exercise was less (40.0± 10.9 min) compared to the FATMAX exercise trial (62.9± 21.9 min; p=0.01).



A)



B)

Figure 3.3 A) Fat oxidation and B) CHO oxidation during acute bout of exercise for the FATMAX and OBLA trials. ± SEM

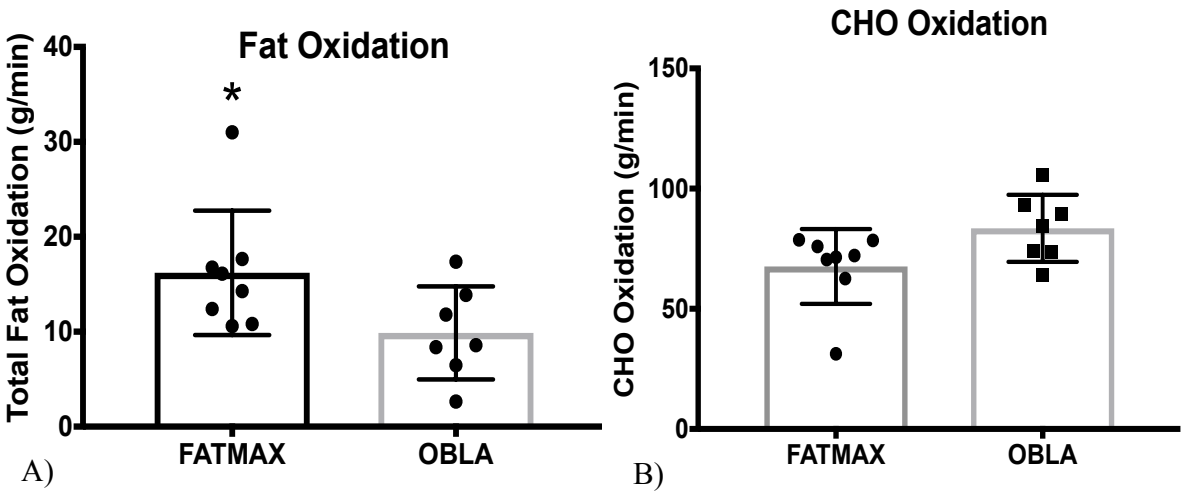
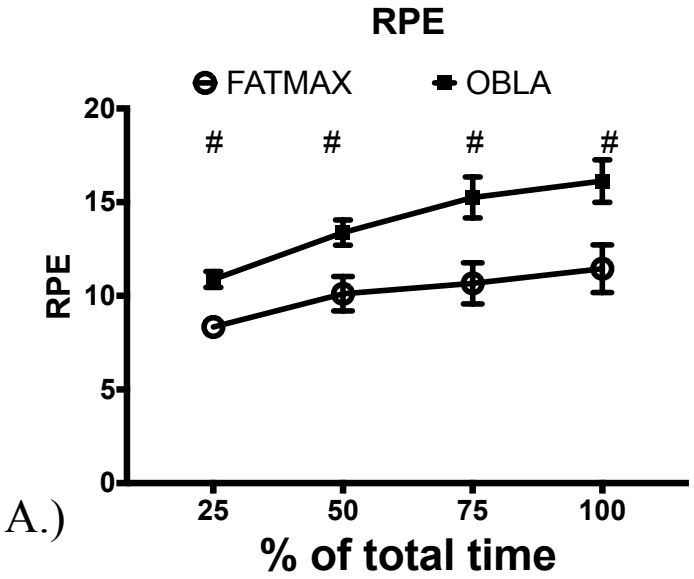


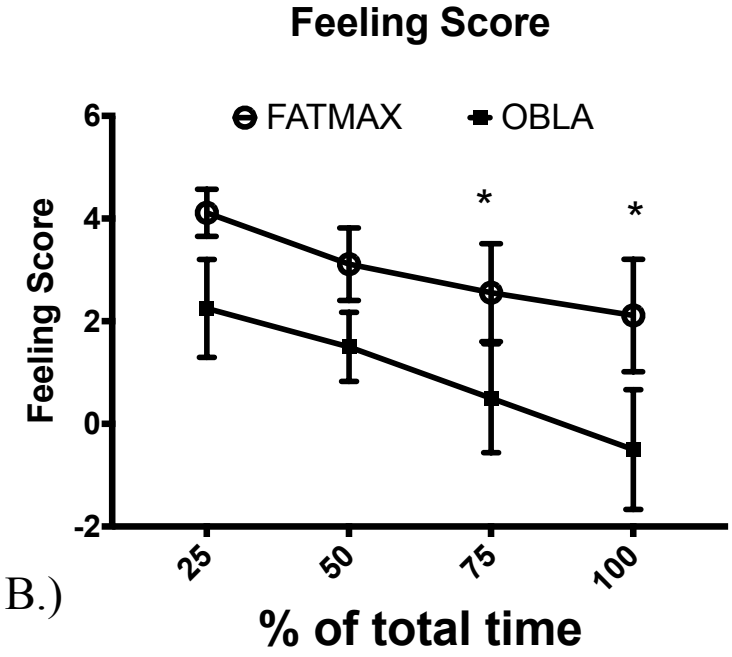
Figure 3.4. Average fat oxidation (A) and total carbohydrate (CHO) oxidation (B) for the FM and OBLA exercise exercise trials. * FM significantly greater fat oxidation ($P<0.05$) during FM trial and carbohydrate oxidation was significantly greater during the OBLA trial. Group means $\pm$ SEM

3.3.2 Affective Response

Participants completing the FATMAX exercise session reported lower RPE (Figure 7a) at all time points (25%, 50%, 75%,100%) compared to OBLA (average RPE FATMAX 10.1$\pm$2.1vs. OBLA 13.9$\pm$2.4; pvalue). Participants completing the FATMAX trial also reported significantly lower Felt Arousal (FA)(Figure 7b) ($p<0.05$) and Feeling Score (FS) (Figure 7c) ($p<0.05$) at the 25 (FATMX 1.6±1.7 vs. OBLA 2.5±1.5) and 50% (FATMAX 1.7±1.1 vs. OBLA 3.0±1.2) time points and 75 (FATMAX 2.5±2.7 vs. OBLA 0.05 ±3.0) and 100% (FATMAX 2.1±3.1 vs. OBLA -0.05±3.3) respectively.


RPE
FATMAX
OBLA
20
15
10
5
0
RPE
#
#
#
#
25
50
75
100
% of total time

A.)


Feeling Score
FATMAX
OBLA
6
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2
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-2
Feeling Score
*
*
25
50
75
100
% of total time

B.)

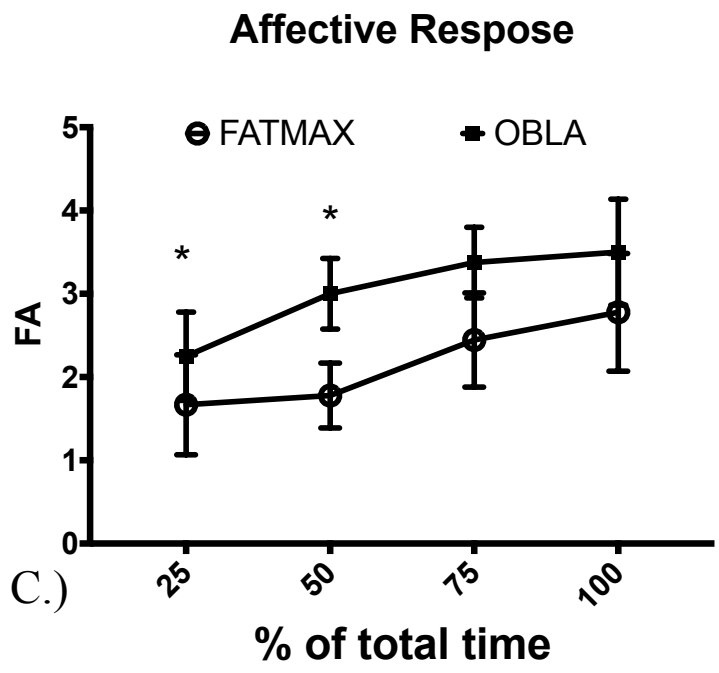

Figure 3.5. A) RPE, B) Feeling Score (FS), and C) Felt Arousal (FA) during FM and LT exercise. *P<0.05 , #P<0.001. ± SEM

3.3.4 Oral Glucose Tolerance

No significant differences were detected for fasting glucose at baseline or before the FATMAX or OBLA exercise conditions (84.2 ±17.0 and 90.0 ± 19.9 mg/dl respectively). Total AUC for glucose (figure 3.6) was higher for OBLA (15126.2±1351.1) exercise than baseline condition (13143.9±909.5; p<0.05). There were no significant differences between AUC for glucose after FATMAX (13411±1721) compared to baseline conditions (p=.58). Additionally, peak plasma glucose did not differ between trials (Baseline=128.8±15.4, FATMAX= 142.2±24.4, and OBLA=148.8±17.1)).

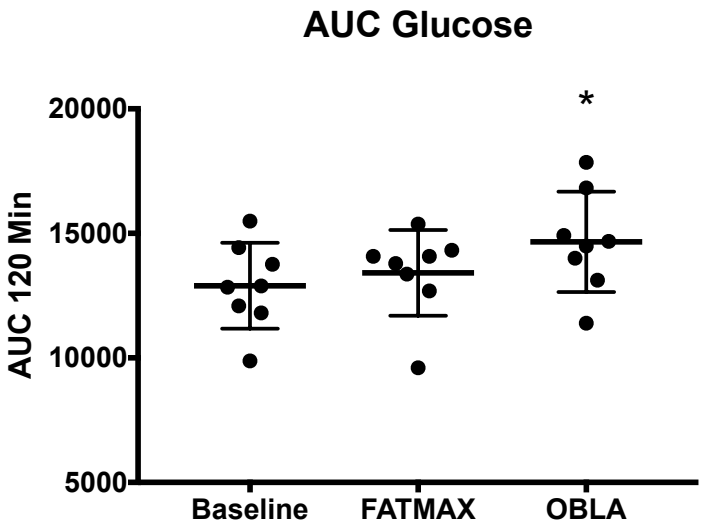

Figure 3.6 Glucose area under the curve (120-0 AUC) for all visits.
*p<0.01 OBLA vs. baseline.

3.3.5 Metabolic Flexibility

No significant differences were detected between the FATMAX (0.089 ± 0.032) and baseline measurements (0.11± 0.04; p<0.005) for the metabolic flexibility (ΔRER 120-0) (figure 3.7). There was however, a lower ΔRER (0.051 ± 0.032) 24-hr post OBLA exercise at the 60-minute time point compared to baseline (values; p<0.05). There were no significant differences between any condition at 120 minutes.

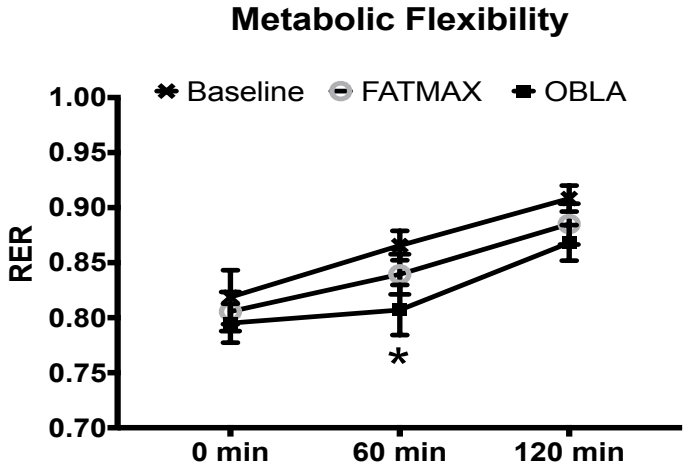

Figure 3.7 Metabolic flexibility measured during the OGTT at baseline and 24hrs after FATMAX and OBLA exercise sessions. *p<0.05 OBLA vs. baseline. ± SEM

3.4 Discussion

The purpose of this study was to investigate the effect of acute isocaloric aerobic exercise at FATMAX and OBLA on the glucose tolerance and metabolic flexibility of young overweight males. Previous work suggests that acute exercise increases insulin sensitivity for up to 24 hrs. post-exercise [7,10,28,29]. However, within these studies there appears to be differential effects of exercise due to intensity, type, meal timing, measurement timing, and diabetic status. Energy expenditure (total kcal) is a modifiable facet of exercise that is a potential source variability that may explain the response to exercise. In the current study, we found that OBLA exercise acutely lowers glucose tolerance and metabolic flexibility under well-controlled exercise conditions. This suggests that the acute period (24 hours) is characterized by delayed glucose disposal and oxidation.

Substrate utilization during exercise is dictated primarily by the intensity of exercise [23,30]. Duration of exercise also affects carbohydrate and fat oxidation especially when comparing trained versus untrained individuals. Klein et al (1990), demonstrated that fat oxidation was higher in trained volunteers (vs. untrained) during 4 hours of treadmill running at a low intensity (~20 ml/kg^{-1}/min^{-1}), despite similar rates of lipolysis and fatty acid uptakes [31]. Fat oxidation is also impacted by carbohydrate availability and the production of lactate [30,32,33]. However, these factors have been demonstrated under long duration (2.5-4 hours) exercise, which may not be clinically applicable.

Fat oxidation can be challenging to measure, in part, to the different sources (adipose and IMTG) of FFA available to the skeletal muscle during exercise. Evidence suggests that IMTG are a major source of FFA during exercise at low to moderate intensity [34,35]. Experimental inhibition of adipose lipolysis does appear to increase IMTG oxidation in healthy and T2D

patients, which suggests IMTG does account for some percentage, albeit varying, amount of fat oxidation [36,37]. As exercise intensity exceeds ~55% of VO_{2max} there appears to be a decrease in fat oxidation rate, especially IMTG oxidation [23,35]. In our study, FATMAX exercise trials were completed at individually calculated FATMAX thresholds (41.1 ± 12% VO_{2max}). During FATMAX exercise (Fig. 1A and 1B) we demonstrated a consistent level of fat oxidation without significant increases in carbohydrate oxidation. This suggest that the dosage of exercise (400 kcal) may be ideal to observe such effects.

As expected, as exercise duration increased during the OBLA trial there was an increase in fat oxidation and the contribution of FA to overall energy expenditure. However, average carbohydrate oxidation (20.8±3.4 g/min) was greater during OBLA exercise suggesting the higher exercise intensity promoted preferential utilization of carbohydrates (78.9±5.3% of total energy expenditure). The increased fat oxidation during OBLA exercise can be explained by numerous factors. As exercise duration increases, the availability of FA increases as does the transport of FA into the contracting skeletal muscle [35,38]. The isocaloric (400 kcal) study design was successful in executing exercise trials that promoted preferential substrate oxidation without exceeding the physiological threshold that would promote greater carbohydrate oxidation in the FATMAX trial and greater fat oxidation in the OBLA trial.

The accretion of IMTG is thought to be a factor in the development of insulin resistance [39]. However, this hypothesis is more specifically focused on the lipid intermediaries (ceramides, DAG, Faty acyl-CoA) [40]. The accumulation of these by-products of incomplete FA oxidation have been shown to interfere with the insulin signaling cascade of the skeletal muscle. The oxidation of IMTG and potentially lipid intermediaries could promote increased insulin sensitivity [41-43]. In the current study, we were not able to label and track specific sources of FA

being oxidized. Therefore, we cannot be certain as to the contribution of IMTG being utilized during the FATMAX trial. Nonetheless, we did not observe a lower glucose AUC 24 hrs post FATMAX exercise, which suggests there was no improvement in insulin sensitivity due to acute FATMAX exercise. Conversely, there was a significant increase in glucose AUC post OBLA exercise. The sympathoadrenal system is stimulated to a greater degree with higher intensity and longer duration exercise. The counter regulator hormones that are responsible for fuel mobilization (glucagon, epinephrine, norepinephrine, growth hormone, etc.) are activated, which increases glycogenolysis and gluconeogenesis [44,45]. A similar study comparing a single bout of exercise at 50% and 70% VO_{2max} did not detect significant difference in endogenous glucose production 30-minutes post exercise, which would suggest that effect does not extend to 24 hrs after exercise [7]. However, the sedentary and overweight status of our participants, could explain the effects of the acute higher intensity OBLA exercise and the duration its metabolic effects.

Peak metabolic flexibility (ΔRER 0.09±0.04) occurred at 120 minutes post oral glucose, but was not significantly improved by FATMAX exercise (0.08±0.04) or OBLA exercise (0.07±0.04). The shift in RER was significantly ($p<0.05$) lower at the 60-minute time point for OBLA exercise when compared to baseline (0.01±0.05) (Figure 3.7). Metabolic inflexibility is a common feature of obesity, impaired glucose tolerance, and T2D [46-48]. Metabolic inflexibility is also a characteristic of the offspring of type 2 diabetics [39,49], who are at a significantly greater risk for future develop of T2D [50]. The current study cohort did not have a positive family history of T2D and did not demonstrate metabolic inflexibility at baseline. The negative effect of OBLA exercise on metabolic flexibility does appear to be related to impairments in glucose tolerance 60 minutes post oral glucose (Figure 3.6). Higher resolution techniques (i.e hyperinsulinemic clamp

and radioactive labeled glucose) would be helpful in elucidating the mechanism behind this phenomenon.

A primary metabolic stimulus for triggering muscle glucose uptake is glycogen depletion [51]. The post exercise period of glycogen resynthesis is characterized by the rapid resynthesis immediately post exercise and the slower insulin mediated resynthesis. Kelley et al (1988) reported that skeletal muscle takes up ~26% of the glucose load after an OGTT and of that 50% is oxidized, 35% is stored as muscle glycogen, and the remaining 15% is released as gluconeogenic precursors (lactate, pyruvate, alanine) [52]. However, after glycogen depleting exercise, muscle glycogen storage can account for up to 40% of total oral glucose uptake [53,54]. These studies were conducted to maximize glycogen breakdown (2 hours of cycling at ~75% VO_{2max}). The amount of time, 40.0±10.9 min for OBLA compared to the FATMAX exercise trial 62.9± 21.9 min, and average intensity 41% vs 68% VO_{2max} likely did not reach the same level of glycogen depletion. Additionally, our sample was sedentary overweight young men, a group, that compared to well-trained individuals, has reduced glycogen storage capacity [53]. Nonetheless, exercise has been shown to increase the partitioning of oral glucose to glycogen storage even in the untrained.

OBLA exercise utilized a greater total amount of CHO (82.8 g) vs. FATMAX exercise (62.4 g), suggesting a greater potential level of glycogen depletion. The higher glucose AUC during the 24-hour post OBLA exercise OGTT, in combination with reduced oxidation of CHO (lower RER), suggests that non-oxidative glucose disposal may be impaired in this cohort. In fact, the post-exercise period in which the OGTT was performed (24 hours post FATMAX or OBLA exercise) is considered primarily insulin-mediated. Therefore, it is reasonable to assume

the effect exercise at OBLA in this group does not promote insulin sensitivity in the time frame we studied.

Participants reported feeling significantly less stressed and/or feeling better during the FATMAX trial compared to OBLA exercise. Considering the significantly lower intensity level (FATMAX 41±12% and OBLA 68±10%) experience during FATMAX exercise this is not surprising. However, this does suggest that this FATMAX intensity exercise provides some positive feedback to participants and does not acutely negatively affect glucose metabolism. These finding may be particularly relevant for certain populations, such as the population in this study (overweight sedentary young men) that are at increased risk for the development of cardiovascular disease.

In conclusion, there appears to be differential effects on glucose tolerance and metabolic flexibility in response to a single bout of FATMAX and OBLA exercise. The potential benefits of exercise may not manifest until sufficient adaptations have occurred. Another potential explanation for the differential response to exercise at FATMAX and OBLA intensity is the time course for measurement of oral glucose tolerance and metabolic flexibility. The period after exercise (immediately post to 24 hours) is characterized by metabolic responses that make the interpretation of health-related outcomes troublesome, therefore the measurements of these outcome may require a fuller understanding of the impact and lasting effects of a single bouts of exercise.

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CHAPTER 4: THE IMMEDIATE AND EXTENDED EFFECTS OF INTENSE INTERVAL EXERCISE ON METABOLIC FLEXIBILITY AND MITOCHONDRIAL FUNCTION

4.1 Introduction

Metabolic flexibility (METFLEX) is defined as the ability to switch from predominantly lipid oxidation and fatty acid uptake in the fasted state to insulin-stimulated suppression of lipid oxidation and high rates of muscle glucose uptake and glycogen synthesis that occurs in the postprandial state [1]. The inability to "shift" (Δ respiratory exchange ratio (RER)) metabolism from lipid to glucose oxidation, termed metabolic inflexibility, is a characteristic of obesity and T2D [2-4] and is implicated in the development of insulin resistance [1,5,6]. However, metabolic inflexibility is present prior to the development of insulin resistance and fasting hyperglycemia in the offspring of T2D individuals [6-8] and remains in previously obese individuals after weight loss [4].

Metabolic inflexibility is associated with impaired fasting lipid oxidation [3,5,9], which suggests that metabolic inflexibility is a reflection of the inability of the mitochondria to switch between fuels.[2] Since lipid oxidation occurs in the mitochondria, it is logical to deduce that impairments in mitochondrial function could alter whole-body lipid oxidation. Adding clarification to this issue, Ukropcova (2007) and van de Weijr (2013) have demonstrated that skeletal muscle mitochondrial function is reduced in metabolically inflexible individuals [8,10]. Recent evidence also suggests that the offspring of type 2 diabetics have impaired METFLEX without the presence of other metabolic risk factors [7]. Moreover, individuals with a biological mother that has T2D are at greater risk of developing T2D, when compared with a father with a positive history [11,12].

Continuous moderate to high intensity exercise training (6-12 weeks) has been shown to improve insulin sensitivity and metabolic flexibility in obese and type 2 diabetic patients, with greater improvements demonstrated with higher intensities [13,14]. Interestingly, the time course of glucose tolerance and insulin action in response to a single bout of exercise reveals periods of increased insulin

sensitivity (immediately post exercise to 3 days) and potential resistance or no improvement (5 to 7 days post exercise) [15,16]. However, the data regarding improvements due to a single bout of exercise or exercise training is more difficult to interpret in the light of the change in body weight (reduction in whole body adiposity)[2,17-19]. Favorable changes in body composition have significant impact on insulin action and METFLEX [2,20,21], thereby complicating the interpretation of the benefits of exercise in the absence of weight loss.

Recent research suggests that 2-6 weeks of high intensity interval training (HIIT) improves insulin sensitivity and skeletal muscle oxidative capacity [22,23]. However, data regarding changes in METFLEX in response to a single bout of high intensity interval exercise (HIIE) are lacking. Arad et al (2015) did not find any differences in fasted or insulin stimulated change in RER after 14-weeks in sedentary, premenopausal, African American women when compared to a non-exercising control group [24]. Careful measures were taken to keep body weight stable during this intervention. The design of the study was focused on measuring the chronic adaptations to HIIT and therefore, reassessment of insulin sensitivity and METLFEX utilizing the hyperinsulinemic-euglycemic clamp method was completed 72 hours after the final training session. This study, and others, suggest that the timing and methodology of measuring METFLEX and insulin sensitivity should be carefully considered. While the clamp technique is considered the "gold standard" for measuring insulin sensitivity the insulin infusion level likely does not reflect the ability of endogenously produced insulin to suppress whole body fat oxidation in the postprandial state.

To date there are no studies investigating the time-course of METLFEX in response to a single bout of HIIE. Characterizing the immediate and extended response of a single bout of HIIE could provide insight into the therapeutic applications of this exercise approach. The purpose of this study was to determine the immediate and extended (48-hr) effects of a bout of high intensity interval exercise prior to a mixed meal on METFLEX in men and women. We sought to test the hypothesis that acute HIIE would improve METFLEX in men and women. Additionally, we were interested in whether young men and

women with a FmHx of T2D, were metabolically inflexible as has been reported in older adults [8,12,25]. We also sought to measure *in vivo* mitochondrial function (OXPHOS) by completing a near-infrared spectrophotometry (NIRS) protocol. Several studies demonstrate that the adult children of mothers with T2D are more likely to develop metabolic inflexibility and that, in part, may be due to the inherited mitochondrial dysfunction passed on through the mother's mitochondrial DNA [8,25-27]. Finally, we aimed to determine the effect of a single bout HIIE on METFLEX in FmHx participants.

4.2 METHODS

4.2.1 Participants

Sixteen apparently healthy, active, normal weight (BMI = 18 to 25 kg/m^2) young (age 19 to 25 y) men and women with (n=8) and without (n=8) a positive family history of T2D participated in this study. A positive family history (FHx) was defined as having a biological mother with T2D. The control participants were considered to have no FHx if both parents and grandparents were free of T2D. All participants reporting being no more than moderately physically active (3 days week of 30 minutes of moderate intensity physical activity) and no participant reported engaging in regular HIIE. Individuals with known cardiovascular, T2D, and/or metabolic disorder were excluded from the study. This study was approved by Louisiana State University's Institutional Review Board. All participants provided written informed consent.

4.2.2 Experimental Design

After an initial screening and confirmation of positive or negative FHx eligible participants were scheduled to complete a baseline maximal graded exercise test, near-infrared spectrophotometry (NIRS) OXPHOS, and mixed meal tolerance test (MMTT) (Figure 4.1). Results from the maximal graded exercise test were used to prescribe a single bout of HIIE performed during a separate week and followed by a MMTT 1 hour post HIIE (1hrPE). Finally, a follow-up MMTT was performed 48 hours after the HIIE day (48hrPE).

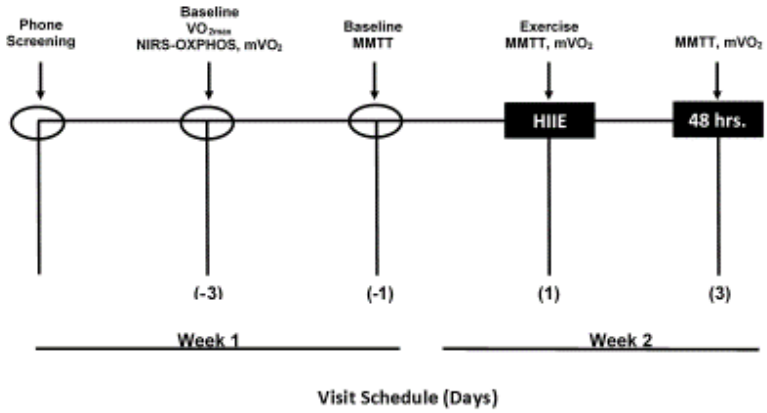

Figure 4. 1. Visit schedule. *After confirmation of study eligibility, participants will undergo a baseline VO_{2max} and mitochondrial function test (OXPHOS). The following week participants will complete a baseline mixed meal tolerance test (MMTT) to determine metabolic flexibility. The next day, participants will return to complete high intensity interval exercise (HIIE) followed by a MMTT with measures of mVO_2. 48 hrs post exercise intervention MMTT and mVO_2 will be repeated.

4.2.3 Experimental Protocol and Measurements

4.2.3.1 Maximal Graded Exercise Testing (VO_{2max})

If participants meet all inclusion/exclusion criteria during the screening visit, a maximal cardiorespiratory test (VO_{2max}) on a cycle ergometer (Velotron, Racermate, Inc., Seattle, WA) was completed during the baseline visit. Participants rode at an initial workload of 25 Watts for 4 minutes. The workload increased by 25 Watts every 4 minutes until respiratory exchange ratio reached 1.05 after which, the workload increased by 25 Watts every 2 minutes until volitional exhaustion. Throughout the test, respiratory gases were analyzed using a metabolic cart (ParvoMedics Inc., Sandy, UT) and fat oxidation rates will be determined using stoichiometric equations[28]. The watts achieved at the time of volitional exhausting was consider 100% watt max.

4.2.3.2 NIRS Protocols to Measure Muscle Metabolism (e.g., NIRS mVO2, blood flow, and OXPHOS)

Study participant arrived at laboratory having refrained from exercise 24 hours prior to the study visit and having fated overnight. Measurements of subcutaneous adipose tissue and muscle depth were collected for the vastus lateralis and vastus medials via use of B-mode ultrasound (LOGIQ e, GE Healthcare, Little Chalfont, UK) Participants were positioned on a table rested in the supine position with knees slightly flexed. The NIRS optodes (Oxymon MKIII, Artinis Medical Sytsems) were placed longitudinally on the non-dominant *vastus lateralis* and *vastus medialis* just anterior to the *iliotibial band* and ~1/3 the distance between the top of the patella and the greater trochanter of the test leg (Figure 4.2). The optodes were secured in place with adhesive tape and Velcro strap. A blood pressure cuff (Hokanson, Bellevue WA) was placed proximal to the optode, and connect the cuff to a rapid cuff inflation system (Hokanson E20, Bellevue WA) that was controlled using an external controller and Labview Software. NIRS signals for oxygenated (O_2Hb) deoxygenated (HHb), and total hemoglobin (tHb) were continuously monitored by NIRS.

Resting muscle blood flow was measured in triplicate using venous occlusions by inflating the blood pressure cuff to (50-60 mmHg) for 20 seconds with at least 120 seconds between each measurement. Blood flow was measured by calculating the slope of the linear increase total hemoglobin (tHb): BF= (((ΔtHb x 60)/ ((([Hb] x1000)/4))x1000/10 in ml^{-1}•min•$100ml^{-1}$. Measurements of resting muscle oxygen consumption (mVO2) were made in triplicate using arterial occlusions by inflating the blood pressure cuff to (250-300 mmHg) for 20 seconds with at least 120 seconds between each measurement and were calculated by measuring the linear decrease in O_2Hb by the following formula: mVO_2=Abs(((ΔO_2Hb x 60)/(10 x 1.04)) x 4) x 22.4/1000) in mlO_2•$min^{-1}100g^{-1}$.

For measurement of muscle oxidative capacity (NIRS-OXPHOS), participants completed a 7-second isometric contraction of the vastus lateralis (by knee extension) immediately followed by a series of 15-20 arterial occlusions (250-300 mmHg) to measure the rate of recovery of mVO2. The occlusions started with a series of 9 occlusions at 5 seconds on and 5 seconds off followed by a series of 11

occlusions 10 seconds on 10 seconds off. This NIRS-OXPHOS protocol was repeat twice with ~5-10 min between each trial. Recovery mVO_2 was calculated for each occlusion and fit to a monoexponential function as follows according to Ryan et al (2014): $y=(end - delta) \times e^{-kt}$ where y is the relative mVO_2 during the arterial occlusion, end is the $mVO2_2$ immediately following the isometric knee extension, delta is the change in mVO_2 from rest to knee extension, k is the fitting rate constant and t is time[29]. Finally, we performed an ischemic calibration procedure and calculate mVO2 corrected for blood volume as previously described [30], which includes a 15-20 second isometric contraction of the vastus lateralis (by knee extension) immediately followed by ~8-10-minute arterial occlusion (250-300 mmHg). According to the methods of Ryan et al (2013) Time constants (Tc) functioned as an index of mitochondrial function [31]. NIRS data was analyzed via a custom written program in MATLAB (Mathworks, Natick, MA). Muscle blood flow and mVO_2 were measured 30 minutes prior to and 30 minutes, 60 minutes, and 120 minutes after ingestion of the mixed meal.

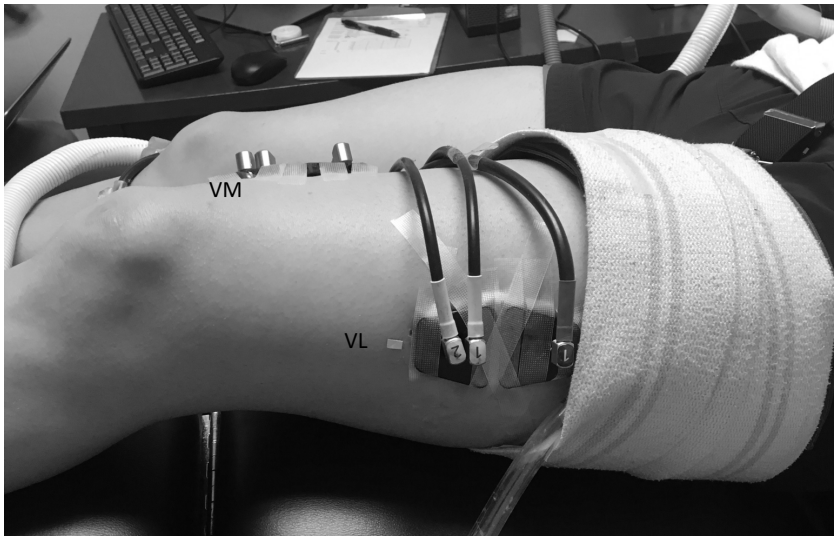

Figure 4.2 A.) Longitudinal placement of NIRS optodes on vastus lateralis (VL) and vastus medialis (VM).

4.2.3.3 Mixed Meal Tolerance Test (MMTT)

Prior to the baseline MMTT visits, participants were asked to refrain from exercise, alcohol, and caffeine, for 48 hours and were ask to provide a record of food intake the day preceding the test. The morning of the test, participants arrived fasted and provided the food record. Upon arrival participants started a 30-minute rest period. During this time, the participants were provided with a heart rate monitor and NIRS probes were attached, as described previously, for measurements of blood flow -30, +30, +60, and +120 post mixed meal ingestion.

After the 30-minute rest period the -30 minute metabolic rate measurement was taken by placing a vented canopy system to measure expired O_2 and CO_2 (ParvoMedics Inc., Sandy, UT) to measure resting energy expenditure (REE; Kcal/day) and calculate carbohydrate and fat oxidation rates using stoichiometric equation [32]. Exhaled air was continuously measured for 30 minutes pre-mixed meal (-30 to 0 minutes,) and post meal (+40 to +70 minutes and 110-140 minutes) while the participate remained still and refrained from sleeping. The last ten minutes of each collection period was averaged and used to report REE, RER, and substrate oxidation rates.

After completion of the fasted pre-mixed meal metabolic rate measurement, the mixed meal drink (Ensure, Abbott laboratories, Lake Forest, Illinois) was prepared by calculating the average REE (kcal/day) of the final 10 minutes of the measurement period and dividing by 3. A member of the study staff then prepared the meal by measuring the grams of powder and mixing into ~150-200 ml of water. Additionally, blood was drawn through an IV catheter immediately before the ingestion of the mixed meal and 5, 10, 15, 20, 30, 60, 90, 120, 150, 180 minutes post meal. All procedures for the MMTT, including the energy content (kcal) of the mixed meal, were replicated for the immediate and 48-hour post exercise visits.

4.2.3.4 High Intensity Interval Exercise (HIIE) and 48 hr follow-up visit

On a separate week from the baseline measurements, participants arrived fasted overnight having replicated the diet 24 hours prior to the baseline MMTT. Participants were also asked to refrain from

alcohol, caffeine, and vigorous exercise 48 hours prior to the visit. Upon arrival participants completed 30 minutes of rest followed by the baseline REE measurement. Participants then completed a 5-minute warm-up on the cycle ergometer (Velotron, Racermate, Inc., Seattle, WA) at 50 watts. Immediately after the warm-up participants then completed alternating intervals of watts corresponding to 90% of the watts achieved at peak oxygen consumption during the VO_{2max} test for 60 seconds followed by 60 seconds at 30% of the max watts for a total of 20 minutes.

After completion of the HIIE intervention (1hrPE) participants returned to the resting laboratory to rest for 60 minutes, after which fasted blood samples were drawn, the mixed meal was provided and sampling proceeded as detailed previously. Forty-eight hours after the HIIE visit (48hrPE) participants returned to the laboratory to complete the final MMTT of the study. Participants were asked to replicate their baseline study diet and refrain from alcohol, caffeine and additional exercise between the HIIE visit and 48-hour follow up.

4.2.3.4 Measurement of Metabolic Flexibility and Calculation of Substrate Oxidation

Metabolic flexibility (METFLEX) was defined primarily as the difference (Δ) in RER 60 minutes post mixed meal ingestion compared the RER at 0 minutes prior to the mixed meal. Additionally, we will define flexibility at the change in rise rate of carbohydrate oxidation and the decrease in rate of fat oxidation (Figure 4.3). REE, carbohydrate (CHO) and fat oxidation rates were calculated utilizing the following equations [32].

1.) $REE= ((3.58 \times VO_2) + (1.448 \times VCO_2)-0.002)) \times 1440$

2.) $CHO\ Ox= (4.55 \times VCO_2) – (3.21 \times VO_2)$

3.) $Fat\ Ox=(1.67 \times VO_2) – (1.67 \times VCO_2)$

Additionally, we will classify the different phases of METFLEX as the early or "1st phase" which was be measured by subtracting the rate of CHO and fat oxidation (mg/kg/min) at the 60-minute time

point by the 0 minute time points. 2nd and 3rd phase will represent the latter phases of METFLEX that correspond to (60-120 minute) and (120-0 minute) calculations respectively.

During the 1st phase of a healthy METFLEX response, insulin should mediate the rapid increase in CHO oxidation and suppression of fat oxidation (Figure 4.3). The 2nd is likely to correspond to a lower, but still elevated levels of CHO oxidation, whereas fat oxidation should start to approach near fasted levels. The 3rd will represent either the residual CHO oxidation and fasted or higher levels of fat oxidation.

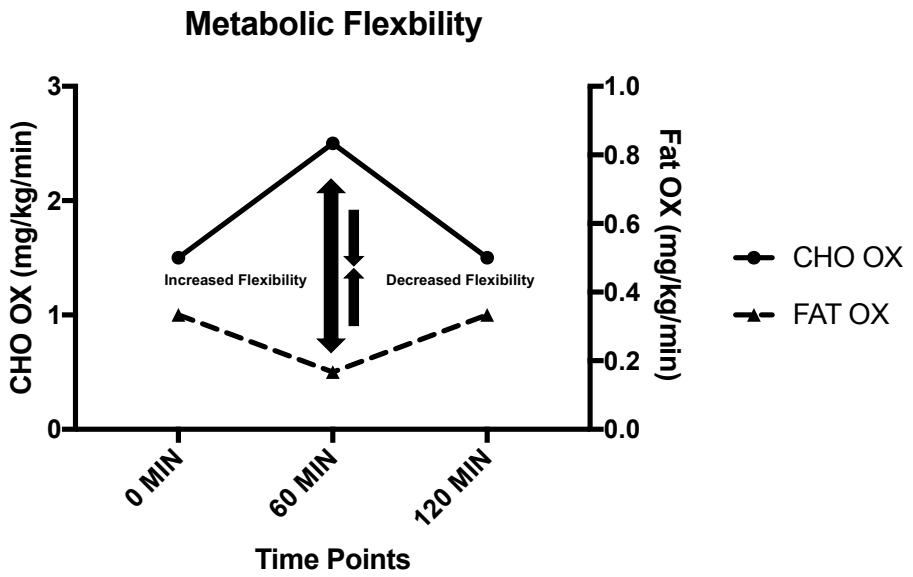

Figure 4.3. Conceptual model of metabolic flexibility based on the rise in carbohydrate oxidation rate and decrease in fat oxidation rate. Outward pointing arrows indicate greater metabolic flexibility. Inward pointing arrows reflect decreased metabolic flexibility.

4.2.3.5 Plasma and Serum Assays

Blood samples were collected in Sodium fluoride/Oxalate EDTA tube and immediately placed on ice. An additional set of tubes without anticoagulant was used for the collection for serum. The tubes were centrifuged at 2000 rpm for 10 minutes thereafter plasma and/or serum were transferred to a plastic microtube and frozen at -80 degrees Celsius. The glucose oxidase technique was used during the MMTT via a bedside glucose measurement system (GL5, Analox Instruments LTD. Lunenburg, MA). The measurement of plasma insulin was completed with the use of an enzyme linked immunoabsorbance

assay (ELISA) kit (Sigma Aldrich, St. Louis, MO). Serum non-esterified fatty acids (NEFA) were quantified by an in vitro enzymatic colormetric assay (Wako chemical, Richmond, VA).

4.2.4 Statistical Analysis

All statistics were performed using JMP statistical software (SAS Institute Inc., Cary, NC). Data analysis will generally follow CONSORT recommendations using General Linear Models and repeated measures analysis of variance (RM-ANOVA), co-varied as needed depending on normality distributions at baseline. An unpaired t-test will be used to compare between group baseline characteristics. In addition, two way RM-ANOVA (exercise session x time point) were used to determine differences in responses in both the primary and secondary outcome variables. Significant effects will be further evaluated using student's t-test post-hoc analyses when appropriate. Data will be reported as mean±SD and differences declared at $P<0.05$.

4.3 Results

4.3.1 Participants

A list of participant characteristics is presented in table 4.1. Participants were (n=16, 6 males, 10 females) young (21.2±1.2 y) with normal body weight (22.6±2.6 m/kg^2) and physically active (≥30 minutes of moderate intensity exercise). All participants had normal fasting plasma glucose levels (5.4±0.5 mmol/l).

Table 4.1 Participant Characteristics

	Total (n=16)	Male (n=6)	Female (n=10)	
Age (y)	21.2±1.2	21.8±1.6	20.8±0.8	
Height (cm)	169.3±11.3	176.6±7.0	165.1±5.3	
Weight (kg)	65.3±11.3	74.3±10.9	59.9±7.8	
BMI	22.6±2.7	23.8±3.2	21.9±2.1	
	Males (n=6)		Females (n=10)	
	Control (n=4)	FmHx (n=2)	Control (n=4)	FmHx (n=6)
Age (y)	22±4	21.5±7	20.7±5	20.83±0.9
Height (cm)	177±8.7	174.6±78.2	164.9±5.5	165.2±5.7
Weight (kg)	72.3±12.7	78.2±7.8	60.0±5.1	59.7±9.6
BMI	22.9±3.7	24.67±1.2	22.0±.15	21.8±2.5

Data are mean ± SD

4.3.2 Baseline Measurements

4.3.2.1 Maximal Graded Exercise Test

Table 4.2 provides the data acquired from the maximal graded exercise test completed on the cycle ergometer. There were significant differences between males and females for VO2max, maximum watts achieved during the test (Max Watts), however, when watts were expressed as watts/kg there were no significant differences between Max Watts. The prescribed exercise intensity for HIIE was 90% of the Max Watts during the work interval and 30% of Max Watts during the recovery interval. There were significant differences according to gender at 90% (men= 187.5 ± 30.7 W vs. women 135 ± 21 W; p=.006) and at 30% (men= 62.5 ± 10.2 vs. women 45 ± 7.0 W; p=.0063). These differences were not significant in terms of watts/kg for the 90% and 30% intervals (p=.674 and p=.1759 respectively).

Table 4.2 Baseline Graded Exercise Test Results

	Total (n=16)	Male (n=6)	Female (n=10)
VO_{2max} (ml/kg/min)	31.7±4.1	35.0±2.0	29.7±3.7
METS	9.34±1.1	10.3±.35*	8.7±1.0
Max Watts	171±39.6	200±35.3*	143.7±12.5
30% Rest Interval (W)	51.5±11.8	62.5±10.2*	45±7.0
90% Work Interval (W)	154.6±35.6	187.5±30.7*	135±21.1
Max Watts/kg	26±0.4	2.8±0.4	2.5±0.3
30% Rest Interval (W/kg)	.78±0.11	.84±0.4	0.75±0.3
90% Work Interval (W/kg)	2.3±0.35	2.5±0.4	2.26±0.3

Table 4.2 *continued*

	Controls Males (n=4)	FmHx Males (n=2)	Control Females (n=4)	FmHx Females (n=6)
VO_{2max} (ml/kg/min)	34.3 ± 2.2	36.5 ± 0.7	28.0 ± 2.6	30.9 ± 4.2
METS	10.3 ± 0.4	10.5 ± 0.2	8.4 ± 0.9	9.0 ± 1.2
Max Watts	200.0 ± 35.4	225.0 ± 35.4	143.8 ± 12.5	154.2 ± 29.2
30% (W)	60.0 ± 10.6	67.5 ± 10.6	43.1 ± 0.8	46.3 ± 8.8
90% (watts)	180.0 ± 31.8	202.5 ± 31.8	129.4 ± 11.3	138.8 ± 26.3
Max Watts/kg	2.8 ± 0.4	2.9 ± 0.7	2.4 ± 0.03	2.6 ± 0.4
30% w/kg	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.0	0.8 ± 0.1
90% w/kg	2.5 ± 0.3	2.6 ± 0.7	2.3 ± 0.4	2.3 ± 0.4

*p<0.05
Data are mean ± SD

4.3.3.2 NIRS OXPHOS

Mitochondrial function (OXPHOS) was significantly (p=0.03) lower in FmHx participants (46.5±25.6 %/s) compared to control participants (28.4±10.3 %/s) (figure 4.4). There were no significant associations between OXPHOS and VO_{2max}.

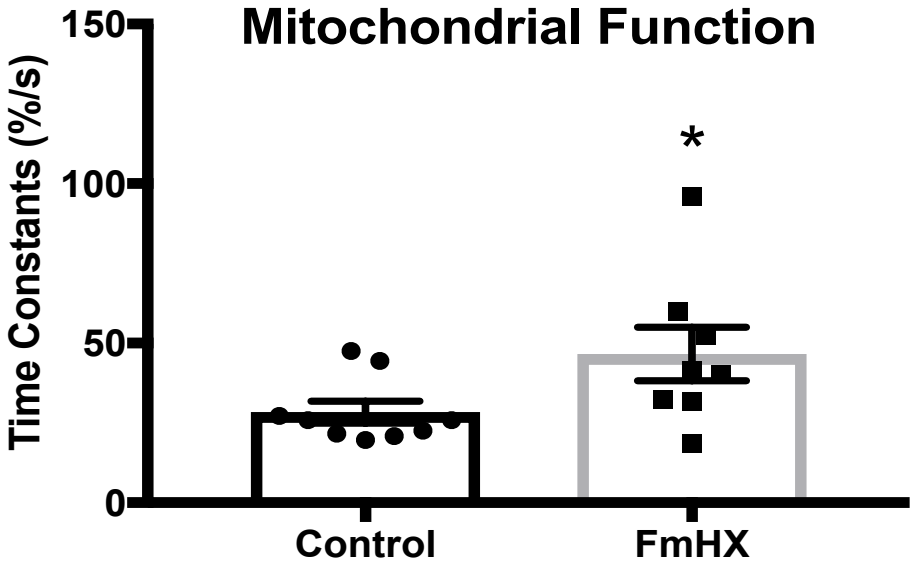

Figure 4.4 Mitochondrial Function measured in control and FmHx participants during baseline assessments. *p<0.05. Data are expressed as mean ± SEM

4.3.3.3 Resting Energy Expenditure and Mixed Meal Composition

Individual baseline REE was used to determine the content of the mixed meal (table 4.3). There were no significant differences in REE and therefore no differences in meal composition. The mean baseline REE was not significantly different between males (1337±234.9 kcal/d) and females (1118.2±198.6 kcal/d). CHO, Pro, and Fat intake were similar across all groups.

Table 4.3 Mixed Meal	Total (n=16)	Males (n=8)	Females (n=8)
REE (kcal/d)	1241.2 ± 219.2	1337.7 ± 234.9	1183.2 ± 198.6
Meal (kcal)	412.2 ± 71.5	445.9 ± 78.3	392.0 ± 62.5
Kcal/kg	6.5 ± 0.8	6.3 ± 1.0	6.7 ± 0.8
CHO (g)	54.4 ± 9.4	58.9 ± 10.3	51.8 ± 8.3
Fat (g)	14.4 ± 2.5	15.6 ± 2.7	13.7 ± 2.2
Pro (g)	14.4 ± 2.5	15.6 ± 2.7	13.7 ± 2.2
CHO(g/kg)	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
Fat(g/kg)	0.2 ± 0.0	0.8 ± 0.1	0.2 ± 0.0
Protein(g/kg)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0

CHO, carbohydrate; Pro, protein.
Mean±SD

4.3.2 Metabolic Response to Mixed Meal and HIIE

Resting energy expenditure (Figure 4.5) was not different between visits. REE increased (23.3%) between the 0 and 60 minute time points. Baseline REE (1241.1±219.1 kcal/d) increased to 1531.1±327.9 kcal/d ($p<0.0001$), but returned to a non-significant ($p=0.72$) rate at 120 minutes (1488.8±338.6 kcal/d). During the 1hrPE REE increased ($p<0.0001$) in a similar manner from 1305.8±224.9 to 1647.7 kcal/d as was the case with the 48hrPE visit as well (1330.9±265.3 to 1539.7±373.0 kcal/d; $p<0.001$). No significant interaction between visit and time point were detected.

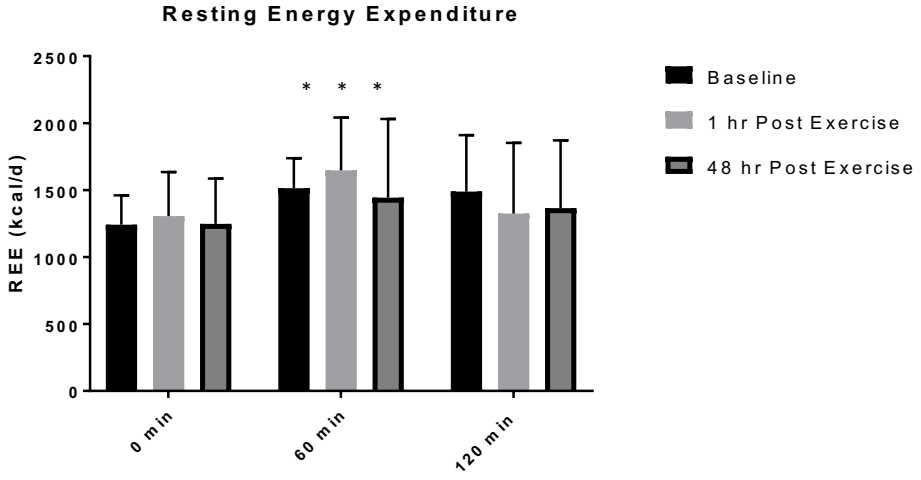

Figure 4.5 REE across time points (0, 60, 120 min) and visits. *<0.001 (0 min vs. 60 minute). REE, resting energy expenditure. Data are presented as mean ± SD

Analysis of the 1st phase ΔRER (ΔRER60-0min) (Figure 4.6) METFLEX response did not reveal any statistical differences between baseline and 1hrPE (p=0.65) and 48hrPE (p=0.31). There was no significant relationship between any visits and the 2nd or 3rd phase shift in RER. There were no significant differences detected between groups for the 1st, 2nd, or 3rd phase shift in CHO oxidation during the mixed meal tolerance (baseline vs. 1hrPE p=0.09; baseline vs. 48hrPE, p=0.65; 1hrPE vs. 48hrPE, p=0.88) or fat oxidation (baseline vs. 1hrPE p=0.91; baseline vs. 48hrPE, p=0.28; 1hrPE vs. 48hrPE, p=0.40).

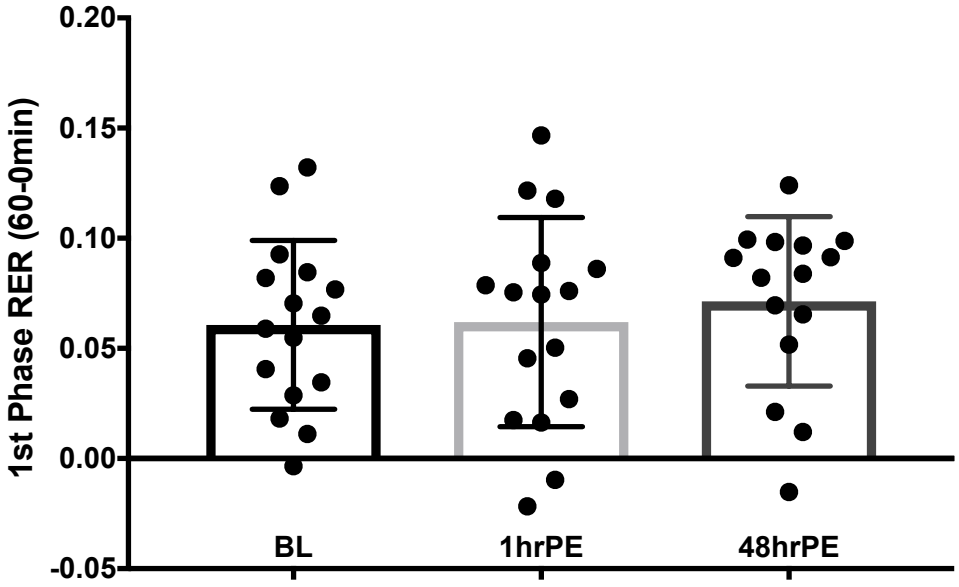

Figure 4.6 Baseline METFLEX measured as the difference between RER at 60 subtracted from RER at 0 min. RER, respiratory exchange ration (VCO_2/VO_2). Data are presented as mean ± SD

Fasted CHO oxidation (1.17 ±0.77 mg/kg/min) was not significantly different between any visits (1hrPE 0.97±0.69 and 48hrPE 0.87±0.56) CHO oxidation (mg/kg/min) was increased (p<.0001) within the baseline trial when transitioning from fasted CHO oxidation to the measured peak at 60 minutes (2.23±0.67) and remained elevated (p=0.04) in the 2nd phase and at 120 minutes (p<0.0001) post mixed meal ingestion. Similar differences (p<0.0001) were detected within the 1hrPE and 48hrPE visits to the 60 minute time point (2.24±0.92 mg/kg/min and 2.11±0.80 mg/kg/min). CHO oxidation rate was not different 1hrPE when comparing the 120 minute time point to the 0 minute (fasted) CHO

oxidation,(1.23±0.58 mg/kg/min), indicating that CHO oxidation levels returned to fasted level more quickly than baseline and for the 48hrPE trial (1.39±0.62 mg/kg/min; p<0.0001).

Fasted fat oxidation rate (figure 4.7) at baseline (0.95±0.32 mg/kg/min) was not significantly different when compared to the fasted "0 min" measurement during the 1hrPE (1.1±0.32 mg/kg/min) visit prior to the HIIE intervention. Fasted fat oxidation rate was elevated (p=0.049) at the 48hrPE visit suggesting a residual increase in fat oxidation due to the HIIE intervention.

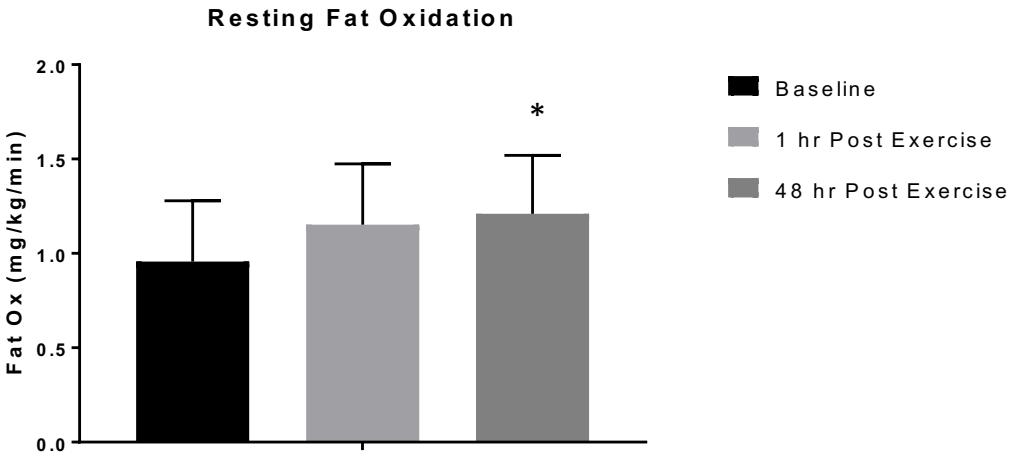

Figure 4.7 Fasted oxidation rate prior to mixed meal and or exercise for all visits. *p<0.05 baseline vs. 48hrPE. Data are presented as mean ± SD

There were no differences (figure 4.8) detected when measuring the transition from fasted to 60 min for the reduction in fat oxidation during this time period. Fat oxidation decreased from fasted rates to 0.83±.032 and 0.98±0.35 mg/kg/min for the baseline and 1hrPE visit respectively. However, fat oxidation rate decreased significantly (p=0.012) within the trial for 48hrPE falling to a rate of 0.94±0.38 mg/kg/min. The fat oxidation rate during the 48hrPE at 120 minutes (1.31±0.39) remained elevated above fasted level (1.31±0.39), but not significantly (p=0.08). The overall METFLEX response for all visit and comparisons to between visits is presented in figure 4.9.

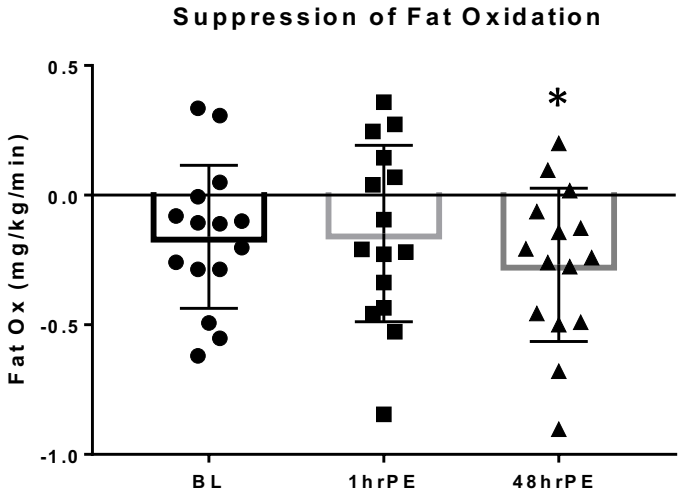

Figure 4.8 Decrease in fat oxidation (1st Phase) in response to the mixed meal. BL, baseline, 1hrPE, 1 hour post exercise, 48hrPE, 48 hours post exercise. *p<0.05 within visit. Data are presented as mean ± SD

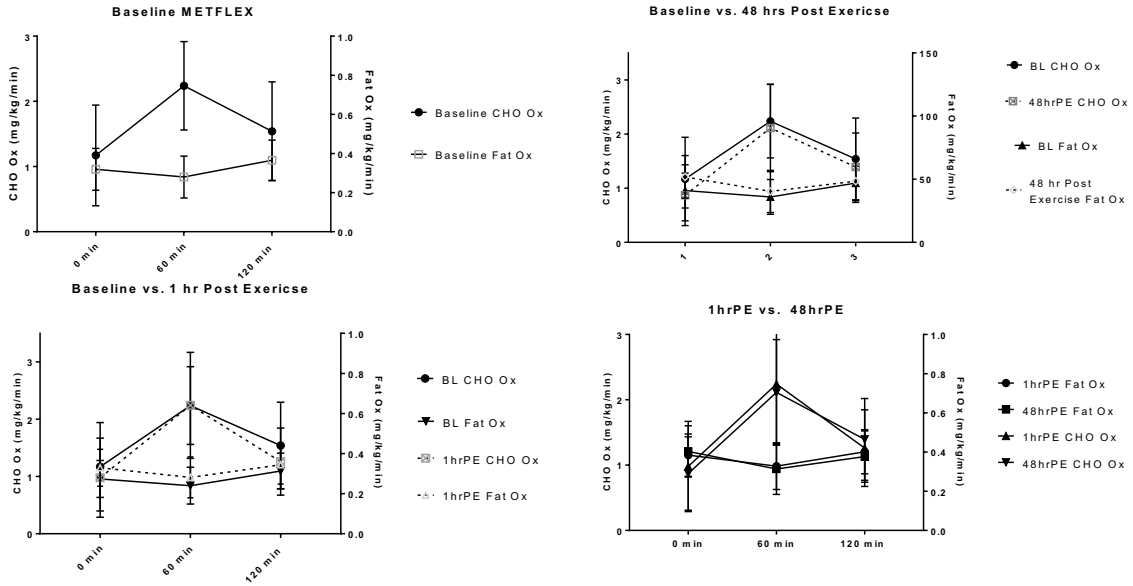

Figure 4.9 METFLEX response for CHO and Fat oxidation alteration at baseline, basline vs. 1hrPE, baseline vs. 48hrPE, and 1hrPE vs. 48hrPE. Data are presented as mean ± SD

4.3.3 Plasma and Serum Analysis

Fasting plasma glucose was increased (p=0.043) in the 1hrPE (5.9±1.3 mmol/l) visit when compared to the baseline visit (5.3±0.5 mmol/l), but not the 48hrPE visit (5.5±0.7 mmol/l; p=0.45). Fasting plasma insulin was not different between baseline (10.7±4.3 uUI/ml) and 1hrPE (9.3±3.3 uUI/ml; p=0.68) and the 48hrPE (7.9±3.1 uUI/ml; p=0.25) visits or between 1hrPE and the 48hrPE (p=0.73) visits. Fasted serum NEFA levels were significantly greater (p=0.006) 1hrPE (1.2±0.2) when compared to baseline (0.76 ±0.2 mmol/l). Fasted NEFA levels returned to near baseline fasted levels at 48hrPE (0.74 ±0.3. The time course of plasma glucose (4.10), plasma insulin (4.11), and serum NEFA (4.12) did reveal significanty differences for the calculated total area under the curve (4.13A) and insulin (4.13B) across all visits. There was no significant difference between time points.

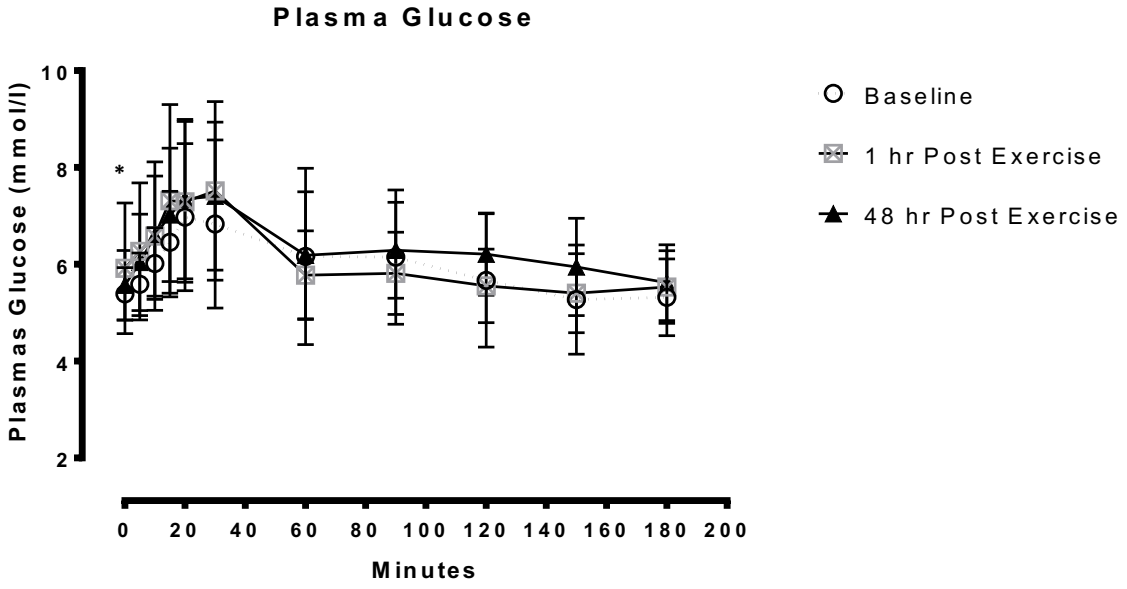

Figure 4.10 Plasma glucose response across time points and visits. *p<0.05 baseline vs. 1hr post-exercise. Data are presented as mean ± SD

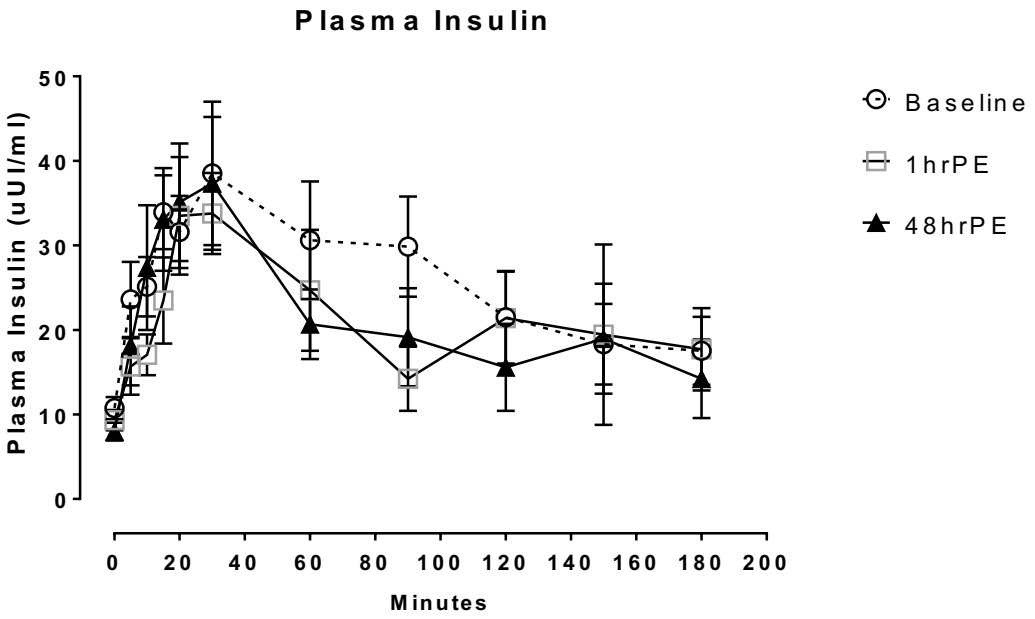

Figure 4.11 Plasma insulin response across time points and visits. Data are presented as mean ± SD

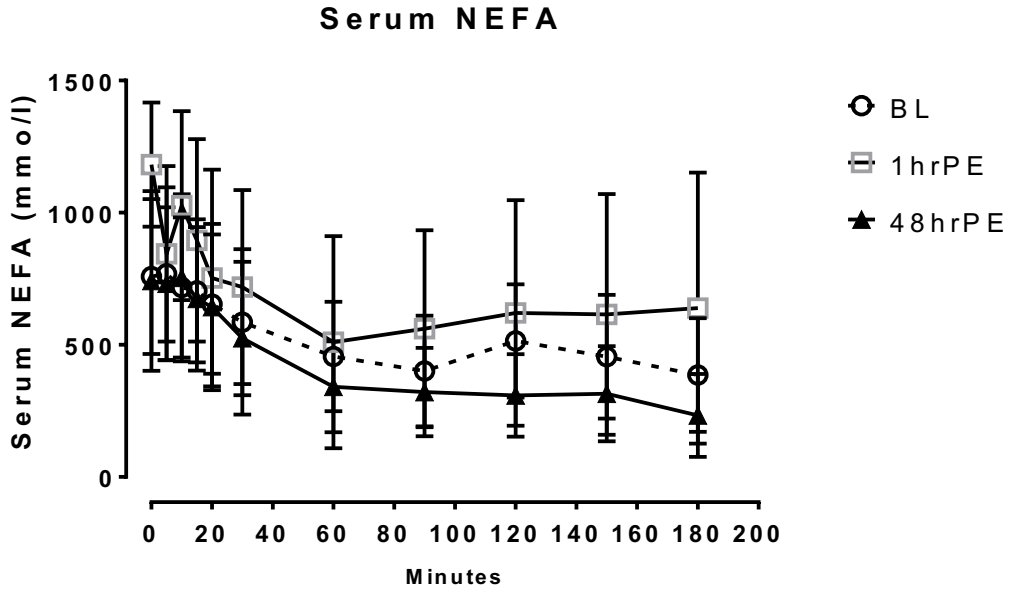

Figure 4.12 Serum NEFA response across time points and visits. Data are presented as mean ± SD

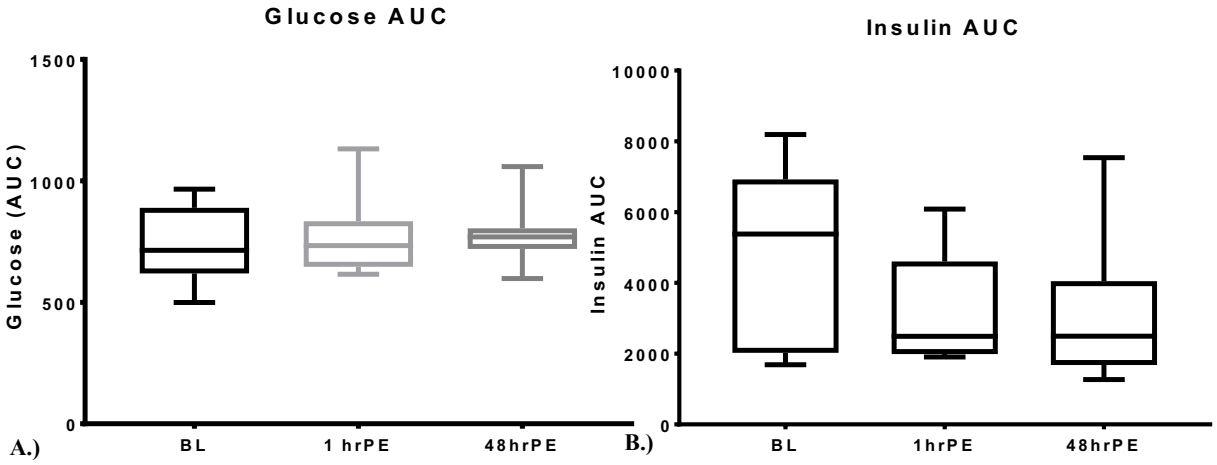

Figure 4.13 A.) Total glucose area under the curve and B.) total insulin area under the curve. Data are presented as mean ± SD

4.3.4 Blood Flow Measurements

Blood flow measurements (Figure 4.14) were conducted prior to ingestion of the mixed meal 0 min, 30 minutes, 60 minutes, and 120 minutes after. Blood flow measurement are expressed in terms of fold change. Blood flow did not increase to a significant degree during the baseline visit between 0 and 30 minutes ($p=0.72$) and at the 60 minute time point ($p=0.20$), but did increase at the 120 minute time point ($p=0.04$). During 1hrPE visit blood flow increased ($p=0.02$) at 30 minutes, 60 minutes ($p<0.0005$), and remained nearly significant ($p=0.07$) at 120 minute post mixed meal. No differences were detected within the 48hrPE trial. Between group analysis revealed an increased fold change during the 1hrPE visit at the 60 minutes (1.83±0.8, $p=0.03$) and 120 minute time point (2.0±1.0; $p=0.04$)) above the corresponding fold change during the baseline visit (1.4±1.01) and 48hrPE visit (1.18±0.5; $p=0.004$).

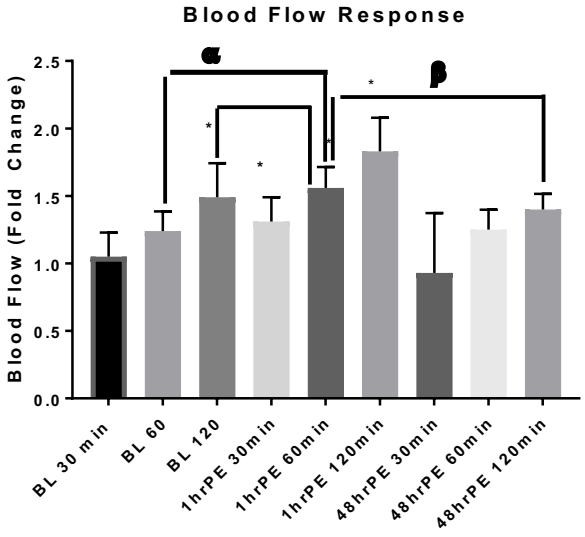

Figure 4.14 Blood flow response reported as fold change from 0 min measurement across visits and time points. *p<0.05 within visit increase in blood flow. α p<0.05 between visits, β p<0.005. Data are presented as mean ± SD

4.3.5 Comparison of Positive Family History Versus No Family History of T2D

A comparison of the fold change (figure 4.15) in CHO oxidation and fat oxidation during the baseline visit revealed differences in the suppression of fat oxidation (0.99±0.08 fold change) at the 60 minute time point between the control group (0.76±0.08) and FmHx (p=0.926). These differences did not extend past to the 120 minute time point for the fold change in fat oxidation. The increase in CHO oxidation at the 60 minute mark did not reach statistical significance (p=0.052) in the FmHx group, whereas the control group had significantly (p=0.02) increased CHO oxidation at the 60 minutes.

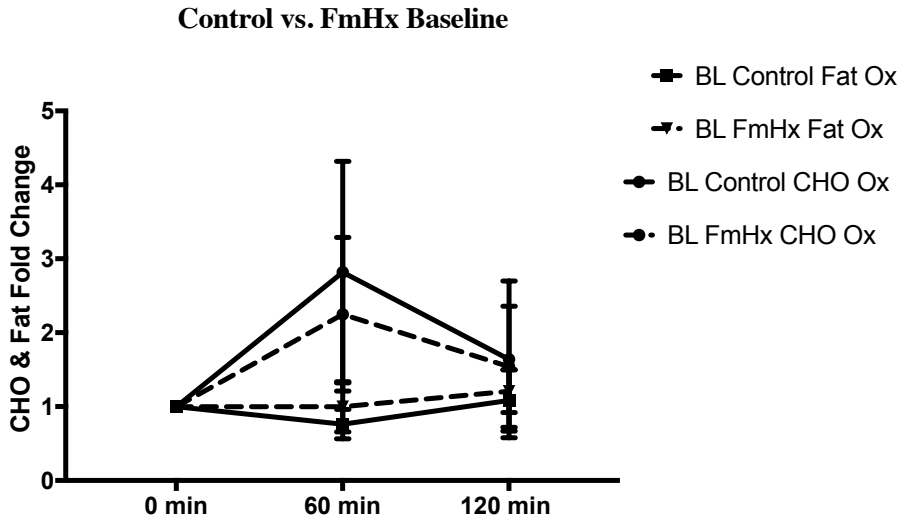

Figure 4.15 Comparison of the fold change in CHO oxidation and fat oxidation between control participants those with FmHx. Data are presented as mean ± SD

Differences were detected (p=0.043) in the ability to suppress fat oxidation in during the 1hrPE visit at the 60 minute time point (figure 4.16). The ability to increase CHO oxidation was improved (p<0.0001) at 60 minutes during the 1hr PE visit, with an approximate 4 fold increase in CHO oxidation compared to a 2.5 increase at baseline. The improvement in CHO oxidation during the 1hrPE visit did not extended to the ability to suppression fat oxidation during any of the time points for the FmHx group during the 1hrPE visit. However, this effect also manifested itself in the control, whereby, the increase in CHO oxidation significantly increased (p<0.0001) without a significant decrease in fat oxidation (60 minute, p=0.043; 120 minutes, p=0.09).

During the 48hrPE visit (figure 4.17)FmHx participants nearly significantly increased CHO oxidation at 60 minutes (p=0.053) and to similar fold change measured during the BL visit (BL 2.25 vs. 48hrPE 2.31). The ability to suppress fat oxidation 48 hours post exercise improved from a fold change 0.99±0.08 to 0.81±0.07. Comparatively, the control group achieved a suppression fold change of 0.78±.08, which did not differ significantly between groups.

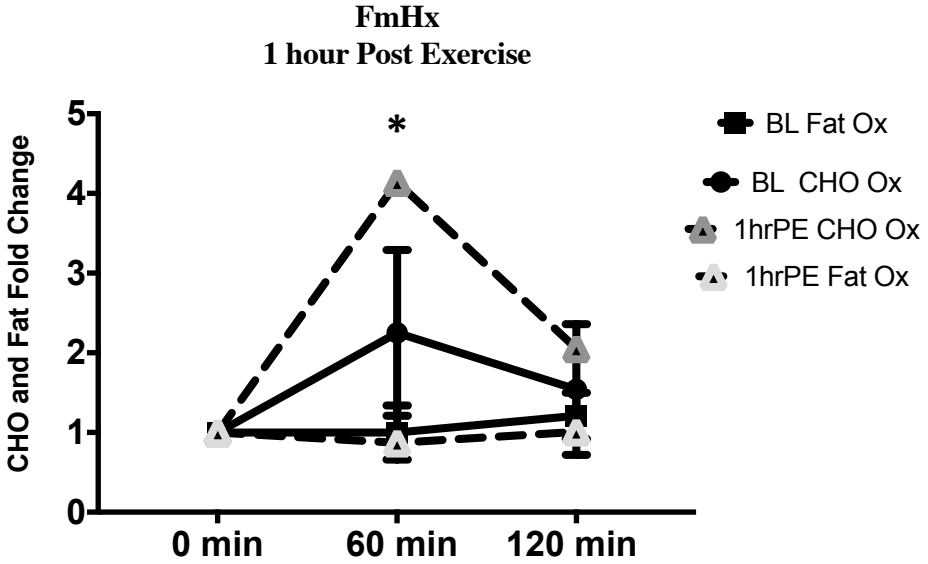

Figure 4.16 The comparison between baseline and 1hrPE METFLEX response within the FmHx group.* $p<0.05$. Data are presented as mean ± SD

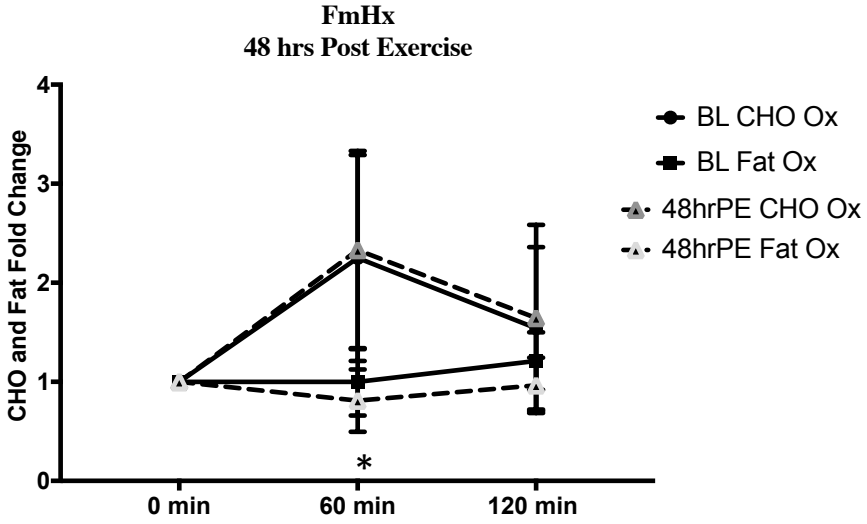

Figure 4.17 The comparison between baseline and 48hrPE METFLEX response within the FmHx group. *$p<0.05$. Data are presented as mean ± SD

4.4 Discussion

The purpose of this study was to determine the effect of HIIE on METFLEX immediately after exercise (1hrPE) and to measure any extended effects 48 hours after exercise (48hrPE) on METFLEX. To establish baseline METFLEX, we implemented a MMTT that was specifically prescribed for the individual participants based on one-third of their measured REE. We assessed METFLEX by measuring the difference (1st phase) in RER, CHO oxidation, and fat oxidation from the fasted 0-minute time point to 60 minutes. METFLEX was reassessed at the 1hrPE and 48hrPE visits to determine if HIIE intervention promoted greater 1st phase METFLEX response. These measurements were conducted to test the hypothesis that acute HIIE would increase the 1st phase response immediately post exercise.

We sought to understand some of the potential mechanisms and links to metabolic flexibility, measuring plasma glucose, plasma insulin, and NEFA in response to the mixed meal to determine to what degree glucose tolerance and insulin sensitivity was affected by HIIE. Additionally, we were interested in whether young men and women with a FmHx of T2D were metabolically inflexible as has been reported in older adults [8,12,25] and if HIIE improved metabolic flexibility. The primary findings of this study were that METFLEX was differentially improved 1hrPE and 48hrPE via increased CHO oxidation and increased suppression of fat oxidation respectively. Additionally, we detected significantly reduced mitochondrial function in participants with a FmHx of T2D. We also established improved METFLEX in FmHx participants in response to HIIE via similar mechanism demonstrated by control participants as previously mentioned.

Metabolic inflexibility is characterized by the inability to increase RER in response to insulin stimulation via either oral glucose administration or hyperinsulemic clamp method [2]. However, recent work has improved our understanding of METFLEX as the inability to partition fuel once it enters the cell [33-35]. It has been suggested that provision of a single macronutrient (glucose) or stimuli does not challenge cells to partition fuel and cannot, therefore, assess METFLEX [33,36]. Therefore, we chose to provide a mixed meal (~412 kcal, 55g CHO, 14g fat, and 14g Pro) to adequately challenge postprandial metabolism

with a mixture of macronutrients. The MMTT is also considered the gold standard test to measure the insulin release, which plays an important role in the postprandial response to METFLEX [37,38].

The metabolic response (figure 4.5) to a mixed meal in our study was evident in the increase in REE at the 60-minute time point. The 23% increase in REE is likely due to a couple of factors. The thermic effect of digestion increases metabolic rate typically proportionally to the energy consumed [39,40]. There are also mechanisms involved in the hypothalamus-pituitary-adrendal axis that work to increase gastric motility that influence REE [40]. The response to the meal did not reveal any differences between study visits (baseline 23% increase, 1hrPE 26% increase, and 48hrPE 15% increase) suggesting differences in RER, CHO ox, and fat ox were likely not due to a simple increase REE.

We did not detect a difference in the 1st phase shift in RER between baseline METFLEX and 1hrPE and or 48hrPE visits. The 2nd phase shift in RER (60min-120min) measures the increase in RER from the peak. This phase represents the balance of residual increases in CHO oxidation and or fat oxidation suppression. Interestingly, the 2nd phase RER response was significantly elevated at the 1hrPE visit. Parsimonious, analysis suggests that the increase in CHO oxidation is the factor that is driving the increase in RER during the phase at the 1hrPE visit. There was an increase, albeit not statistically significant ($p=0.09$), in the 2nd phase response at the 1hrPE visit compared to baseline.

Exercise at higher intensity (>75% VO_{2max}), in particular intermittent or interval-based exercise, alters the relationship of hepatic glucose output (HGO) and glucose uptake in the muscle [41,42]. Typically, during steady state exercise HGO meets the demands of the working skeletal muscle, however, during very intense exercise HGO typically exceeds the rate of demand [42]. As exercise ceases, glucagon, epinerphrine, and norepiphrine remain elevated, which leads to the increase in HGO and resulting hyperglycemia[42-44]. Elevated glucose levels in healthy people increase insulin levels in order return to euglycemia. Insulin, in this scenario, will promote glucose uptake, oxidation, and storage to varying degrees [45].

In the current study, we identified a significant increase in plasma glucose 1 hour post HIIE when compared to baseline and 48hrPE visits. The increase in plasma glucose concentration is a likely mechanism to promote the oxidation of glucose in addition to the carbohydrate ingested as a part of the mixed meal. The fact that fasted insulin levels were not increased significantly prior to the mixed meal, during the 1hrPE visit, may be due to the fact that blood was not taken until immediately before the mixed meal was given (60 minutes after HIEE). This point is supported by a study conducted by Marliss et al (2002), whereby insulin levels had returned to fasted levels 60 minutes after a 40-minute bout of moderate intensity exercise and return to baseline within 20 minutes after a 15-minute bout of exercise at 87% VO_{2max} [42].

Glucose AUC was not different between baseline, 1hrPE, and 48hrPE visits. Insulin AUC was also not affected by the HIIE at 1hrPE or 48hrPE visits. In current, young healthy cohort, reports of a lack of improvement in glucose tolerance not entirely surprising. Several studies have indicated with mixed meal feeding a lack of effect in terms of glucose tolerance and insulin sensitivity in response to various intensities of exercise [46-50]. Potentially, the benefit of utilizing the mixed meal to measure metabolic flexibility may not be optimal for testing glucose tolerance and therefore, may indicate the two are not as closely related as previously thought [51].

An interesting finding of our study was the increase in resting-fasting fat oxidation rate (mg/kg/min) at the 48hrPE visit. Important to note, REE was not elevated at 48hrPE visit. The lasting effect of post-exercise oxygen consumption (EPOC) is due to many factors (fitness level, intensity and duration of exercise) [52-55], however, in most cases the effect an exercise bout on has REE has diminished within 24 hours. The specific increase in fat oxidation as a result of HIIE is of interest and coincides with existing literature that demonstrates increased skeletal muscle mitochondrial enzyme levels after 2-6 weeks of HIIE training [23,56]. While there is a paucity of data regarding the effects of a single bout of HIIE, a study conducted by Little et al (2011) demonstrated that a single bout of HIIE (4x30s maximum

effort or ~500% peak power) increased mitochondrial biogenesis via the increase in the nuclear abundance of PGC1-α [57].

The HIIE protocol utilized in the aforementioned study by Little et al (2011) was a wingate-based model. The demands (specialized equipment, hard to tolerate, unsafe for some individuals) of the wingate-based model reduced its practicality and applicability. The HIIE protocol utilized in the current study (60s at 90% Wmax followed by 60s at 30%Wmax x 10) was also developed in the lab of Martin Gibala and Jonathan Little and is referred to as the "practical model". Interestingly, similar mitochondrial adaptations have been shown using this protocol in 2 weeks of training [22]. The results of the current study may potentially provide physiological evidence that some mitochondrial adaptations occur within 48 hours of a single bout practical model HIIE.

The increase in fasting fat oxidation rate at 48hrPE also corresponded to a significant suppression in fat oxidation rate after ingesting the mixed meal during the 1st phase METFLEX response. An important function of insulin is the suppression of lipolysis and fat oxidation in the postprandial period [36,51,58]. Fat oxidation rate decreased significantly at the 48hrPE visit (~26% reduction), whereas, similar levels of suppression were not measured at baseline (10% reduction) or during 1hrPE visit (~12% reduction). This phenomenon was not robust enough to significantly increase RER during 1st phase METFLEX, however, it does emphasis an important component of METFLEX in altering the balance of increasing CHO oxidation and decreasing fat oxidation in the postprandial period.

The levels of NEFA in the blood stream reflects the balance of lipolysis systemically. 1hrPE fasted serum NEFA levels were significantly elevated when compared to baseline and 48hrPE. As was the case with HGO, the hormones epinephrine, growth hormone, and hormone sensitive lipase are secreted in an intensity-dependent manner [59-61]. Therefore, it is expected that NEFA levels would be elevated after one hour HIIE. Increasing NEFA levels has been experimentally shown to induce metabolic inflexibility in lean sedentary individuals and lean endurance trained individuals by reducing CHO oxidation [62]. However, the lean endurance- trained participants increased non-oxidative glucose disposal (i.e. glycogen

storage) in response to the lipid infusion. These results were linked to the superior mitochondrial performance measured in the endurance-trained compared to the sedentary participants.

In the current study, the increased NEFA levels did not reveal any impairment in the ability to increase CHO oxidation. Although we did not purposely maintain elevated NEFA levels, as did the previous study, we simply demonstrated an intact mechanism for insulin to reduce lipolysis and lower fat oxidation in the postprandial period. An intriguing mechanism that has emerged from these data involves increased fasting fat oxidation as an integral component to improve the function of insulin to increase CHO oxidation and suppress fat oxidation. This is not a trivial finding, as elevated NEFA have been shown to inhibit insulin action and glucose transport in the skeletal muscle. [58 63]

Impaired postprandial blood flow is considered a potential factor in the actions of insulin and control of glucose levels [64,65]. Additionally, metabolic syndrome is often associated with a reduced sensitivity to beta-adernergic stimulation thereby reducing blood flow to the skeletal muscle and adipose tissue [66]. METFLEX is potentially affected by blood flow alterations in the postprandial period considering that a large amount of glucose will be oxidized in the skeletal muscle. Utilizing NIRS (figure 4.14) we measured the change in blood flow during the MMTT at baseline, 1hrPE, and 48hrPE. Significant increases were measured 120 minutes after ingestion of the mixed meal during the baseline visit. Blood flow increased 1.3, 1.5, and 1.7-fold 30 minutes, 60 minutes, and 120 minutes post mixed meal during the 1hrPE visit. The increases in blood flow were greater than the comparative changes at 60 minutes during baseline. The changes in blood flow were also evident at 120 minutes 1hrPE when compared to 48hrPE. These finding demonstrate the HIIE is effective at increasing blood flow postprandially.

A secondary aim of this study was to investigate whether young participants with a FmHx of T2D were metabolically inflexible and had reduced mitochondrial function, as has been reported in older adults [7,8], and if HIIE improved METFLEX. Baseline mitochondrial function (figure 4.4) was significantly lower in FmHx participants when compared to controls. Reduced mitochondrial function is

frequently demonstrated as a potential component of metabolic inflexibility and insulin resistance [25,67,68]. In our study control participants and FmHx particiapants were matched BMI, activity level, and fitness (VO_{2max}) all of which may have be closely related to mitochondrial function. These results indicate that mitochondrial dysfunction may play a role in the early manifestation of metabolic inflexibility without the presence of insulin resistance or increase adiposity.

There were no detected interactions between FmHx and the 1st phase RER, CHO oxidation, and fat oxidation METFLEX response, however, there were differences within each visit that were not detected in our control group. At baseline FmHx did not significantly reduce fat oxidation (fold change) (Figure 4.15) in the 1st phase (60-0 min) and the capacity to increase CHO did not reach statistical significance (p=0.052). 1hrPE (Figure 4.16) FmHx significantly increased CHO oxidation during the 1st phase, however, fat oxidation suppression was unaltered. This phenomenon appears to be a uniform response across groups. The robust increase in CHO oxidation during the 1hrPE visit suggests the effect exercise persists at least 2 hours post-HIIE. Additionally, the reduced suppression in fat oxidation in the 1st phase during the 1hrPE visit may be due to the increased flux of fatty acids during that time point, and under such circumstances would dictate great fat oxidation.

During the 48hrPE (Figure 4.17) visit there was a significant improvement in 1st phase fat oxidation suppression which was not present during the baseline visit or 1hrPE visit. Importantly, this degree of fat oxidation suppression was similar the control group. There was a difference in the resting fat oxidation between controls (1.23±0.3 g/min) and FmHx group (1.8±g/min) at the 48hrPE visit. The relationship may point to a relationship between the actions of insulin and rate of fat oxidation.

This study was limited in terms of the ability to determine the rate of HGO as a factor in glucose tolerance and or the METFLEX response to exercise. Aside from its role in increasing CHO oxidation, glycogen storage, and fat suppression insulin also works to reduce HGO. Understanding the role of the liver in METFLEX is a potentially critical component that we were not able to elucidate. Another limitation of this study was the inability to assess the level of glycogen depletion during HIIE and to

determine if the METFLEX response was associated with the degree of depletion. The rate of glycogen storage is a primary determinant for METFLEX [36,51,62] and insulin sensitivity [69,70]. The determination of factors involving the time frame of glycogen resynthesis after exercise and in the day after hold considerable potential. The amount of calories and carbohydrate in the mixed meal may have been another potential limitation. It is possible that a greater total amount may have produced a response that could be evaluate under the influence of exercise.

In conclusion, the immediate effects (1 hour) of HIIE on METFLEX are primarily directed on the increase in carbohydrate oxidation and blood flow in response to a mixed meal. The extended effects (48 hours) appear to work via fat oxidation or mitochondrial mechanisms by increasing resting fat oxidation rate and improving the suppression of fat oxidation in response to a mixed meal. The lack of difference in RER under these conditions demonstrates the need to assess the alteration in fuel oxidation, whereas RER does reflect the balance of CHO and fat oxidation it does not identify the influencing factor. Finally, HIIE improved METLFEX in participants with a FmHx of T2D, that demonstrated metabolic inflexibility and reduced mitochondrial function, by improving the suppression of fat oxidation in the postprandial period.

4.5 References

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CHAPTER 5. CONCLUSION

The purpose of this dissertation was study the time-course of diet and exercise and its effects on METFLEX. Three studies were conducted to investigate molecular and clinical aspects of diet and exercise and how these stimuli may alter the metabolic response to fuel. These include work using both animal models and human participants. Additionally, we sought to understand the METFLEX response in individuals with FmHx of T2D and to determine the effects of exercise in the group. The first study was conducted on obesity-prone, Osborne-Mendel (OM) and obesity-resistant S5B/Pl (S5B) rats. This experiment investigated inherent differences in energy expenditure (EE), metabolic rate, MF and skeletal muscle markers of metabolism, lipid storage and lipid oxidation between OM and S5B, as well as HFD-induced strain differences in these parameters. The consumption of a HFD in S5B rats increased metabolic rate and EE and decreased metabolic rate and EE in OM rats. These strain differences were not due to the differences in activity or food intake. These results suggest that HFD-induced differences in metabolic rate and EE may be mediated by HFD-induced differences in pAMPK and PPARγ expression and lipid accumulation in the gastrocnemius muscle of OM and S5B rats.

The second study was designed to investigate the effect of acute aerobic exercise at an intensity that maximizes the rate of fat oxidation (FATMAX) on glucose tolerance, insulin action, and METFLEX compared to acute aerobic exercise at the onset of blood lactate accumulation (OBLA) resulting in greater carbohydrate (CHO) oxidation. OBLA exercise appears to have deleterious effects on oral glucose tolerance and metabolic flexibility acutely, however, FATMAX exercise does not confer improved MF. These results suggest that predominate substrate utilization does not promote improved glucose tolerance and metabolic flexibility in young overweight men.

The third and final study of this dissertation was designed to investigate the immediate and extended of a single bout of high intensity interval exercise (HIIE) on METFLEX and mitochondrial function. In study 3, participants were recruited with a family history (FmHx) of T2D (n=8) and without a

family history of T2D (n=8). Participants completed baseline (no previous exercise) mixed tolerance tests (MMTT) to assess MF. On a separate week participants completed a single bout of HIIE follow by an immediate (1 hour later) MMTT and follow-up MMTT (48 hours later) MMTT. The immediate effects (1 hour) of HIIE on METFLEX are primarily directed on the increase in carbohydrate oxidation and blood flow in response to a mixed meal. The extended effects (48 hours) work via the reduction fat oxidation by increasing resting fat oxidation rate and improving the suppression of fat oxidation in response to a mixed meal. Particiapants with a FmHx of T2D demonstrated reduced mitochondrial function and some impairments in metabolic flexibility. HIIE improved METFLEX in a similar time course and mechanism as mentioned previously.

In summary, the potential benefits of exercise may be contained within a certain time frame after the completion of exercise. The degree to which exercise perturbs metabolism often dictates the length of the response and therein the benefits of exercise on health. The susceptibility to develop metabolic inflexibility appears to be linked to mitochondrial dysfunction. The early manifestation of this phenomenon in our cohort demonstrates the important role that mitochondrial metabolism plays in the pathogenesis of metabolic inflexibility. The extend and duration to which exercise improves mitochondrial function will be of considerable importance in the future study of metabolic health.

APPENDIX

1.1 General Exam

MYOKINES AND THE ANTI-INFLAMMATORY EFFECTS OF EXERCISE

A General Examination

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Kinesiology

by
Timothy Allerton
M.S., Northeastern University 2007
B.S., Louisiana State University, 2005
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Introduction

Inflammation is a fundamental component of a living organism's response to noxious or infectious stimuli. The immune system is comprised of specialized organs (thymus, bone marrow, lymph nodes, etc.) and cells (T cells, B cells, macrophages) and responds to eliminate an invading parasite, virus, or bacteria from the host. In some cases, the host's initial innate barriers are overwhelmed and infection can occur. In order to defend against these pathogens the host's immune system works to increase inflammation in order to isolate and kill the pathogen by recruiting specific immune cells. If inflammation persists, the physiological function of the affected tissue can be destroyed [1] . Chronic inflammation is linked to many costly chronic conditions such as obesity, type 2 diabetes (T2D), and cardiovascular disease [2-4]. In fact, some suggest that chronic inflammation plays a major role in the development of obesity and insulin resistance [5,6].

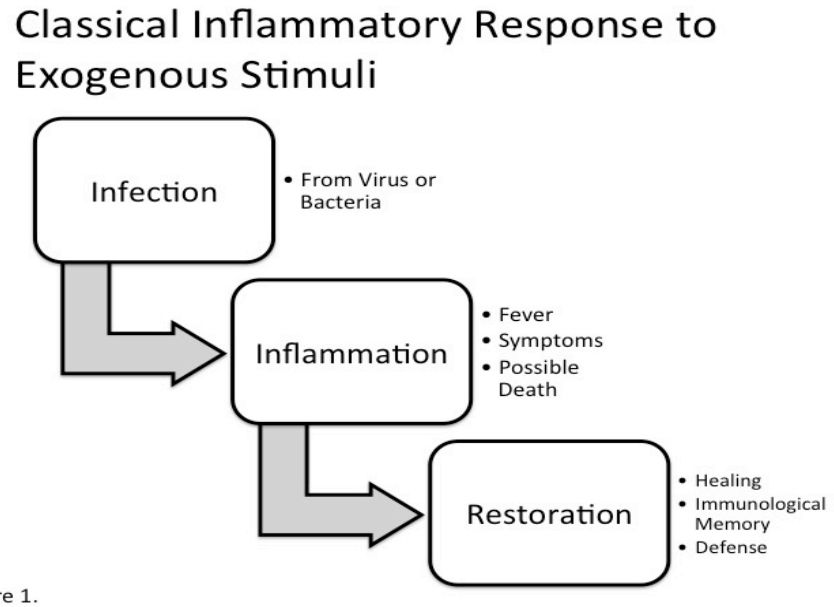

Figure 1.

Obesity is a chronic disease characterized by impaired energy balance, which favors the accumulation and expansion of adipose tissue during periods sustained energy excess. The global prevalence of obesity has doubled from 1980 to 2008. According to the world health organization (WHO) 205 million men and 297 million women over the age of 20 are obese (BMI >30 kg/m^2) [7]. Obesity is not only strongly correlated with T2D, this disease is considered to be a primary driver in the development T2D. T2D is a chronic disease that occurs due to lack of insulin production by the pancreas or the inability to utilize the insulin produced in the periphery. The global prevalence in 2000 was estimated to be 2.8% for all age groups [8]. The 2030 models predict that 4.4% of global population will have T2D. These reports suggest that the T2D epidemic will continue even if obesity levels remain consistent [8-10].

Decreasing amounts of physical activity and exercise have coincided with the increasing rates of obesity and T2D [11]. Currently, 50% of US adults do not meet the recommended guidelines for weekly physical activity and 25% report no regular physical activity (cdc.gov/nccdphop/dnpa/physical). The relationship of physical inactivity and increased morbidity and mortality rates is indisputable and physical inactivity is a major risk factor for the development of a cluster of metabolic disorders (obesity, insulin resistance, dyslipidemia, and hypertension) called metabolic syndrome [12-15]. Moreover, structured exercise programs can significantly slow the development and progression of these diseases [16-18]. In this review, I will explore the mechanism of chronic inflammation initiated by metabolic dysfunction and how skeletal muscle can mediate the anti-inflammatory effects of exercise.

Obesity and Type 2 Diabetes

The expansion of adipose tissue is the defining characteristic of obesity. For many years, adipose tissue was thought to be an inert tissue that simply served as storage site for triglycerides [19]. The discovery of leptin, a fat derived hormone, demonstrates that adipose tissue plays a role

in systemic metabolism by producing hormones that have downstream effects on the liver and skeletal muscle. Serum leptin levels positively correlate with increases in body fat [20] and *in vitro* research demonstrated that leptin plays a direct role in skeletal muscle fatty acid metabolism [9]. The obesity related reductions in leptin production, are thought to contribute to the development of skeletal muscle insulin resistance [10].

Skeletal muscle insulin resistance is argued to be the primary defect in T2D [21]. In humans, under euglycemic hyperinsulinemic conditions approximately 80% of glucose uptake occurs in the skeletal muscle [22]. In the skeletal muscle of healthy individuals, glucose that enters the cell is rapidly converted to glycogen or oxidized into carbon dioxide and water. The natural progression from insulin resistance to frank T2D is characterized by inability to convert glucose to glycogen. Like obesity, there are many contributing factors to the development of insulin resistance. Obesity itself significantly increases the risk of becoming insulin resistant. However, there is a cohort of individuals that are not classified as obese or overweight that demonstrate insulin resistance. These lean individuals demonstrate impaired insulin mediated glucose disposal in skeletal muscle and are likely to develop T2D if the condition persists[23].

Histological analysis of obese adipose tissue demonstrates an increase in the presence of inflammatory cytokine producing macrophages [2,24]. In the skeletal muscle of type 2 diabetics, there is increased concentration of inflammatory cytokines, particularly tumor necrosis factor alpha (TNF-α). Elevated systemic markers of inflammation, such as C-reactive protein (CRP), accompany the local inflammation present in adipose and skeletal muscle during metabolic dysfunction [1,25-27]. This evidence demonstrates that the metabolic and immune systems are closely linked in disease progression.

Exercise and Chronic Inflammation

Regular exercise reduces levels of chronic inflammatory markers (1) and also promotes both pro and anti-inflammatory responses within the skeletal muscle (8). The type and intensity of exercise appears to modulate the balance between the two responses. Exercise has been shown to be an effective treatment for obesity and T2D [18]. In addition exercise also has anti-inflammatory qualities that promote metabolic health [28-30]

The mechanism responsible for anti-inflammatory effects of exercise remains controversial. On a gross level, exercise induces hormonal fluctuations that have effects on immune function (23, 9). Exercise reduces chronic inflammation, in part, by the production of muscular derived IL-6, which then stimulates the release of anti-inflammatory cytokines (IL-1rα and IL-10) and a beneficial reduction CRP [31]. Additionally, exercise is efficacious at reducing chronic inflammation, even without the loss in body fat [32]. However, the mechanisms associated with this positive effect are unclear. In this portion of the review, I will provide a more in depth examination of chronic inflammation.

Acute vs. Chronic inflammation:

Inflammation is the response mounted by the immune system characterized by the recruitment of leukocytes to areas of the body that have been damaged or infected by foreign pathogens [33]. Initially, recognition of infection is modulated by tissue resident macrophages and mast cells. These immune cells secrete inflammatory cytokines, chemokines, and lipid molecules that dictate the inflammatory response. Inflammatory cytokines act in countless ways throughout the body. For example, during an acute infection, the inflammatory cytokines TNF-α and interleukin-1 (IL-1) work to increase the vascular permeability of the affected tissue and to increase the activation of vascular adhesion molecules, such as vascular cell adhesion molecule-1

(VCAM-1). Circulating neutrophils and monocytes gain access to the inflamed tissue via interaction with their cell surface receptors for VCAM-1. Once inside the inflamed tissue these innate immune cells attempt to eliminate the foreign pathogen by their own effector function and in conjunction with the activation of the adaptive immune system [33] .

If the source of the inflammation (exogenous) is eliminated by an effective immune response then levels of inflammation will subside. A classical inflammatory response is necessary for host defense. Physical injury of a tissue is also a potent activator of the inflammatory response and is necessary to initiate tissue repair. Prolonged periods of tissue dysfunction or stress also represent an inflammatory trigger. This inflammatory response is the host's response to restore the organ to balance [34]. However, in chronic conditions such as obesity and T2D, the prolonged inflammation can eventually destroy normal physiological function of metabolically active tissues [1].

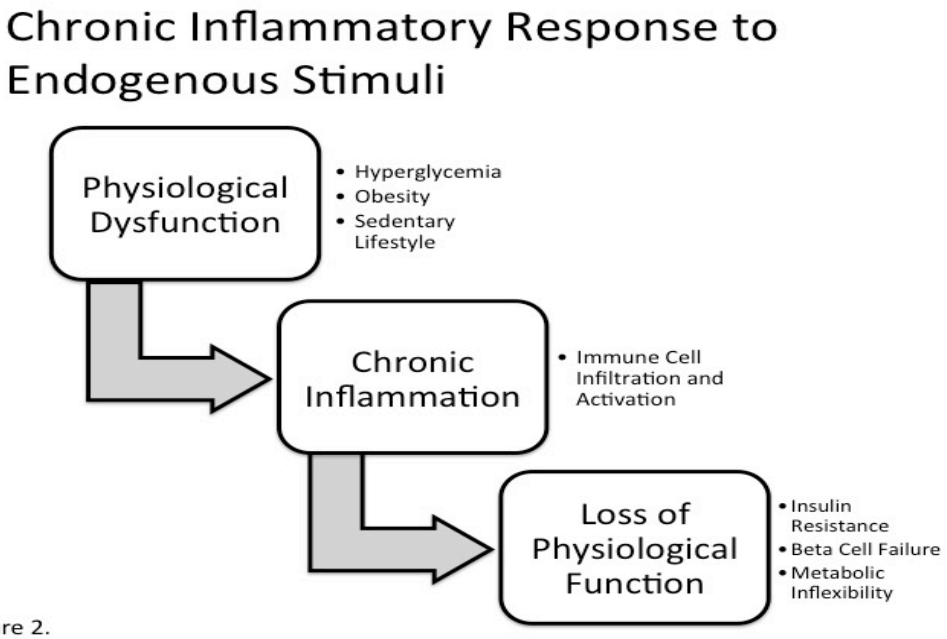

Figure 2.

Pro- and Anti-inflammatory cytokines

Cytokines are soluble glycoproteins that aid in communication between both immune and non-immune cells and act to mediate inflammation [35]. The benefits of regular exercise are believed to be modulated via the reduced production of inflammatory cytokines and or lowered expression of toll-like receptors (TLR) on monocytes and macrophages [36]. The pro- or anti-inflammatory effects of a cytokine depend on the environment in which it is activated, the cell type from which secreted, and the specific cell-signaling cascade utilized [33,37,38].

Tumor Necrosis Factor-Alpha (TNF-α)

Tumor necrosis factor-alpha TNF-α is a 212 amino acid cytokine produced as a transmembrane (mTNF*α*) and soluble (sTNF-*α*) factor mainly by monocytes and macrophages. TNF- α is considered a pro-inflammatory cytokine because it is released in response to lipopolysaccharide, which is the product of gram-negative bacteria in order to induce the acute phase response. TNF-α acts throughout the body in many different ways. During an inflammatory response the increased concentration of TNF-α acts to recruit additional monocytes and neutrophils to the affected area. TNF-α is associated with a number of acute and chronic conditions. Septic shock is an acute life-threatening condition whereby mass amounts of TNF-α are released into the blood stream causing systemic vasodilation resulting in death. Chronically elevated levels of TNF-α is also associated with muscle wasting conditions [39].

Hotamasligil and colleges [6] were the first to report the metabolic link between obesity-induced insulin resistance and TNF-*α.* This initial observation was followed by a series of experiments that demonstrated that adipose tissue of obese diabetic mice had increased expression of TNF- *α* [40,41]. The specific function of TNF-α was then studied by generating a TNF-α knockout mouse model (TNF-$\alpha^{-/-}$) that was fed a high fat diet (HFD) to induce obesity

and glucose intolerance [42,43]. These high fat fed TNF-$\alpha^{-/-}$ mice did not demonstrate the same degree of glucose intolerance when compared to obese wild type mice (with the intact TNF-α gene). The TNF-$\alpha^{-/-}$ animals were not protected from developing obesity and still demonstrated some glucose intolerance, albeit greater than wild type mice, suggesting that TNF-α is not solely responsible for the development of obesity-related insulin resistance.

In humans, serum concentrations of TNF-α and sTNF-α receptors levels have been shown to be elevated in obesity [44,45]. These effects have also been observed within the adipose tissue itself. For example, TNF-α mRNA content was measured in the adipose tissue of obese premenopausal women before and after weight loss [25]. When compared to lean controls, obese women had significantly increased TNF-α mRNA and protein from adipose and secretion from adipose. Adipose tissue TNF-α mRNA was also significantly correlated with plasma insulin ($r=0.82$, $P<0.001$), glucose ($r=0.031$, $P=0.06$), and triglycerides ($r=0.40$, $P=0.02$). After diet induced weight loss both adipose tissue TNF-α mRNA and plasma insulin concentrations were reduced. However, the weight loss period failed to induce a significant reduction in the circulating levels of TNF-α [25]. Consequently, some have suggested that TNF-α may act in a paracrine-autocrine manner, as opposed to an endocrine manner, on nearby by tissues.

In vitro and in *vivo* data suggest that TNF-α can induce insulin resistance by reducing the expression of key molecules involved in the insulin-signaling cascade [46,47]. Specifically, obese mice with intact TNF-α signaling have decreased muscle glucose transporter type-4 (GLUT4), insulin receptor substrate-1 (IRS-1), and insulin receptor phosphorylation. Insulin receptor phosphorylation is restored to significant degree when TNF-α function is ablated [42]. However, Stephens et al (1997) studied the effects of prolonged TNF-α exposure (96 hrs) on 3T3-L1 adipocytes and demonstrated no impairment in GLUT4 translocation to the plasma membrane.

Rather, the transcriptional activity of GLUT4 and IRS-1 were inhibited which reduced the total available protein [47]. Given these findings, it is apparent that TNF-α can induce insulin resistance by inhibiting the transcription factors responsible for the synthesis of insulin signaling intermediaries.

Although glucose transport into adipose is an important physiological phenomenon, it pales in comparison to the role that skeletal muscle plays in glucose disposal. In fact, there is a 50% reduction in skeletal muscle glucose uptake during the last hour of insulin clamp studies in diabetic subjects [48]. Saghizadeh et al (1990) demonstrated that expression of TNF-α, in skeletal muscle, was increased by four fold in insulin resistant and diabetic subjects compared with insulin sensitive subjects [45]. Using a hyperinsulinemic euglycemic clamp technique these researchers were able to show that, in insulin sensitive subjects, there was a significant inverse linear relationship between glucose disposal rate and muscle TNF-α [45].

The key substrates involved in the skeletal muscle insulin-signaling pathway appear to be altered by elevated TNF-α. In healthy humans, TNF-α infusion induces insulin resistance in skeletal muscle by increasing the phosphorylation of p70 S6 kinase, extracellular signal-related kines-1/2, and c-Jun NH2-terminal kinase in addition to increased serine and reduced tyrosine phosphorylation of IRS-1 [46]. TNF-α also promotes insulin resistance by altering skeletal muscle fatty acid oxidation by suppression of activated AMP-kinease (AMPK) activity through TNFR1 signaling [49]. Suppression of fatty acid oxidation in skeletal muscle causes the accumulation of fatty acid intermediaries (DAG, acyl co-A, ceramides) [50,51]. These bioactive molecules can activate the serine/threonine kinase cascade and lead to disruption of proper insulin signaling[51-54].

Interleukin-6 (IL-6)

IL-6 is a pleiotropic cytokine, primarily synthesized by monocytes/macrophages, endothelial cells, and fibroblasts that serves both pro-and anti-inflammatory functions throughout the body and plays a significant role during infection or illness [55]. IL-6 is pivotal in the synthesis of acute phase proteins that are released in an effort to restore homeostasis to the infected/injured tissue/organ [56]. IL-6 also promotes inflammation through the proliferation and activation of T cells and the differentiation of B cells [33] .

IL-6 signals through the IL-6 receptor complex consisting of IL-6R (80 kDa) and 130 kDa glycoprotein gp130 which acts as a signal transducer [57]. Once binding occurs between IL-6 and gp130 a Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling cascade is initiated [38,58]. Additionally, IL-6 possesses a soluble receptor that binds to IL-6 in circulation to form an IL-6/sIL-6R complex extending the half-life of IL-6 [59]. This complex can activate the JAK/STAT pathway through membrane bound gp130. The improved stability and intact functional capability allows IL-6 to interact with many different cell types [59].

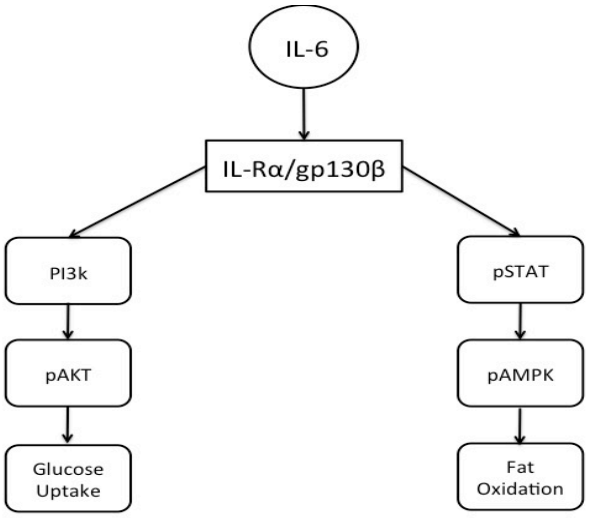

Figure 3. IL-6 Signal Transduction pathways

Elevated IL-6 concentrations are commonly associated with increased risk of T2D, obesity, atherosclerosis, hyperinsulinemia, and low HDL-Cholesterol [60-62]. When dietary measures are taken to reduce body fat there is a reduction in circulating IL-6 [63]. Until recently, the mechanisms related to IL-6 production and the progression of obesity induced insulin resistance has been speculative and dependent on the tissue of study. *In vitro* studies demonstrate that adipocytes [64,65] and hepatocytes [66]become insulin resistance when cultured with IL-6. Additionally, Klover et al (2003) demonstrated that chronic infusion of IL-6 similar to levels observed during obesity induced hepatic insulin resistance in mice[67].

Priceman et al (2013) recently determined that IL-6 signaling through STAT3 in the T helper 1 (T_H1) and regulatory T cells (T regs) cells that reside within visceral adipose depots was essential in diet-induced obesity (DIO) induced insulin resistance. The results of this study demonstrated that STAT3 was elevated in obese visceral adipose tissue (VAT) and in VAT T cells. Ablation of STAT3 in T cells reduced VAT inflammation, DIO and improved insulin sensitive and glucose tolerance. Interestingly, loss of STAT3 signaling shifted the VAT resident macrophage population from M1 classically activated to M2 alternatively activated [68].

Circulating concentrations of IL-6 are directly related to increased concentrations of CRP, which suggest its involvement in the acute phase response. During an infection IL-6, is released primarily from monocytes and macrophages to target hepatocytes to release CRP [56,69]. CRP is one of the strongest predictors of cardiovascular events and is thought to play a direct role in the development of atherosclerotic lesions [70,71]. The relationship of IL-6 and CRP and the overwhelming evidence that increased adiposity contributes to elevated serum IL-6 demonstrate that metabolic dysregulation can perturb the immune system to cause chronic systemic inflammation.

Anti-inflammatory Cytokines:

During an inflammatory response, pro-inflammatory cytokines increase the circulating number of lymphocytes, monocytes, and granulocytes. Once the inflammatory cascade is set into motion the compensatory anti-inflammatory response is initiated via the production of anti-inflammatory cytokines [37]. IL-10 is an anti-inflammatory cytokine produced by cells of both the innate and adaptive immune system [72]. The primary producers of IL-10 are T helper 2 cells (T_H2) cells and M2 macrophages. IL-10 acts to regulate and, in many cases, diminish the inflammatory response initiated by myeloid and lymphoid cells. Genetic deficiencies in IL-10 are commonly associated inflammatory disease such as systemic lupus erythromatosis, inflammatory bowel disease and bechet's disease[73-75]. Additionally, IL-10 knockout mice reportedly demonstrate chronic enterocolitis [76]. These data clearly demonstrate that IL-10 is necessary to promote tolerance meditated through an appropriate T_H2 cell response [73-75,77].

Low circulating levels of IL-10 are associated with obesity and metabolic syndrome in humans [78]. The mechanism of IL-10 release from T cells and M2 macrophages works to downregulate the chronic inflammatory response that is potentiated by the adaptive immune system [77]. IL-10 reduces the secretion of IL-12 and TNF-α from macrophages and dendritic cells in order to reduce inflammation. Reductions in IL-12 cause the downregulation of MHC II-peptide presentation on antigen presenting cells (APCs) and the reduction of the co-stimulatory molecules CD80 and CD86 [79].

In murine models muscle specific overexpression of IL-10 prevents lipid induced insulin resistance [80]. Increased IL-10 expression in skeletal muscle increases circulating systemic levels, but was not associated with decreased weight gain compared to wild type mice fed a HFD[81]. The overexpression of IL-10 significantly increases glucose infusion rate, whole-body

glucose turnover, and skeletal muscle glucose uptake in both chow and HFD fed animals compared to wild type. Immunohistochemical analysis demonstrates that greater expression of IL-10 decreases the infiltration of CD68, F4/80, and total macrophage infiltration in skeletal muscle. Muscle IL-10 transgenic mice prevented macrophage infiltration by reducing the expression of macrophage recruiters CCR2 and MCP-1. It is apparent that IL-10 regulates the inflammatory response by modulating the intracellular signaling molecules of macrophages and resident tissues.

The nuclear factor kappa-B (NF-κB) pathway is an essential pathway that mediates inflammatory responses [80]. IL-10 binding with its receptor (IL-10r) inhibits the NF-κB pathway in macrophages and that prevents release of interferon-Y, (INF-γ), IL-2, interleukin-3 (IL-3), TNF-α and granulocyte macrophage colony stimulating factor (GM-CSF) [81,82]. The transcription regulation of IL-10 is controlled by the gene regulators GATA3, E4BP4, and to a lesser degree MAF [81]. In a similar fashion to IL-6, IL-10 binds its receptor and initiating the JAK/STAT signaling pathway by phosphorylating the tyroinse kinases JAK2 and Tyk2, which then phosphorylate and activates STAT1, STAT2, STAT3, and STAT5 [82].

Interleukin-1 receptor antagonist (IL-1ra) is anti-inflammatory cytokine that binds IL-1 and IL-1β receptors [83]. As a result the activity of IL-1 and IL-1β are significantly reduced. In terms of systemic chronic inflammation, IL-1ra appears to play a role in blocking IL-1β which plays a primary role in the inflammatory macrophage-adipocyte cross talk [84]. IL-1ra is often referred to as a soluble factor and not an active cytokine *per se*. IL-1ra was originally called IL-1 inhibitor because it blocks the ability of IL-1 to stimulate synovial PGE_2 inflammatory response [85]. *In vitro*, IL-1ra binds to T cells and fibroblast at the same infinity as IL-1. Remarkably, this binding does not induce any of the agonist activity of IL-1[86].

Many of the negative metabolic consequences that arise from systemic chronic inflammation are associated with elevated levels of TNF-α. TNF-α binding with its receptors TNF receptor I and II (TNFRI) works to increase the inflammatory response [41]. Under certain circumstances the ectodomain of the 55-kda transmembrane TNFRI can be proteolytically cleaved or remain intact packed into exosomes to be released as a functioning soluble receptor (sTNFRI) [87]. Cells of the immune system will shed their TNF receptor to neutralize the function of TNF-α without provoking an inflammatory response [88].

Sources of inflammation:

Adipose Tissue:

Adipose tissue is a complex tissue composed mainly of adipocytes. However, other cells, such as pre-adipocytes, fibroblast, and endothelial cells comprise the stromal vascular fraction (SVF) are usually present. Immune system cells also occupy the SVF of adipose primarily in the form of macrophages called adipose tissue macrophages (ATMs). Adipose acts a major endocrine organ that regulates energy expenditure and food intake and secretes regulatory hormones like leptin, estrogen, and resistin. Adipose tissue increases the expression and secretion of inflammatory cytokines, primarily IL-6, during the development of obesity. These cytokines are primarily produced by ATMs, which comprise approximately 40% of the adipose tissue of obese individuals [2,89].

Adipose tissue, as a whole, can be subdivided into subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). VAT, also referred to as abdominal fat, resides between abdominal organs. VAT significantly increases during obesity and is positively associated with insulin resistance and T2D [62,90]. As is the case with all tissue, adipose contains a resident

population of immune system cells that provide surveillance against foreign microbes. Resident tissue macrophages in SAT and VAT are essential for the removal of apoptotic cells without increasing inflammation [91]. However, if the stress is extreme or prolonged, as is the case in obese adipocytes, ATM may recruit addition immune system cells that promote inflammation. Kanada et al (2006) demonstrated that obese mice with malfunctioning adipocytes secrete monocyte chemoattractant protein-1 (MCP-1) to recruit additional macrophages into the adipose tissue [92]. The physiological mechanism for this signal appears to be linked to tissue stress induced by hypoxic environment [93].

Several animal and human studies demonstrate that as adipose tissue mass expands, there is decreased partial pressure of oxygen (PO_2) to white adipocytes [94-97]. In fact, in lean individuals, blood flow to the adipose is increased postprandially while there is no change in obese individuals [98,99]. As adipocytes expand from energy imbalance, the diffusion of oxygen into the adipose becomes impaired, thereby lowering the O_2 saturation of the tissue. This represents a fundamental physiological stress on adipose tissue. The hypoxic environment of the adipocyte can trigger the regulator of cellular O_2 homeostasis hypoxia inducible factor-1 (HIF-1). HIF-1 regulates the cells response to hypoxia by increasing chemoattractants (MCP-1 and CXCL12) to the stressed tissue [93]. Immunohistochemistry of the adipocytes of high fat fed C57/BL6 mice confirms that hypoxic areas had the greatest accumulation of macrophage and cytotoxic T cells [100].

Excessive energy intake for prolonged periods of time without increases in energy expenditure tips the scale in favor of fat storage into adipocytes. As adipocytes reach their maximal storage capacity the cellular environment becomes stressed, which leads to the increased secretion of chemokines (MCP-1 and CCL5) and the cell surface expression of

adhesion molecules (α4β7) to recruit and navigate monocytes to the area. Adipocytes that have been stressed in this manner can undergo apoptosis and their cellular contents (glycerol) spill into the interstitial space attracting more macrophages. This cycle of tissue expansion, hypoxia, and metabolic stress work to overwhelm the resident tissue macrophage and activate the innate immune system in an attempt to restore homeostasis [34,93,96].

Inflammatory Macrophages:

Dendritic cells, granulocytes (neutrophils, basophils, eosinophils, mast cells), and macrophages comprise the three phagocytic cell classes of the innate immune system. Dendritic cells function primarily to ingest antigens and migrate to peripheral lymphoid tissues where they can present peptide to the cells of the adaptive immune system. Granulocytes are highly phagocytic cells that confer immediate short-live response to pathogens. Macrophages serve a primary role in immune surveillance and orchestrating the inflammatory response [33,101].

Macrophages are long-lived cells that mature from circulating monocytes and populate tissues. Macrophages that encounter a pathogen or microbe are stimulated to release pro-inflammatory cytokines. During a classical, pathogen initiated immune response these cytokines promote increased blood flow, cell-adhesion molecules expression, and vascular permeability. The purpose of the inflammatory cascade is to increase the number of effector cells to the initiating site and to promote the destruction of the pathogen. The cytokine, macrophage colony-stimulating factor (M-CSF) promotes the maturation of monocytes into macrophages that will continue to promote the inflammatory response or become tissue resident macrophages[2].

Tissue resident macrophages serve a central role in immune surveillance and the clearance of cellular debris from apoptotic cells. An important aspect of immune surveillance is the "silent" removal of cellular debris that does not stimulate the release of inflammatory

cytokines or noxious substances. ATMs in lean mice ($F4/80^{+}$) are considered to be "alternatively activated" (M2). This differs from classically activated macrophages (M1), which are induced by T helper 1 cell (T_H1) cytokines INF-γ and TNF-α to initiate a classical inflammatory response. M2 macrophages, on the other hand, can be induced by T_H2 cytokines IL-4 to secrete anti-inflammatory cytokines.

In mice, the development of obesity stimulates the recruitment and conversion of the ATM population to switch from M2 "anti-inflammatory" to M1 "pro-inflammatory" macrophages [102]. These M1 macrophages expressed the surface protein CD11c and have been shown to represent 90% of the ATM population in obese adipose tissue in mice [103]. Humans do not demonstrate the same polarization in macrophage differentiation. Human ATMs during obesity presents a mixed profile exhibiting variable qualities of both anti-and pro-inflammatory macrophages [24,104]. An analysis of ATMs from lean and obese individuals, utilizing flow cytometry, revealed that ATMs from obese humans produced pro-inflammatory cytokines (IL-6, TNF-α, IL-23, MCP-1, IL-8, and cyclooxygenase-2) and anti-inflammatory cytokines (IL-10, TGF-β, and cyclooxygenase-1)[24].

Human ATMs are considered to be positive for CD45, CD14, HLA-DR, CD31, and CD206 when analyzing cell surface markers [104]. Within the population of ATMs there are markers for differentiation. For example, CD206, the manose receptor, is highly expressed on the surface of differentiated macrophages and, along with other scavenger receptors, promote a pro-phagocytic phenotype. Additionally, the ATMs increase expression of matrix metalloproteinase-9 (MMP-9), which is key protease involved in the breakdown of the extracellular matrix promoting angiogenesis [24,105]. These studies demonstrate that human ATMs exhibit distinct

macrophage subpopulations that cannot be solely categorized on cell surface makers, but rather cellular behavior [24,104,105].

Macrophages *in vivo* demonstrate high degrees of plasticity in their differing states of activation. This makes segregation of human ATMs into distinct classes and subsets unrealistic. In murine models of DIO adipose tissue there is a marked increase in the M1 macrophage that is temporally associated with insulin resistance. There is substantial evidence in favor of obese adipocytes undergoing apoptosis and recruiting monocytes from the circulation to be classically activated macrophages. MCP1$^{-/-}$ mice exhibit significantly less adipose tissue macrophage infiltration and subsequently have improved insulin sensitivity and hepatic steatosis when compared to wild type mice on HFD [106].

Obesity can lead to the activation of the cytostolic multiprotein complex NLRP (NOD-LLR-and pyrin domain containing), adaptor molecule ASC, and caspase-1 effector molecule termed the inflammasome [91]. The inflammasome can be activated in macrophages, and other cells of myeloid lineage, by pattern associated molecular patterns (PAMPs), reactive oxygen species (ROS), and endogenous ligands such bioactive lipid molecules (i.e. ceramides) [91,107]. The result of activation of the inflammasome leads to the cleavage of pro-caspase-1 into caspase-1, which then leads to processing and release IL-1β. IL-β is considered a master inflammatory cytokine and a potential candidate for macrophage induced insulin resistance. Elevated levels of IL-1β and IL-6 are positively associated with increased risk of T2D. Gao et al (2014) found that IL-1β is a critical cytokine facilitating macrophage-adipocyte cross-talk, which detrimentally effects insulin signaling (i.e. significant reductions in GLUT4, IRS-1, IRS-2) [108]. This study also demonstrates that inhibition of IL-1β results in increased protein expression insulin signaling

molecules GLUT4, IRS-1, PI3K p85α, and completely reverses the lipolysis initiated by the macrophage cytokine treated media.

To summarize, ATMs represent a diverse subset of macrophages that may or may not represent a distinct lineage of circulating monocytes attracted to areas undergoing metabolic stress. The difference between human and murine ATM behavior may be representative of the models as a whole. The murine immune system has more distinct roles and relationships to certain cytokines, whereas the human immune system is clearly more pliable. Human ATMs are more hybrid macrophages that possess qualities of both pro-and anti-inflammatory (M1 and M2) macrophages. Murine models of DIO elicits a clear transition in macrophage activation from "M2" alternatively activated macrophages that promote a toleragenic environment capable to quietly clearing cellular debris to a "M1" classically activated macrophage that secretes pro-inflammatory cytokines. Considering these observations it is clear that macrophage infiltration into obese adipocytes promotes chronic inflammatory state [2,91,104].

Human ATMs do not provide the same "smoking-gun" in their relationship to obesity related insulin resistance and chronic inflammation. There is currently no consensus on all the cell surface markers to distinguish the activation state of human ATMs. However, in obese adipocytes, ATMs produce cytokines that promote insulin resistance, proteases that remodel the extracellular matrix, and induce adipocyte differentiation. The ATMs in obese humans appear to promote inflammation in order to rebuild the adipocyte structure in order to store more lipids. Insulin resistance, due to the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β, is potentially a adaptive response to limit the uptake of glucose into adipocytes that are overwhelmed [103,108 109].

There is now little doubt that adipose tissue is the source of increased pro-inflammatory cytokines in circulation that defines obesity and chronic inflammation. Measurements of arterio-

venous blood flow across subcutaneous adipose tissue beds demonstrate that approximately 30% of circulating IL-6 is produced by the adipose [89]. While TNF-α is typically not detected in the serum of obese individuals, its protein expression is significantly increased in the adipose of the obese [89,110,111]. This pro-inflammatory environment works not only to increase the recruitment of monocytes/macrophages, but also increases their phagocytic activity. Increased uptake of necrotic adipocytes and lipids by macrophages increases the presentation of those molecules by both major histocompatibility complex I and II (MHC I and MCH II) to cells of the adaptive immune system [89,110].

Skeletal Muscle Inflammation:

Adipose tissue has been the primary focus of research in metabolically derived chronic inflammation. However, in recent years skeletal muscle has been receiving more interest as an affected tissue during chronic inflammation and as a potential contributor to inflammation. Indeed, there are many conditions under which skeletal muscle becomes inflamed. Duchene's Muscle dystrophy and myasthenia graivs are characterized by an intense inflammatory response and repair process that promote fibrosis [112,113].

Activating inflammatory pathways within the skeletal muscle interferes with insulin signaling pathways and therefore insulin stimulated glucose uptake. Several *in vitro* studies demonstrate impaired glucose uptake when skeletal muscle cells are exposed to inflammatory cytokines [114-116]. However, these data do not fully support the role of inflammatory cytokines in the development of obesity. For example, attempts to reverse insulin resistance by administering neutralizing antibodies against inflammatory cytokines has been largely unsuccessful [34].

Skeletal Muscle Macrophages

The infiltration of inflammatory macrophages into expanding adipose tissue during obesity has been repeatedly demonstrated in both animal and human models and is positively associated with chronic inflammation [1,91]. The primary disease states associated with obesity are insulin resistance and frank T2D [21]. T2D is largely considered a disease of the skeletal muscle due to the fact that the majority dietary glucose is disposed within skeletal muscle [48]. Therefore, it is logical to speculate that inflammatory macrophages may be increased in the skeletal muscle of obese individuals and type 2 diabetics.

To address this, Fink et al (2013) obtained biopsies from lean normal glucose tolerant (NGT) individuals and overweight and obese individuals with and without T2D [117]. The results of this study demonstrate that CD11c+ macrophages are positively correlated with T2D in addition to HbA1c and fasting plasma glucose. The genes for alternatively activated anti-inflammatory macrophages (CLEC10A, MRC1, CD163) are inversely associated with the same parameters for T2D. The investigators of this study stated that when corrected for age these relationships did not reach significance [117]. These results demonstrate that muscle-residing macrophages might play a role in the preserving or impairing insulin sensitivity.

Limited methodological analysis of inflammatory macrophage infiltration in skeletal muscle has created a discrepancy in the theory that inflammatory macrophages infiltrate skeletal muscle and contribute to insulin resistance. Several of these studies cite low relative number of macrophages in muscle when compared to adipose tissue [118-120]. However, these studies failed to determine the inflammatory activity and polarization of macrophages in muscle in obesity. Studies by Varma et al (2009) and Fink et al (2014) utilize a multitude of techniques (flow cytometry, qPRC, and immunohistochemistry) to analyze the population and behavior of

macrophages in the muscle of mice fed a HFD and obese humans alike [26,121]. These data demonstrate a differential immune cell response in muscle in response to obesity. In response to a HFD human and mouse muscle have proportionally fewer T cells than adipose tissue, but showed increased neutrophils. Importantly, MCP-1 (CCL2) increases in response to a HFD, in mice and humans, and is necessary to increase macrophage numbers within muscle [121].

The cytokines and chemokines released by skeletal muscle macrophages and by the muscle fibers themselves appear to act synergistically to promote inflammation in response to HFD feeding in mice [5,26]. In obese and type 2 diabetic humans, there is a similar response that up-regulates the expression of MCP-1 to recruit monocytes from circulation and populate the stressed muscle. This state of chronic inflammation initiates the cross-talk between skeletal muscle tissue and its resident immune cells that promotes insulin resistance and fibrosis [26,117,122].

Chronic Inflammation and the Adaptive Immune System:

Innate immune system responses are mounted against extracellular and intracellular pathogens. As mentioned previously, monocytes are immature leukocytes that reside within the vascular endothelium and in circulation. Monocytes, in the presence of inflammation due to infection or tissue injury, mature to become macrophages. These phagocytic macrophages consume and lyse pathogens so that their peptide components can be "presented" on their surface by major histocompatibility complex class I and class II (MHC I and MHC II) [33].

Vital to the survival of an organism is the ability to differentiate "self" from "not self". Nearly all cells of the body display "self" peptides in conjunction of MHC I and II molecules. When an antigen-presenting cell has consumed and killed a pathogen by phagocytosis it will then present the pathogen to T cells and B cells that have specific receptors for that peptide-MHC complex. Early in the development of human and mice there is an education of the immune

system, whereby the T cells and B cells undergo both positive and negative selection. T cells are a subset of lymphocyte that mature in the thymus. During that early maturation T cells that are auto-reactive or bind to strongly to "self" are deleted from population [33,123].

The presentation of digested adipocytes and lipids through MHC I and MHC II molecules on the surface of macrophages functionally builds a bridge between the innate and adaptive immune system. A major aspect the chronic inflammatory response is the persistent presentation of antigen via macrophages. The T cell's role in host protection is primarily in cellular defense, whereas, B cells contribute to humoral defense. Due to this fact T cells will be the primary focus of this portion of the review [124].

T Cells

T cells are CD3+, but also demonstrate other markers of differentiation such as CD8 and CD4. CD8+ T cells are called cytotoxic T cells that recognize antigen in the form of peptide-MHC I and are major protectors against intracellular pathogens. CD4+ T cells recognize antigen in the context of peptide-MHC II. Upon recognition of the peptide-MHC complex T cells terminally differentiate into cytotoxic T lymphocytes (CTLs) and T helper cells (T_H1, T_H2, T_H17, T_H9) respectively. It is at this point that T cells are capable of executing their effector function [33]

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A tolerogenic environment, in which the immune system does not attack its host, is fundamental to a healthy immune system. The foreign microbes of the gastrointestinal tract do not cause an inflammatory response in healthy individuals. The explanation for this phenomenon stems from regulatory T cells (T regs) that promote tolerance by secreting anti-inflammatory cytokines IL-10 and TGF-β. T regs are positive for forkhead/winged-helix transcription factor

(FoxP3), CD25, and CD4. FoxP3$^+$ T regs occur commonly at sites of recently cleared infection to terminate the inflammatory response allowing the tissue to be restored to homeostasis.

In C57BL/6 mice at 30 weeks of age approximately 50% of CD4$^+$ T cells are CD25$^+$ FoxP3$^+$ T reg cells. *In vitro* suppression assays demonstrate that T reg cell from the VAT of mice poorly suppress CD$^+$ T cells with no deficiency in proliferation when compared to T regs taken from subcutaneous adipose[125]. These VAT T regs had increased gene transcripts for chemokines that recruit leukocytes and promote extravasation (CCR1, CCR2, CCR9, CCL6, integrin αV, CXCL10, and CXCL2). These cells also produce large of amounts of IL-10 and its receptor IL-10R. Studying the function of the fat resident T reg cells has proven to be technically difficult. Therefore the precise role that these tolerogenic cells play in the development of obesity associated inflammation remains to be elucidated [126].

The chronic inflammation that is associated with long-term obesity is sustained by the adaptive immune system. In support of this, CD3 gene transcripts have been shown to be elevated in humans VAT during obesity most of which are CD8$^+$ and CD4$^+$ [127]. The majority of T cells in VAT in obese individuals are effector memory CD8$^+$ CD44$^+$ CD62L$^-$. These CD8 T cells, in contrast to naïve CD8$^+$CD44$^-$CD62L$^+$T cells, are capable of promoting a long-term inflammatory response by recruiting and activating macrophages into the adipose tissue [128]. Effector CD8+ T cells or CTLs release cytotoxic granules once binding with their cognate peptide-MHC complex. Cells that are exposed to cytotoxic granules will start to undergo program cell death called apoptosis [109].

In adipose tissue, the CD4 T cell population is mainly comprised of T_H1 and T_H2 T cells [128]. T_H1 cells are primary producers of the pro-inflammatory cytokines INF-γ. INF-γ is a macrophage-activating factor that also stimulates the natural killers (NK) cell population.

However, it is the action of the macrophage that initially stimulates T_H1 cells [127]. The presentation of peptide-MHC II and release of IL-1 and IL-12 from macrophages that have encountered an extracellular pathogen stimulates T_H1 cell activation. T_H1 cells are notably primary producers of INF-γ, which is a potent activator of macrophages in vivo and in vitro [129,130].

The recruitment of additional macrophages into the adipose tissue by INF-γ produced by T_H1 cells is a potential mechanism for the role of the adaptive immune system in chronic inflammation. In support of this, Rocha (2008) and Winer (2009) independently demonstrated that obese mice have greater total number of CD4+ and CD8+ T cells than when compared to lean controls[131,132]. *In vitro* stimulation of T cells from obese mice produce significantly greater amounts of INF-γ and display a skewed TCR-Vα repertoire suggesting that expanding adipose tissue drives T cell biased towards adipose. These mice also show increased MHC II ($I\text{-}A^b$) presentation [131,132]. These data support the theory that obesity elicits a specific T cells receptor repertoire response to adipose tissue that potentially displays self-epitopes.

The T_H2 cytokines, IL-4 and IL-13, serve a role to promote the activation of anti-inflammatory M2 macrophages [133]. As stated previously, M2 macrophages promote a tolerogenic environment via the production of anti-inflammatory cytokines and through the production of arginase-1[101]. Arginine, the primary substrate for arginase-1, is necessary to sustain a CD4 T cell response. The arginase-1 produced by M2 macrophages depletes arginine and is sufficient to reduce the CD4 T cell response [134]. The potential protective role for a T_H2 cell response in the protection of obesity induced inflammation and insulin resistance, however, is still up in question, as T reg cells also promote tolerance and reduce inflammation [135].

It is important to note that many other cells of the immune system have been implicated in the pathogenesis of chronic inflammation. Eosinophils are granulocytes that are major producers of IL-4 and IL-13, which have been shown to promote M2 macrophages activation in VAT. Genetic deletion of eosinophils increased adiposity and insulin resistance in mice fed a HFD when compared to eosinophil intact mice [136]. Conversely, mast cell deficient mice were protected from DIO and insulin resistance when compared to wild type mice [137]. However, the validity of mast cell deficient mice has been the subject of some scrutiny and care should be taken into consideration when interpreting the metabolic implications of mast cell deficient models [138].

Anti-Inflammatory treatments to ameliorate metabolic disease:

Evidence of increased acute phase proteins and inflammatory cytokines has linked metabolic dysfunction with the immune system response [27]. The inflammation associated with these conditions is a low-grade subclinical level. It is not yet clear if inflammation is responsible for organ dysfunction or chronic over-nutrition and metabolic stress is the impetus for the inflammation of the organ. Much research has been dedicated to reversing the perceived harmful effects of inflammation on metabolically stressed organs by treating patients with anti-inflammatory compounds (salsalate) and antibodies medications (infliximab, entanercept, tocilizumab) [139-142]. The results of these trials have failed to demonstrate efficacy in improving glucose tolerance and insulin resistance [34].

The shortcomings of anti-inflammatory therapies aimed to improve glucose homeostatsis emphasize the critical role that inflammation plays in the body's response to metabolic stress. It is possible that the early response to nutritional overload and expanding adipose tissue is the increase in inflammation that acutely works to maintain energy homeostasis. However, if the

stimuli for inflammation (over-nutrition, glucotoxicity, and physical inactivity) are not altered, than the inflammatory cycle promotes a negative adaptation that fundamentally alters the function of the organ [1].

Exercise is a potent anti-inflammatory therapy that reduces chronic inflammatory biomarkers associated with T2D and obesity [16,28-30,143]. However, exercise, in particular resistance training, promotes a transient inflammatory response that increases the circulating levels of inflammatory cytokines (ex. IL-6). This transient spike is followed by an increase in anti-inflammatory cytokines[144]. The degree of improvement in the biomarkers for chronic inflammation (ex. CRP) are positively associated with the intensity of the exercise [17]. Muscle produced myokines present a novel a mechanistic explanation for the anti-inflammatory effects of exercise [145]. The remainder of this review will focus on the various myokines and their relationship to exercise.

Skeletal Muscle as a Mediator of Inflammation:

IL-6 Myokine

Regular exercise has been shown to reduce levels of chronic inflammation [146]. The anti-inflammatory mechanisms of exercise have focused on the production of pro-and anti-inflammatory cytokines. Observations by Ostrowski et al (1998) first suggested that the elevation in IL-6 from prolonged running was a result of exercising muscle [147]. Since that time extensive research has been conducted to confirm the muscle origins of increased IL-6 during exercise. This phenomenon of skeletal muscle production and release of myokines into circulation suggests that skeletal muscle itself may mediate the anti-inflammatory effects of exercise [146]. IL-

6 is the most extensively studied myokine to date. Therefore IL-6 will provide the conceptual framework to understand how skeletal muscle can act as an anti-inflammatory endocrine organ.

Northoff and Berg (1991) were amongst the first to identify the elevation in cytokines in response to strenuous exercise in humans[148]. However, observations by Ostrowski and colleagues provided the bulk of the evidence for increased cytokines in circulation in response to prolonged running [147,149]. These early studies demonstrate that in marathon runners IL-6 levels peak immediately post-race and remain elevated for up to 4 hours. Work from this laboratory also demonstrates significantly increased circulating IL-6 levels at 30 minutes of treadmill running at 75% VO_2max.

Ostrowksi et al (1998) postulated that skeletal muscle is the primary producer of IL-6 measured in circulation during exercise [149]. To test this hypothesis, muscle biopsies and blood were collected before, immediately after, and two-hours post marathon. Comparative polymerase chain reactions were conducted to detect the mRNA levels of IL-6 and other cytokines in skeletal muscle and blood mononuclear cells (BMNC). IL-6 mRNA was only detected immediately and 2hrs post-marathon in skeletal muscle sample and not PBMC [149].

The primary source of IL-6 in circulation, during conditions like sepsis, is leukoycytes (monocytes). Ostrowski et al (1998) provided indirect evidence to the contrary as it applies to increased IL-6 during strenuous exercise [149]. Starkie et al (2000) utilized flow cytometry to rule out circulating monocytes as the producer of IL-6 [150]. Using a similar cohort and study design these researchers measured IL-6 and TNF-α levels and found no increase in the number of cells producing IL-6 or TNF-α or the fluorescent intensity of each cell. This study directly excluded PBCM as the producers of IL-6 during exercise[150].

IL-6 production and release from skeletal muscle

In order to determine that skeletal muscle was in fact the source of IL-6 during exercise Steensberg et al (2000) measured IL-6 concentration during single leg extension exercise [151]. In this landmark study, subjects completed 5 hrs of single leg extension exercise only against concentric resistance while blood flow was collected from catheters placed in the femoral artery of one leg and the femoral vein of both legs (arterio-venous difference method). IL-6 concentrations only increased in the blood from the exercising limb with no release from resting muscle and the non-exercised leg. This is the first study to provide strong evidence for IL-6 as a contraction mediated myokine.

The criticism of a-v difference method is that it does not indicate if other cells residing within skeletal muscle (macrophages, fibroblast, endothelial cells, etc). To address this Jonsdottir et al (2000) showed that in rodent models, electrical stimulation of skeletal muscle increased IL-6 mRNA as measured by quantitative-PCR [152]. This effect could not be recapitulated by infusion of epinephrine at doses that reached peak exercise conditions [153,154]. Together, these data indicate that contraction is an essential component of IL-6 release from the skeletal muscle and not the influence of circulating hormone on muscle.

Utilizing immunohistochemistry and in situ hybridization IL-6 protein and mRNA respectively was detected in biopsies taken from male subjects before and after 120-minutes of continuous cycling at significantly higher levels post exercise compared to pre-exercise sections [155]. More recently, Lauritzen et al (2013) identified IL-6 containing vesicles in a mouse quadriceps muscle that was transfected with enhanced green fluorescent protein (EGFP), which was reduced upon muscle contractions [156].

Initial observations from Brauusgaard (1997) indicate the IL-6 released from the skeletal muscle was due to damage that occurs during eccentric muscle contractions [149,157]. This hypothesis was based from studies that compared the concentration of IL-6 in men after completing two separate exercise trials. In the first bout, 9 male subjects completed 30 minutes of concentric cycling at 65% of VO2 max. After two weeks the same group of men completed a bout of eccentric cycling that matched the workload achieved by the concentric cycling bout. As expected, the results of this study demonstrate that eccentric exercise significantly increases markers for muscle damage (ex. creatine kinase) when compared to concentric exercise[157]. These levels persist even up to 7 days after the exercise bout. IL-6 is significantly increased only after the eccentric training bout. The authors of this study concluded that IL-6 was likely increased due to exercise as a result of muscle damage and was linked the inflammatory response. However, there was no significant difference in the concentration of blood monocytes or the composition of leukocytes between exercise bouts. Taking this response into account, the authors suggest that the source of IL-6 was still completely unknown as it pertains to exercise [157].

Further work to demonstrate that muscle damage was a primary component for elevated IL-6 circulation during exercise does not support this hypothesis [158-160]. Coisier et al (1999) provides compelling evidence that muscle damage is not a necessary component for IL-6 production during exercise [161]. In this trial IL-6 and myoglobin were measured immediately after 2 bouts of eccentric exercise, thereafter subjects completed 5 training sessions during a three-week period. Subjects were then tested again to measure IL-6 and myoglobin. The second testing bout at the end of the study was at the same workload as the pretest. Therefore the markers for muscle damage were reduced in the post-test. However, IL-6 levels from the pre-and post-test were similar.

IL-6 and glycogen

The highest measurements of circulating IL-6 during exercise have been recorded during prolonged running with elevations as high as 128-fold [144]. These data shifted the focus from muscle damaging exercise to glycogen depletion as the mechanism for skeletal muscle IL-6 production. Nehlsen-Cannrella et al (1997) made the observation that carbohydrate intake (6%) attenuated the IL-6 response to 2.5 hrs of running (~75% VO_2max) when compared to placebo [162]. Previously mentioned work by Starkie et al (2000) demonstrated that carbohydrate intake influenced IL-6 release and that circulating monocytes were not the source of secretion [150]. Bishop et al (2001) demonstrated that exercising in the glycogen-depleted state exacerbates the cytokine response [163]. Due to the fact that carbohydrate intake during prolonged exercise preserves muscle glycogen [164] it is logical to speculate that IL-6 release from skeletal muscle is in some way modulated by those levels.

To test this hypothesis, Steensberg et al (2001) conducted a study on 7 recreationally active men to determine if pre-exercise glycogen levels influence the skeletal muscle mRNA and circulating IL-6 levels [165]. Subject performed 2 hrs of single legged extensions for 1 hr followed by 1 hr of two-arm ergometery. The investigators stated that workload for single leg extension was sufficient to deplete muscle glycogen from the exercised leg and that upper body exercise prevented resynthesis in the depleted muscle from endogenous glucose production. Prior to exercise muscle glycogen was depleted 40% compared to control leg. As measured by the a-v difference across the exercising limbs the circulating levels of IL-6 produced by the depleted leg were significantly greater at throughout the exercise bout and up to three hours post-exercise. Skeletal muscle IL-6 mRNA showed a similar significant relationship[165].

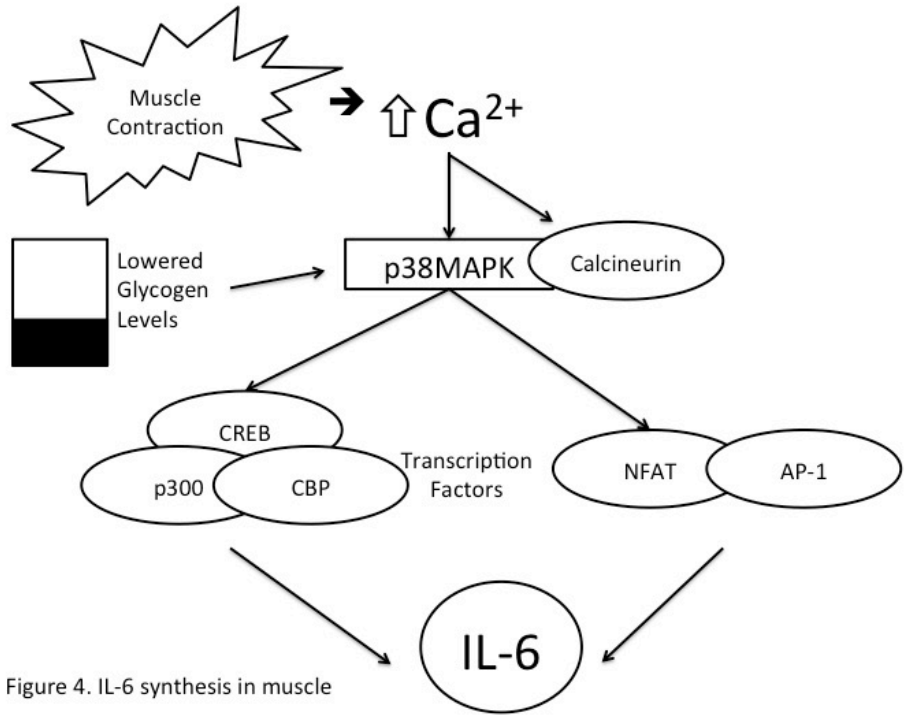

Figure 4. IL-6 synthesis in muscle

In a similar study Keller et al (2001) depleted muscle glycogen levels by having 6 male subjects undergo two identical 180-minute bouts of dynamic extension exercise [166]. Subjects were glycogen depleted the night prior and given a high-carbohydrate or low-carbohydrate meal. When participants were given the low-carbohydrate meal muscle glycogen levels were depleted by 40% compared to the high-carbohydrate condition plasma IL-6 levels were significantly elevated during exercise at 120-and 180-minute time points. Measurement of isolated muscle nuclei IL-6 mRNA transcript also revealed significantly higher IL-6 expression in the low-glycogen condition during exercise. It is important to note that in both trials by Steensberg and Keller that pre-exercise IL-6 levels were not different between low-and normal glycogen levels. It was not until exercise commenced that the levels of IL-6 increased at the greater level in the glycogen-depleted condition. This indicates a clear role for muscle contraction as a primary inducer of IL-6 transcription and protein release during exercise[165,166].

During intense or prolonged exercise hormonal factors are primary regulators of skeletal muscle glucose metabolism. Skeletal muscle expresses a large amount of β2-adergnic receptors to which epinephrine [167,168], norepinephrine, and cortisol [169] may bind and contribute to increased endogenous glucose production (EGP) during exercise and increase during intense or prolonged exercise. Interestingly, as glycogen levels decrease during exercise sympathoadrenal activity increases, as do circulating levels of IL-6. Therefore, epinephrine induced increases in IL-6 is a factor that may confound the relationship between IL-6 and glycogen levels.

Ex vivo studies utilizing rat skeletal muscle have demonstrated that physiological doses of epinephrine (10-100 nmol) do not increase mRNA or protein release of IL-6 from incubated rat skeletal muscle [153]. Only supraphysiological doses were shown to increase IL-6 mRNA into supernatant. Infusions of physiological doses (8 ng·kg·min^{-1}) of epinephrine that mimic the levels experienced during exercise were shown to produce a 6-fold increase in plasma IL-6, whereas during exercise treadmill running for 2.5 hrs at 70% VO_2max plasma IL-6 increased 29-fold [154]. The authors of these studies conclude that epinephrine plays only a minor role in the production and release of IL-6 from skeletal muscle during exercise.

IL-6 and Calcium (Ca^{2+})

During exercise the signal for muscle contraction is initiated by an abrupt increase in the release of Ca^{2+} from the sarcoplasmic reticulum (SR) [170]. Ca^{2+} is a second messenger that acts as a transcription factor that controls gene expression and cellular function and for that purpose intracellular Ca^{2+} levels are tightly regulated within all mammalian cells [171]. The Ca^{2+} ionophore, ionomycin, transports Ca^{2+} across the plasma membrane to raise intracellular Ca^{2+} concentrations [172]. Multiple studies have demonstrated that ionomycin is capable of increasing IL-6 gene expression in rat and human skeletal muscle [153,173,174].

The signaling pathways mediated by Ca^{2+} involve protein phosphatase calcineurin, which is activated during exercise [175] and electrical stimulation [176]. Calcineurin is of interest because it dephosphorylates and therefore activates nuclear factor of activated T cells (NFAT). NFAT is involved in the integration of mechanical stress response from exercise and remodeling that occurs as a consequence, which may explain why NFAT levels are 10-times higher in myocytes when compared to other cell types [177,178]. Activation of NFAT induces its translocation to the nucleus where it increases transcription of cytokines most notably IL-6 and IL-2 [33]. Muscle cells cultured with ionomycin and a selective inhibitor of calcineurin, cyclosporine A (CSA), significantly reduce IL-6 mRNA production when compared to ionomycin alone [174]. The mRNA expression of TNF-α is not reduced, but rather increased in these conditions indicating the IL-6 in skeletal muscle is linked to calcium/calcineurin signaling.

Interestingly, NFAT can be retained in the cytosol by phosphorylation via serine/threonine kinases, such as glycogen synthase kinase 3 (GSK3) [33]. This supports the theory that skeletal muscle contraction, which increases the influx of calcium within the muscle cell, promotes mRNA transcription immediately upon exercise and that appreciable levels of IL-6 are detected in plasma as muscle glycogen levels are depleted. In addition to NFAT, p38 MAPK is a stress-activated protein kinase that is activated during muscle contractions [179,180]. In order to link IL-6 to upstream transcription factors that correspond to glycogen level, Chan et al isolated nuclear fractions of muscle samples pre-and post-exercise in subject with normal and low-glycogen levels [181]. This important study demonstrates that low glycogen levels in exercising muscle cause increased phosphorylation of nuclear p38 MAPK and that IL-6 is likely induced by this phenomenon. L6 myotubes cultured with ionomycin phosphorylated nuclear p38 was increased significantly along with IL-6 mRNA. P38 MAPK is significantly reduced in muscle

cells treated with ionomycin and the p38 MAPK inhibitor (SB203580) and IL-6 mRNA is nearly undetectable [181].

Anti-inflammatory effects of IL-6

The beneficial anti-inflammatory response to exercise is multi-factorial. Reduction in inflammatory visceral adipose tissue, inhibition of macrophage infiltration in adipose tissue, reduced expression of toll-like receptors, and increased catecholamine levels all in some way account for the anti-inflammatory effects of exercise. However, skeletal muscle release of IL-6 plays an important biological role in the induction of the anti-inflammatory cytokine cascade.

During an acute infection TNF-α, IL-1β, and IL 6 are initially detected in that order followed by IL-1ra, sTNFR1 and IL-10. Exercise, of a sufficient intensity, can induce 100-fold elevations in IL-6 without increasing TNF-α levels significantly. Muscle derived IL-6 induces the production of IL-10, IL-1ra, and sTNFR1. After marathon running IL-10 peaks immediately post exercise (27-fold), but unlike IL-6 declines rapidly post exercise [144]. IL-1ra and sTNFR1 are increased 1.5 to 2.5 told, but peak approximately 1.5 hrs post exercise [145].

IL-6 functions as an anti-inflammatory myokine that not only stimulates the release of anti-inflammatory cytokines, but also stimulates the release of cortisol. To demonstrate the acute

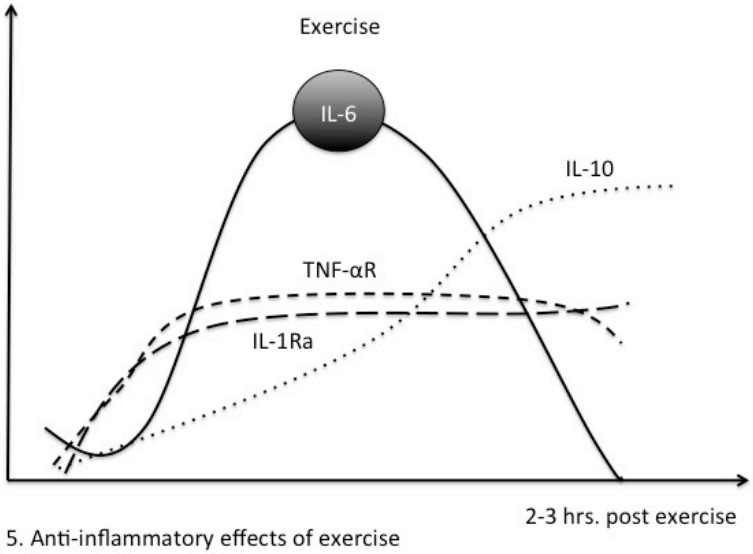

Figure 5. Anti-inflammatory effects of exercise (adapted from Pedersen 2005)

anti-inflammatory effects of exercise Starkie et al (2003) injected LPS (0.6 ng/kg bw) into resting controls, exercising subjects (2.5 hrs treadmill running), and rhIL-6 infusion and measure TNF-α levels for up to 5.5 hrs [182]. Exercising subjects demonstrate a blunted TNF-α response to the endotoxin injection when compared to controls. Importantly, rhIL-6 infusion mimics the effects of exercise by reducing the production of TNF-α *in vivo* [182]. These results demonstrate that muscle derived IL-6 promotes an anti-inflammatory environment.

IL-6 is typically designated as an inflammatory cytokines often associated with a high prevalence of obesity and T2D. This observation has led many researchers to believe that IL-6 is associated with negative metabolic consequences which potentially feedback to activate the immune response. However, this belief is not support by recent studies utilizing IL-6 knock out mice that develop late onset obesity and impaired glucose tolerance that is reversed when IL-6 is exogenously administered [183]. Furthermore, clinic trials blocking IL-6 to treat chronic inflammation increase cholesterol and plasma glucose levels.

The positive metabolic effects of IL-6 appear to be mediated by activated-AMP kinase (AMPK). AMPK is a metabolic energy sensing enzyme that regulates catabolic and anabolic signaling by detecting shifts in cellular AMP:ATP ratios. IL-6 signaling through its gp130 receptor transduces signaling that activates AMPK. The metabolic consequences of this interaction affect both glucose and FA metabolism. IL-6 treated muscle cells have increased basal and insulin stimulated glucose uptake due to increased GLUT4 trafficking and insertion into the plasma membrane [184]. *In vivo* work demonstrates that IL-6 infusion increases glucose infusion rate without altering hepatic glucose responsiveness [185].

AMPK is also an important regulator of FA oxidation that may modulate IL-6 metabolic interactions [186]. In fact, within skeletal muscle FA oxidation increases when infused with IL-6 (5000 pg/ml) by 50%, whereas TNF-α infusion at the same concentration does not affect FA oxidation, but rather increases the uptake FA and increases the accumulation of DAG by 45% [187]. *In vivo* experiments demonstrate that IL-6 is also a potent inducer of whole-body lipolysis and FA oxidation even at low levels (140pg/ml) without any detectable increase in lipolytic hormones [187,188].

The relationship of IL-6 and AMPK activation is supported by research that demonstrates the robust lipolytic effects of IL-6 is reversed when AMPK function is ablated [185]. These finding demonstrate that IL-6 has potentially synergistic effects of exercise metabolism whereby substrates can be mobilized an oxidized at greater rates. From an inflammatory standpoint there appears to be a significant benefit from regular exercise and regular exposure to elevated IL-6 levels. However, it is difficult to tease apart role of exercise in reducing inflammation that leads to improved metabolic health and vise versa.

Irisin: A PGC1-α derived myokine

The transcriptional co-activator peroxisome proliferator-activated receptor (PPARY) coactivator-1α (PGC1-α) is a key regulator of mitochondrial biogenesis and fatty acid and glucose metabolism in skeletal muscle and adipose tissue [189,190]. The use of transgeneic mice that overexpress PGC-1α promotes an oxidative skeletal muscle phenoytype (type I and type IIa) and

increases total muscle oxidative capacity [191]. These transgenic mice have also been shown to have improved insulin-sensitivity and resist age-related fat gain and muscle loss [192].

In wild type mice, cold exposure increases mRNA content of PGC1-α and robustly increases the transcriptional activity of PPAR-γ and the thyroid hormone receptor on the uncoupling protein (UCP-1) promoter[193]. UCP-1 is involved in thermogenesis by directing protons back through the mitochondria to generate heat rather than promote ATP synthesis via ATP synthase [194]. UCP-1 content is significantly higher in brown fat than in white fat cells. Considering that brown fat contains significantly more mitochondria and less ATP synthase than white fat it represents an important metabolic depot [194,195].

Many of the beneficial effects of exercise appear to be mediated by the activation of PCG1-α in skeletal muscle [191,192]. PGC1-α is increased acutely after exercise and chronically due to regular endurance exercise training. A common adaptation to endurance exercise occurs in skeletal muscle, whereby muscle fibers undergo an observable shift to an oxidative profile [191]. Generally, the health related benefits of exercise are associated with increased skeletal muscle oxidative metabolism. In resting skeletal muscle, levels of PGC1-α are elevated in type I and type IIA fibers compared to more glycolytic IIb/IIx fibers. Transgenic PGC1-α (PGC1-α $^{+/+}$) mice demonstrate an oxidative muscle phenotype shift and increased endurance exercise capacity when compared to PGC1-α knockout mice (PGC1-α$^{-/-}$), which demonstrate a more glycolytic shift and decreased exercise capacity compared to wild type mice.

Fibronectin domain-containing protein 5 (FNDC5) is a membrane-bound protein that is highly expressed in skeletal muscle [196,197]. In an attempt to identify secreted muscle factors that increased due to PGC1-α Boström et al (2012) demonstrate that FNDC5 undergoes proteolytic cleavage and releases a peptide fragment named irisin [198]. Irisin represents the N-terminal

domain of FNDC5 that is released in response to exercise, into the extracellular matrix and can be detected in plasma, serum [199], urine, and saliva [200]. FNDC5/irisin is 100% biologically identical between mice and humans [198].

The application of nanomolar concentrations of irisin on white fat cells increases UCP1 by 50-fold demonstrating a browning effect. Viral delivery of irisin stimulates a 3-fold increase in circulating levels; however, UCP1 level increase between 10-20 fold [198]. Irisin administration was shown in this important study to improve glucose tolerance and increased energy expenditure in mice fed a HFD. The authors of this study speculate that the levels of irisin, as stimulated by viral administration, which improve metabolic parameters, is similar to those measured during exercise and therefore irisin must be at least in part responsible for the beneficial effects of exercise [198].

Irisin is considered a myokine that is secreted from the muscle by the cleavage of FNDC5 after activation of PGC1-α[198]. Lecker et al (2012) confirmed that FNDC5 mRNA increased in relation to the increase of PGC1-α mRNA in skeletal muscle of subjects with a high aerobic exercise capacity, but not in subjects considered to have a low exercise capacity [201]. Human studies demonstrate that acute exercise increases circulating irisin levels [199,200,202,203]. Recent studies of acute exercise demonstrate that plasma irisin peaks immediately post exercise and correspond to increased exercise workloads and return to baseline 10-20 minutes post exercise [202,204].

Irisin levels appear to be increased in response to acute exercise bouts, however, there is currently no consensus on irisin response to chronic exercise training [205]. Many studies that have demonstrated the acute increase in irisin failed to demonstrate a positive training response. Timmons et al (2012) demonstrated FNDC5 mRNA was not different between sedentary and

active subjects and that analysis of approximately 200 muscle biopsies revealed that only a high active cohort of elderly subjects has increase muscle FNDC5 mRNA [205,206]. Analysis of another relatively large cohort did not detect an increase in FNDC5 expression or serum irisin levels in response to chronic or acute exercise. [207]

Factors not related to exercise appear to also modulate the production of irisin in the human body. A significant positive relationship between body weight and plasma irisin has been reported on numerous occasions [199,206,208]. However, these studies do not provide direct evidence that increased muscle mass is responsible for increased irisin levels as adipose tissue can produce irisin as well [209].

T2D is caused, in part, by impaired skeletal muscle glucose homeostasis [21] therefore the reported benefit on glucose tolerance of irisin on rats fed a HFD is of great interest [198]. *In vivo* measurement of irisin showed a significant negative correlation in relationship to 2 hr plasma glucose measured in newly diagnosed type 2 diabetics [210]. In support of this several other studies have documented a similar relationship [211-213]. However, some investigators have demonstrated a positive relationship with irisin levels and homeostatic model of insulin resistance (HOMA-IR) in obese humans [214]. In this particular study, irisin levels were positively correlated with metabolic syndrome. In calorie restricted rats that improved insulin sensitivity did so without any significant increase plasma FNDC5/irisin protein levels [215].

In order to clarify the complexity that surrounds the relationship between irisin and T2D studies Kurdiova et al (2014) recently conducted a clinical study that measured circulating irisin levels across glycemic status [216]. This study confirmed that type 2 diabetics have lower circulating irisin levels than non-diabetics. However, FNDC5 mRNA levels were increased significantly in prediabetic muscle compared to lean muscle, but not in T2D. When muscle cells

from type 2 diabetics were cultured a decrease in irisin was not observed, rather FNDC5 gene expression and secreted irisin was increased. When cells were cultured with excess palmitate and glucose, the levels of irisin and FNDC5 were reduced. It is reasonable to speculate that factors involved in the *in vivo* diabetic environment contribute to the reduced irisin secretion [216,217].

Myonectin/C1q Tumor Necrosis Factor-α Related Protein 15 (CTRP15)

Myonectin is characterized by an N-terminal peptide, a collagen repeat domain, and C terminal C1q-like globular domain. Some confusion exists regarding the classification of myonectin as a member of the C1q tnf-α family [218,219]. Work by Park et al (2009) originally described a protein CTRP5 discovered during a search for nuclear genes that were differentially expressed as a result of altered mitochondrial DNA (mtDNA) content [220]. Marked reductions in mtDNA and PGC1-α have been measured in the insulin resistant offspring of T2D parents and is also associated with obesity [221]. Therefore the identification of factors that are associated with increased or decreased mtDNA are important to understand the mechanisms of metabolic dysfunction in T2D and obesity. CTPR5 is described in this study has having 40% sequence homology with adiponectin, an adipokine that improves insulin sensitivity.

In cell culture (L6 myotubes) CTRP5 activates energy utilization pathways by increasing phosphorylation of AMPK and acetyl-CoA carboxylase [220]. Glucose uptake, measured by 2-deoxyglucose (2-DG), uptake and FA oxidation, as measured by palmitate oxidation, is significantly increased in myocytes treated with myonectin. The increase in GLUT4 translocation is accomplished without a measured increase in the phosphorylation of IRS-1 and AKT. Rather, GLUT4 translocation was related to an increase in AMPK, ACC, and p38 MAPK. AMPK phosphorylation of ACC is required for skeletal muscle FA oxidation and insulin sensitivity [222]. These results indicate that CTRP5 functions in similar manner to 5-amino-4-

imidazolecarboxamide ribonuleoside (AICAR) and adiponectin to increase FA oxidation and glucose uptake. However, it is important to note that ablation of the adiponectin receptors (AdipoR1 and AdipoR2) did not ameliorate the phosphorylation of myonectin treated cells. This indicates that myonectin is a novel protein functioning through a novel receptor [220].

The loss of mtDNA and mitochondrial function is implicated in the etiology of insulin resistance [223-225]. The benefits derived from exercise are known to involve transcriptional factors and co-activators such as PGC1-α and PGC1-β that stimulate mitochondrial biogenesis [191,226]. When a cell undergoes mitochondrial stress, such as reduced mtDNA, nuclear transcription factors are induced to aid in recovery [227]. It is believed that mitochondrial rescue is mediated through PGC1-α via calcium/calmodulin signaling pathways [228]. As previously mentioned Park et al (2009) demonstrated elevated circulating levels of CTRP5 in diabetic (db/db) obese rats (ob/ob) and mtDNA depleted rats (OLETF) rats [220]. A follow-up study in humans confirmed that circulating myonectin levels were positively correlated with HOMA-IR [229]. Fifty-six women underwent 10-weeks of moderate intensity exercise (60% VO_2max) for 1 hr three times a week, where after, significant increases in VO_2max, mtDNA density, and adiponectin were observed. These improvements coincided with significant reductions in serum CTRP5 [229].

These the studies demonstrated that CTRP5 is related to insulin resistance and mitochondrial dysfunction. Lim et al (2012) erroneously designated CTRP5 “myonectin”, however, work by Seldin et al (2012) reported that CTRP15 is preferentially expressed in muscle and secreted by muscle, whereas CTRP5 is produced by predominantly adipocytes, suggesting that CTRP is a novel proprietary myokine [230]. It is accepted that CTRP15 is now referred to as myonectin [231].

Myonectin mRNA and protein secretion increases in cultured myotubes in response to the exercise memetics ionomycin, forskloin, and epinephrine, but not to insulin or AICAR *in vitro.* In mice, two weeks of wheel running significantly increased serum myonectin and mRNA in the plantaris and soleus muscles. Remarkably, myonectin levels are suppressed in skeletal muscle and serum concentration during periods of fasting and drastically increase upon refeeding. This response was not preferentially induced by either glucose of emulsified intralipid gavage. Muscle cells were treated with glucose or palmitate also increase their expression of myonectin mRNA and secretion into media, which indicates that myonectin production is linked to nutrient flux though the cell[230].

Injection of myonectin (sufficient to raise myonectin levels 60-70% above resting levels) does not lower blood glucose in mice, however, it was sufficient to reduce non-esterfied fatty acids (NEFA) by 30%. Similar levels of myonectin cultured with 3T3-L1 adipocytes and H4EII hepatocytes increase FA uptake by 50% above non-treated cells and equal to 50nM insulin exposure. The measurements coincided with a significant increase in FA transporters CD36, FATP1, Cav1, and Fabp4. The level of FA uptake was in range with experiments that induce overexpression of currently measured FA transporters [232].

Myonectin is secreted in response to exercise and nutrient flux through the muscle cell via food intake. Recent work demonstrates that myonectin functions in an endocrine manner by activating mammalian target of rapamycin (mTOR) and AKT pathway to suppress liver autophagy in a similar manner to insulin [231]. Myonectin peaks approximately 3 hrs. after food intake or glucose gavage. This suggests that myonectin provides a postprandial signal that acts long after the effects of insulin. These data provide evidence that myonectin is secreted in

response to nutrient flux through the muscle and released into the circulation to act in an endocrine manner by regulating autophagy in hepatocytes [231].

Literature Review Summary

Chronic inflammation is a unique phenomenon that represents a disruption in metabolic homeostasis. Adiposity due to poor diet and exercise habits can contribute to the induction of an immune system response. The fundamental function of the immune system is to protect the host from pathogens and noxious stimuli. In order to fulfill this responsibility, inflammation is a necessary component of the survival and repair of cells, tissues, and organ systems that have been affected. Typical inducers of inflammation such as bacteria and viruses can elicit strong inflammatory response. However, metabolic dysfunction is also a strong inducer of inflammation

Obesity and T2D represent metabolic insults to adipose tissue and skeletal muscle. These tissues demonstrate increased infiltration of inflammatory macrophages and T cells. These immune system cells receive signals from the cellular milieu to increase their secretion of inflammatory cytokines. Since obesity and T2D are chronic conditions, these conditions will likely continue unless considerable action is taken to restore the organism back to metabolic homeostasis. If this inflammatory environment persists, the physiological function of the tissue organ system can be damaged further.

Exercise has potent anti-inflammatory effects that are mediated through metabolic perturbations, which regulates the expression and behavior of the cells of the immune system. Physical inactivity is associated with numerous disease conditions such as obesity, T2D, Alzheimer's disease, and some cancers. Furthermore, prolonged bed rest has well characterized effects on muscle atrophy and the loss of type IIx muscle fibers. This functional decline in muscle mass coincides with a marked increase in insulin resistance. Inflammation has an

underappreciated role in this process. Increased inflammatory cytokines (TNF-α, IL-6, IL-1β) as well as acute phase proteins (CRP) are significantly elevated during prolonged physical inactivity.

Muscle contractions are a fundamental component of exercise. Failure to adequately engage muscle fibers has serious deleterious effects on health and longevity. Therefore, the discovery of myokines provides reasonable speculative grounds that the beneficial effects of exercise are mediated through factors produced by the skeletal muscle during exercise (i.e. myokines). Skeletal muscle has a well-understood role in metabolism during rest and exercise. However, the discovery that a classical cytokine, IL-6, is produced by and secreted from skeletal muscle forms a bridge between the metabolic and immune system.

The focus of this review was to establish the grounds for chronic inflammation that is induced by metabolic dysregulation and to provide a framework to understand the burgeoning field of "immunometabolsim". The discovery and study of myokines has recently been dealt a considerable blow. Key researchers involved in myokine research have been convicted of fraud and several papers have been retracted. Investigation into irisin has yielded few of the positive results first reported by Bostöm et al (2012) casting doubt on the importance of this novel myokine. However, several independent laboratories have reported the discovery of hundreds of currently unidentified myokines that appear to be regulated by muscle contraction.

In conclusion, skeletal muscle represents a major target for the disposal of glucose during insulin-stimulated conditions. Insulin resistance is considered to be the initial defect in the pathogenesis of T2D. Insulin sensitivity and chronic inflammation are inversely correlated *in vivo* whereas *in vitro* inflammatory cytokines have been shown to cause insulin resistance. Exercise that improves insulin sensitivity to the greatest degree also appears to have the greatest

impact on reducing chronic inflammation. Therefore, the study of myokines that are produced in relation to the improvements in insulin sensitivity would be of great importance.

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1.2 Study Protocol

Title of Study: THE IMMEDIATE AND EXTENDED EFFECTS OF HIGH INTENISTY INTERVAL EXERCISE ON METABOLIC FLEXIBILITY AND MITOCHONDRIAL FUNCTION

Overview

Metabolic flexibility is defined as the ability to switch from predominantly lipid oxidation and fatty acid uptake in the fasted state to insulin-stimulated suppression of lipid oxidation and high rates of muscle glucose uptake and glycogen synthesis that occurs in the postprandial state [233]. The inability to "shift" (Δ respiratory exchange ratio (RER)) metabolism from lipid to glucose oxidation, termed metabolic inflexibility, is a characteristic of obesity and T2D [234-236] and is implicated in the development of insulin resistance [233,237,238]. However, metabolic inflexibility is present prior to the development of insulin resistance and fasting hyperglycemia in the offspring of T2D individuals [238-240] and remains in previously obese individuals after weight loss [236].

Metabolically inflexibility is associated with impaired fasting lipid oxidation [235,237,241], which suggests that metabolic inflexibility is a reflection of the inability of the mitochondria to switch between fuels.[234] Since lipid oxidation occurs in the mitochondria, it is logical to deduce that impairments in mitochondrial function could alter whole-body lipid oxidation. Adding clarification to this issue, Ukropcova (2007) and van de Weijr (2013) have demonstrated that skeletal muscle mitochondrial function is reduced in metabolically inflexible individuals [240,242]. Recent evidence also suggests that the offspring of type 2 diabetics have impaired metabolic flexibility without the presence of other metabolic risk factors [239]. Moreover, individuals with a biological mother that has T2D are at greater risk of developing T2D, when compared with a father with a positive history [243,244].

Continuous moderate to high intensity exercise training (6-12 weeks) has been shown to improve insulin sensitivity and metabolic flexibility in obese and type 2 diabetic patients, with greater improvements demonstrated with higher intensities [245,246]. Interestingly, the time course of glucose tolerance and insulin action in response to a single bout of exercise reveals periods of increased insulin sensitivity (immediately post exercise to 3 days) and resistance (5 to 7 days post exercise) [247,248]. Recent research suggests that 2-6 weeks of high intensity interval training (HIIT) improves insulin sensitivity and skeletal muscle oxidative capacity [249,250]. However, data on the immediate and extended effects of a single bout of high intensity interval exercise area lacking. To date there are no studies investigating the time-course of metabolic flexibility in response to a single bout of high intensity interval exercise (HIIE). Characterizing the immediate and extended response of a single bout of HIIE could provide insight into the therapeutic applications of this exercise approach.

Purpose and Procedures

The purpose of this research is to determine the immediate and extended (48-hr) effects of a bout of high intensity interval exercise prior to a mixed meal on metabolic flexibility and mitochondrial function in men

and women with and without a family history of T2D. The offspring of type 2 diabetics without other risk factors (obesity, hyperglycemia, sedentary lifestyle) demonstrate the signs of metabolic inflexibility.

Twenty apparently healthy, active, normal weight (BMI = 18 to 25 kg/m^2) young (age 18 to 40 y) men and women with (n=10) and without (n=10) a positive family history of T2D will be recruited to participate in this study. After an initial screening visit, graded exercise test, muscle oxidative capacity (OXPHOS), and mixed meal tolerance test (MMTT), participants will undergo 1 acute exercise session followed by a MMTT immediately after and 48 hours post exercise. Results from the graded exercise test will be used to determine the peak power output at $VO_{2max}$to prescribe a practical low-volume, high intensity interval program. Dietary intake will be recorded 24 hours before the initial MMTT and replicated 24 hours prior to the exercise session and the post-exercise MMTT. The primary outcome of the study is the change in substrate oxidation (carbohydrate and fat) in response to the MMTT immediately and 48 hours post exercise. Secondary outcomes include measures of insulin action (plasma glucose, insulin, and free fatty acid responses). Skeletal muscle OXPHOS, blood flow, on oxygen consumption using near infrared spectroscopy (NIRS) will also be measured.

Inclusion/Exclusion Criteria: The inclusion/exclusion criteria are summarized in Table 1. Active, college-age men and women with a normal weight (BMI 18-25 kg/m2), with and without a family history of T2D will be recruited. Participants will be frequency matched to control for race. Women that are enrolled in the study will be tested at least 10 days after the last day of their menstrual cycle. Individuals with known cardiovascular disease, T2D, and/or metabolic diseases will be excluded to reduce the likelihood of confounding circumstances regarding the exercise testing, acute aerobic exercise, and MMTT.

Table 1. Summary of the Inclusion/Exclusion Criteria

Inclusion Criteria	
Age	18-40 years
Sex	Men and Women
Positive Family History of T2D	Biological Mother with T2D or biological mother and father with T2D
BMI	18-25 kg/m^2
Informed Consent	The capability and willingness to give written informed consent, to understand the exclusion criteria, and to accept that the randomized group assignment is required
Exclusion Criteria	
Significant CVD or disorders	Including but not limited to serious arrhythmias, cardiomyopathy, congestive heart failure, stroke or transient ischemic attacks, peripheral vascular disease with intermittent claudication, acute, chronic or recurrent thrombophlebitis or myocardial infarction
Physically Inactive	Currently participating in aerobic exercise <20 minutes on <3 days per week and no resistance training.
Diabetes	Fasting glucose >125 mg/dL or 2 hour glucose of >200 mg/dL during MMTT
Blood pressure	Systolic blood pressure >130 and diastolic blood pressure >90.
Other significant medical conditions	Including but not limited to chronic or recurrent respiratory, gastrointestinal, neuromuscular, neurological, or psychiatric conditions. Hospitalization for mental illness within the past 5 years or currently undergoing treatment for severe mental illness. Musculoskeletal problems interfering with exercise. Autoimmune or collagen vascular diseases. Any other medical condition or disease that is life-threatening or that can interfere or be aggravated by exercise

Primary Outcomes – Shift in Substrate Metabolism and Insulin Action: The primary outcome for this study is metabolic flexibility determined as ΔRER during an MMTT, which suggests a shift in substrate

metabolism. In addition, insulin action will be assessed by examining glucose, insulin and free-fatty acid responses during the MMTT. The MMTT will be conducted at baseline, immediately post-exercise and 48-hours after HIIE session (total of 3 MMTT; Figure 1). The ΔRER in response to a MTTT was selected for the following reason: the MMTT is a more cost-effective and physiologically relevant stimulus for assessing metabolic flexibility compared to the gold-standard hyperinsulinemic-euglycemic clamp.

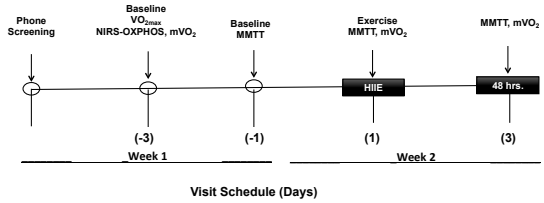

Figure 1. Visit schedule. *After confirmation of study eligibility participants will undergo a baseline VO_{2max} and mitochondrial function test (OXPHOS). The following week participants will complete a baseline mixed meal tolerance test (MMT) to determine metabolic flexibility. The next day participants will return to complete high intensity interval exercise (HIIE) followed by a MMTT with measures of mVO_2. 48 hrs post exercise intervention MMTT and mVO_2 will be repeated.

Measurement of Metabolic Flexibility and Insulin Action: Resting metabolic rate will be measured via indirect calorimetry while the subject is in the fasted state. An intravenous (IV) line will be placed in an antecubital vein for blood drawing purposes and will remain there throughout the testing. During each collection time point, 5 ml of blood (~1 teaspoon) will be drawn and stored for future analysis. A resting, fasting blood sample will be drawn after which the mixed meal will be prepared using a powered Ensure ® and 250 ml of water measured to one-third the resting metabolic rate (RMR). Additional blood samples will be drawn at the 5, 10, 15, 20, 30, 60, 90, 120, 150, 180 minute time points after drinking the mixed meal. Blood samples obtained during the MMTT will be analyzed for plasma glucose, insulin, and free-fatty acid concentrations using commercially available methods (Glucometer and/or Spectrophotometric assays and ELISA).

Muscle Measurements: NIR spectroscopy will be used to measure skeletal muscle OXPHOS at baseline. NIR spectroscopy will measure resting skeletal muscle oxygen consumption (mVO_2) and blood flow before and during the MMTT. The NIRS protocols developed and described by Kevin McCully's Laboratory will be implemented with slight modifications as discussed below [251-253].

High Intensity Interval Exercise Sessions: The HIIE prescription will be based on the established practical low volume, high-intensity technique using a cycle ergometer (Velotron, Racermate, Inc., Seattle, WA) [249]. Prior to exercise, participants will complete a 5-minute warm-up at 50 watts. Based on the results of the VO_{2max} test participants will engage in work intervals at 90% of the power output at VO_{2max} for 60 seconds followed by rest intervals at 30 watts for 60 seconds. Participants will complete 10 total intervals (~25 minutes).

Resting Metabolic Rate during the MMTT: Indirect calorimetry will be performed prior to and during each MMTT. Resting energy expenditure and substrate utilization will be assessed using the ParvoMedics, TruOne 2400 metabolic cart (Sandy, UT) calibrated with standard gas mixtures. A transparent plastic hood connected to the metabolic cart will be placed over the participant's head and calculations of energy expenditure and carbohydrate and fat oxidation rates will be made from expiratory gases diluted to produce a constant fraction of expired carbon dioxide (~1.0%). Resting metabolic measurements will be assessed before the MMTT and at 10 to 30, 40 to 60, 70 to 90, 100 to 120, 130 to 150 and 160 to180 minutes after ingestion of the mixed meal. The average of the 20minute measurement will be used to calculate resting metabolic rate and substrate oxidation using the equations of Jequier et al [254]. Participants will remain motionless and awake during the entire protocol.

Visits and Timeline (Figure 1)

Recruitment and informed consent: Word of mouth, Facebook ®, and strategically placed fliers on the Louisiana State University campus will be utilized to recruit for this study. Interested individuals will meet with study personnel and be provided a full description of study (structure of the study, study-related procedures, risks, benefits, etc). Individuals who are still interested in participating in the study will be offered informed consent and the opportunity to ask questions. If a participant chooses to sign the consent, he or she will continue on to study screening for inclusion/exclusion criteria including a medical history questionnaire, assessment of cardiovascular risk for exercise testing (PAR-Q), medication inventory, and anthropometric measures (height, weight, and resting blood pressure).

VO_{2max} testing: If participants meet all inclusion/exclusion criteria during the screening visit, they will undergo a maximal cardiorespiratory test (VO_{2max}) on a cycle ergometer (Velotron, Racermate, Inc., Seattle, WA). Participants will ride at an initial workload of 25 Watts for 4 minutes. The workload will be increased by 25 Watts every 4 minutes until respiratory exchange ratio reaches 1.05 after which, the workload will increase by 25 Watts every 2 minutes until volitional exhaustion. Throughout the test, respiratory gases will be analyzed using a metabolic cart (ParvoMedics Inc., Sandy, UT) and fat oxidation rates will be determined using stoichiometric equations[255]..

Mixed Meal Tolerance Test (MMTT): The baseline MMTT will occur ~1 week after the screening visit and VO_{2max} test. Participants will record all food and beverages ingested and physical activity levels for the 24-hour period prior to the baseline MMTT. Participants will arrive fasted for at least 10 hours and refrained from moderate/vigorous exercise for at least 48 hours. mVO_2 and muscle blood flow will be measured prior to and 1 and 2 hours post mixed meal (see NIRS Protocols for further details). The baseline MMTT will also complete the exclusion criteria for high fasting glucose (> 125 mg/dL) and abnormal 2-hour glucose (> 200 mg/dL). Additional MMTTs will be conducted immediately (1 hour) post exercise and 48 hours after the HIIE session. Prior to the post-exercise MMTTs, participants will replicate the 24-hour food and beverage diary recorded prior to the baseline MMTT. In addition, both MMTTs, will be conducted in the morning hours after a 10 hour fast.

NIRS Protocols to Measure Muscle Metabolism (e.g., NIRS mVO2, blood flow, and OXPHOS): Subjects will rest in the supine position with knees slightly flexed. The NIRS optode (Oxymon MKIII, Artinis Medical Sytsems) will be placed on the *vastus lateralis* just anterior to the *iliotibial band* and ~1/3 the distance between the top of the patella and the greater trochanter of the test leg. The optode will be secured in place with adhesive tape and Velcro strap. A blood pressure cuff (Hokanson, Bellevue WA) will be placed proximal to the optode, and connect the cuff to a rapid cuff inflation system (Hokanson E20, Bellevue WA) that will be controlled using an external controller and Labview Software. We measure resting muscle blood flow in triplicate using venous occlusions by inflating the blood pressure cuff to (50-60 mmHg) for 20 seconds with at least 60 seconds between each measurement. We will make measurements of resting muscle oxygen consumption (mVO2) in triplicate using arterial occlusions by inflating the blood pressure cuff to (250-300 mmHg) for 20 seconds with at least 60 seconds between each measurement. For measurement of muscle oxidative capacity (NIRS-OXPHOS), subjects will complete a 20 second isometric contraction of the vastus lateralis (by knee extension) immediately followed by a series of 15-20 arterial occlusions (250-300 mmHg) to measure the rate of recovery of mVO2 as depicted in Table 1. We will repeat this NIRS-OXPHOS protocol three times with ~5-10 min between each trial. Finally, we will perform an ischemic calibration procedure and calculate mVO2 corrected for blood volume as previously described, which includes a 15-20 second isometric contraction of the vastus lateralis (by knee extension) immediately followed by ~5-6 minute arterial occlusion (250-300 mmHg). NIRS signals for oxygenated, deoxygenated, and total hemoglobin will be continuously monitored by NIRS. Prior to testing, we will measure adipose tissue thickness by skin fold thickness and/or B-mode ultrasound (LOGIC e, GE) at the location of the optode. Before and after the MMTT we

Cuff Occlusions	On	Off
1-6	5	5
7-10	7	7
11-14	10	15
15-20	10	20

Table 1. Arterial Occlusion Protocol

will measure resting blood flow and mVO2 as described above and at the time points indicated in the description of the MMTT.

Continuous Glucose Monitoring: Participants will be given continuous glucose monitors (Dexcom G5, San Diego, CA). CGM use a sensor inserted under the skin by the participant to check glucose levels in the interstitial fluid. A transmitter sends sensor glucose readings to a display device. The participant will be required calibrate the CGM with a finger stick reading from blood glucose monitor. Participants will be provided with two sensors (1 for the baseline visits and 1 for the week of the exercise bout and 48hr visit).

Heart Rate Monitoring: Heart rate will be monitored during the exercise bouts and oral glucose tolerance testing with a telemetric heart rate monitor (Bioharness, Annapolis, Maryland). The monitor captures time intervals between R-R waves and can produce valid measures of heart rate variability.

Statistical Analysis: All statistics will be performed using JMP statistical software (SAS Institute Inc., Cary, NC). Data analysis will generally follow CONSORT recommendations using General Linear Models and repeated measures analysis of variance (RM-ANOVA) co-varied as needed depending on normality distributions at baseline. An unpaired t-tests will be used to compare between group baseline characteristics. In addition, two way RM-ANOVA (exercise session x time point) will be used to determine differences in responses in both the primary and secondary outcome variables. A power analysis conducted indicated that to measure a difference RER of .04 would require 20 participants (α=.05, β=.80) Significant effects will be further evaluated using Tukey's HSD post-hoc analyses were appropriate. Data will be reported as mean±SD and differences declared at $P<0.05$.

Expected Outcomes: Based on previous work we expect that the offspring of type 2 diabetics will have impaired metabolic flexibility [239]. We hypothesize that high intensity exercise will cause a greater shift in substrate metabolism in response to a mixed meal when compared to no prior exercise. We expect that high intensity interval exercise will result in a greater glucose tolerance and result in higher peak insulin levels, but lower area under the curve (AUC). Additionally, we expect that greater mitochondrial function will be associated with greater fasting fat oxidation and lower AUC for the change in RER in response to a mixed meal.

1.3 LSU IRB Approval

ACTION ON PROTOCOL APPROVAL REQUEST

Institutional Review Board
Dr. Dennis Landin, Chair
130 David Boyd Hall
Baton Rouge, LA 70803
P: 225.578.8692
F: 225.578.5983
irb@lsu.edu | lsu.edu/irb

TO: Neil Johannsen
Kinesiology

FROM: Dennis Landin
Chair, Institutional Review Board

DATE: March 23, 2016

RE: **IRB#** 3696

TITLE: The Immediate and Extended Effects of High Intensity Interval Exercise on Metabolic Flexibility and Mitochondrial Function

New Protocol/Modification/Continuation: New Protocol

Review type: Full __X__ Expedited ____ **Review date:** 2/12/2016

Risk Factor: Minimal _____ Uncertain ___X_____ Greater Than Minimal_____

Approved _____X_____ **Disapproved** _____

Approval Date: 2/12/2016 **Approval Expiration Date:** 2/11/2017

Re-review frequency: (annual unless otherwise stated)

Number of subjects approved: 20

LSU Proposal Number (if applicable)**:**

Protocol Matches Scope of Work in Grant proposal: (if applicable)_____

By: Dennis Landin, Chairman _____

PRINCIPAL INVESTIGATOR: PLEASE READ THE FOLLOWING –
Continuing approval is CONDITIONAL on:

1. Adherence to the approved protocol, familiarity with, and adherence to the ethical standards of the Belmont Report, and LSU's Assurance of Compliance with DHHS regulations for the protection of human subjects*
2. Prior approval of a change in protocol, including revision of the consent documents or an increase in the number of subjects over that approved.
3. Obtaining renewed approval (or submittal of a termination report), prior to the approval expiration date, upon request by the IRB office (irrespective of when the project actually begins); notification of project termination.
4. Retention of documentation of informed consent and study records for at least 3 years after the study ends.
5. Continuing attention to the physical and psychological well-being and informed consent of the individual participants, including notification of new information that might affect consent.
6. A prompt report to the IRB of any adverse event affecting a participant potentially arising from the study.
7. Notification of the IRB of a serious compliance failure.
8. SPECIAL NOTE:

**All investigators and support staff have access to copies of the Belmont Report, LSU's Assurance with DHHS, DHHS (45 CFR 46) and FDA regulations governing use of human subjects, and other relevant documents in print in this office or on our World Wide Web site at http://www.lsu.edu/irb*

1.4 Study Consent Form

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

INFORMED CONSENT

Title of Study: THE IMMEDIATE AND EXTENDED EFFECTS OF HIGH INTENISTY INTERVAL EXERCISE ON METABOLIC FLEXIBILITY AND MITOCHONDRIAL FUNCTION

We give you this consent form so that you may read about the purpose, risks and benefits of this research study.

- The main goal of a research studies is to gain knowledge that may help future patients.
- You have the right to refuse to take part, or agree to take part now and change your mind later on.
- Please review this consent form carefully and ask any questions before you make a decision.
- Your participation is voluntary.
- By signing this consent form, you agree to participate in the study as it is described.

1- Investigators:

The following investigators will be available for questions about this study:

Principal Investigator: Neil M. Johannsen, Ph.D.
Phone: 225-578-5314
Email:njohan1@lsu.edu

Co-Investigators: Tim Allerton, MS
Phone: 504-919-3799
Email: taller2@lsu.edu
Brian Harrell, M.D.,
Phone: 337-292-0431,

Email: brian.harrell@brgeneral.org

Brian Irving, PhD

Phone:225-578-7179,

Email: brianirving@lsu.edu

Guillaume Spielmann, PhD

Phone: 225-578-2926 gspielmann@lsu.edu

Neil Johannsen, Ph.D. directs this study. We expect about 20 people from one site will be enrolled in this study. The study will take place over a period of 4 visits and each participant will be enrolled for ~2 weeks.

2- Performance Site:

This study will take place at Louisiana State University-Baton Rouge Campus.

3- Purpose of the Study:

The purpose of this research is to determine the immediate and extended (48-hr) effects of a bout of high intensity interval exercise prior to a mixed meal on the ability to use nutrients after consuming a meal in men and women with and without a family history of T2D.

4- Participant Inclusion:

Study staff will discuss with you the requirements for participation in this study. It is important that you are completely truthful with the staff about your health history. You should not participate in this study if you do not meet all the qualifications.

You are eligible for this study if you are:

- Capable and willing to give written informed consent, understand exclusion criteria and accept the randomized assignment
- Male or Female age 18-40 years
- Have a biological mother with type 2 diabetes
- Healthy (no uncontrolled disease) with a BMI between 18 and 25 kg/m^2 and/or a waist circumference $\leq$ 35 inches for women and < 40 inches for men.
- Currently active (greater than 30 minutes/day, 3 or more days/week of moderate intensity, structured physical activity)

- Willing to record food intake and replicate for the 24 hours before each testing period.
- Willing to participate in VO_{2max} testing on a treadmill.
- Willing to participate in an intense bout of interval based exercise (~20 minutes)
- Willing to participate in 3 mixed meal tolerance tests with ingestion of 250-400 calories
- Willing to have blood drawn through an IV catheter and by finger stick
- Willing to have a blood pressure cuff inflated (50-250 mm Hg) around your upper thigh
- Willing to wear a continuous glucose monitor

You are **NOT eligible** for this study if you have any of the following conditions:

- HIV, Hepatitis B, or Hepatitis C
- Uncontrolled CVD/ arrhythmia
- COPD, Emphysema, Exercise Induced asthma/bronchospasm
- Cerebral Palsy, Multiple Sclerosis, Amyotrophic lateral sclerosis, Cystic Fibrosis
- Diabetes (Type 1 or 2); fasting glucose >125 mg/dL or 2 hour glucose >200 mg/dL during the baseline mixed meal tolerance test
- Systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 90 mmHg
- Uncontrolled thyroid disorder (controlled = 6 months of medication)
- Epilepsy
- Osteo/Rheumatoid arthritis
- Unresolved orthopedic injury of any kind
- Any other medical, psychiatric or behavioral factors that in the judgment of the Principle Investigator that may interfere with study participation or the ability to follow the protocol

<u>Medications/Non-Drug Therapies:</u>

- Blood pressure medication
- Diuretics
- Beta-blocker
- Anti-inflammatory drug (corticosteroid/anabolic steroid/NSAID)
- Antipsychotic
- Other medications that may affect glucose and or fat metabolism

<u>Lifestyle:</u>

- Consume > 3 drinks/day of any alcoholic beverage
- Smoker (Former smokers must be smoke free for 12 months)
- Donated blood within the past 6 weeks

5-Study Procedures:

The study for which you are volunteering will take about 2 weeks to complete and will include 4 visits

1. Screening Phase – 1 Screening Visit (Visit 1)
2. Baseline Testing Phase – 1 Baseline Visits (Visit 2)
3. Follow-up Phase – 2 Visits (3 and 4)

Summary of Visit Schedule

	Screening Baseline OXPHOS, VO_{2max}	**Baseline Metabolic Flexibility**	**Exercise Intervention**	**48 hours Post-Exercise**
	Visit 1	Visit 2	Visit 3	Visit 4
Procedure (Time)	Day 0	Day 1	Day 2	Day 4
Informed Consent	X			
PARQ and Medical History	X			
Height	X			
Weight	X			
Body Composition and skin folds	X			
Vital Signs	X	X	X	X
Medication Inventory	X			
NIRS-blood flow and mVO_2	X	X	X	X
NIRS-OXPHOS	X			
Questionnaires	X		X	X
Food Recall	X	X	X	X
Physiological Monitors	X	X	X	
VO_{2max} test	X			
Blood Samples		X	X	X
Mixed Meal Tolerance Test		X	X	X

Substrate Metabolism		X	X	X

Study Timeline

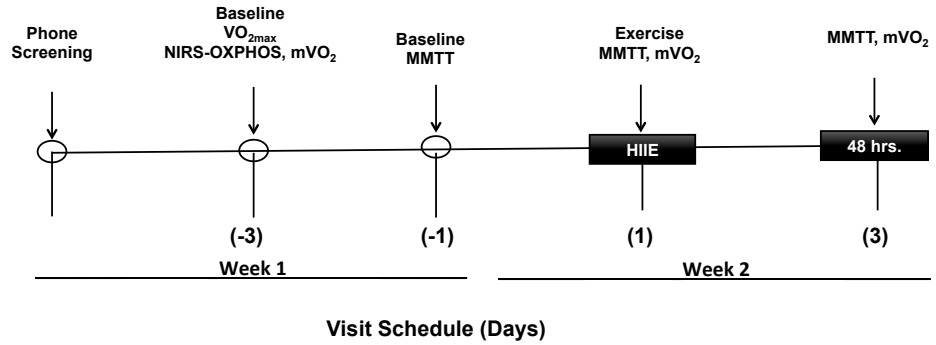

Figure 1. Visit schedule. *After confirmation of study eligibility participants will undergo a baseline VO_{2max} and mitochondrial function test (OXPHOS). The following week participants will complete a baseline mixed meal tolerance test (MMT) to determine metabolic flexibility. The next day participants will return to complete high intensity interval exercise (HIIE) followed by a MMTT with measures of mVO_2. 48 hrs post exercise intervention MMTT and mVO_2 will be repeated.

Screening Visit –(Phone or In-person)

At this visit, the informed consent will be explained to you by our study staff and procedures are performed. If you choose to sign the informed consent, the following tests and procedures will be performed to determine if you qualify to participate in this research study:

- You will be asked questions about your:
  - Demographics
  - Medical history
  - Medication use

- You will be asked to perform the following medical assessments:
  - Blood pressure and heart rate
  - Height and weight
  - Skin Folds for body composition

Volunteers who are meet the inclusion/exclusion criteria at the screening visit will be scheduled for baseline visits and intervention and follow-up visits.

Baseline Visits: Visit will last approximately 1 ½ hour

Baseline visit 1 (This visit may be divided into two separate days) :

(Non-Fasting visit: Please refrain from caffeine for at least 5 hours prior to visit.)

- You will be asked to return to the Clinical Exercise Testing Laboratory at LSU for Baseline Testing Visit 1. For 24 hours prior to Baseline Visit 2, you will be asked to record your food and drink intake. You will be asked to replicate this record before follow-up visit 1 and 2. The following tests will be performed:
    - Blood pressure and heart rate – by a cuff placed around your arm and monitor worn around your chest
    - Weight
    - Resting Muscle Oxygen Consumption and Oxidative Function measurements (NIRS OXPHOS and NIRS $mVO_{2)}$
    - Graded Exercise Test (VO_{2max})
    - Given continuous glucose monitor for self application

The time involved with the VO_{2max} test depends on your fitness level. Your aerobic fitness will be assessed while you pedal on a cycle ergometer. The workload of the ergometer will increase at regular increments (every 2 to 4 minutes) until you are unable to continue pedaling. Your tiredness will in most cases be due to your muscles becoming too tired to perform the work required of the stage and you may feel short of breath. Both or a combination of both are normal responses to this type of test. The purpose of this test is to determine your fitness level in order to prescribe the exercise intensities during the high intensity interval exercise bout (Visit 3).

During the exercise test, the volume of oxygen and carbon dioxide you are breathing will be measured continuously using an instrument known as a metabolic cart. The test requires that you breathe through a mouthpiece, similar to a snorkel, with a nose clip on. The mouthpiece is attached to a long tube, which is then attached to the metabolic cart. The breath gasses collected during the exercise test allow us to accurately measure your energy expenditure and type of fuel (carbohydrate or fat) you are using at each stage of the test. Your heart rate will be monitored continuously through a wireless heart rate monitor (Bioharness 3.0). At the end of each stage, you blood lactate levels will be measured by finger stick blood analysis.

In order to measure glucose levels throughout the study period you may or may not be asked to wear a continuous glucose monitor. The monitor is self-applied by inserting the sensor under the skin by use of the sensor applicator. Instruction will be provided on site in addition to the manufactures guidebook.

To measure muscle metabolism and blood flow, we will place a monitor on the upper leg secured with adhesive tape and a Velcro strap. A blood pressure cuff will be placed above the monitor and connected to a rapid inflation device. Triplicate measurements of resting muscle blood flow will be taken by inflating the blood pressure cuff to 50-60 mm Hg (venous occlusion) for 20 seconds and releasing the pressure with at least 60 seconds between each measurement. Triplicate measurements of resting muscle oxygen consumption (mVO_2) will be taken by inflating the blood pressure cuff for ~20 seconds to ~250-300 mm Hg (arterial occlusion) and releasing the pressure with at least 60 seconds between each measurement. For muscle recovery measurements, you will complete ~20 second of maximum isometric knee extension exercise followed by 15-20 arterial occlusions. You will complete this muscle recovery protocol three times with ~5-10 min between each trial. You will also complete a 15-20 second maximum isometric knee extension exercise immediately followed by a ~5-6 minute arterial occlusion to calibrate the measurements. Prior to testing, we will measure the fat tissue thickness under the monitor by skin fold thickness and/or ultrasound.

Baseline Visit 2: Visit will last approximately 3 ½- 4 hours

(Fasting Visit- Please arrive at 10 hours fasting and refrain from caffeine at least 5 hours and refrain from vigorous exercise 48 hours prior to study visit)

- You will be asked to return to the Clinical Exercise Testing Laboratory at LSU and the following tests will be performed:

    - Mixed meal tolerance test – ingestion of 250-400 calorie Ensure drink.
    - Blood samples – An IV catheter will be placed in your arm vein for blood draw purposes and will remain there throughout the testing period. Blood will be drawn at specific times. About 5 teaspoons of blood will be collected each trial and measured for glucose, insulin, and free fatty-acids. The remaining blood will be stored for this study and for future research if you consent.
    - Respiratory measures - a plastic, transparent hood will be placed over your head before and periodically during the oral glucose tolerance test to measure resting energy expenditure and the type of fuel you are using

(carbohydrate or fat). The hood will be attached to the metabolic cart similar to the exercise test.
    - Resting muscle oxygen consumption and blood flow measurements as described during testing visit 1 will be performed before and 1 and 2 hours after starting he mixed meal tolerance test.

Exercise Intervention: Visit will last approximately 4 hours

(Fasting visit – please refrain from eating 10 hours alcohol and exercise for 48 hours and caffeine for 5 hours before this visit.)

- You will be asked to return to the Clinical Exercise Testing Laboratory at LSU and the following tests will be performed:
    - Heart rate – by a cuff placed around your arm and monitor worn around your chest
    - Perform repeated bout of cycling lasting 1 minute followed by one minute of recovery for 20 minutes total.
    - Mixed meal tolerance test will be initiated 1 hour after the acute bout of exercise.
    - Resting muscle oxygen consumption and blood flow measurements as described during testing visit 1 will be performed before and 1 and 2 hours after starting he mixed meal tolerance test.

Follow-up 48 hour Recovery Visit: Visit will last approximately 3.5- 4 hours

(Fasting visit – please refrain from eating 10 hours alcohol and exercise for 48 hours and caffeine for 5 hours before this visit.)

- You will be asked to return to the Clinical Exercise Testing Laboratory at LSU and the following tests will be performed:

    - Mixed meal tolerance test – ingestion of 250-400 calorie Ensure drink.
    - Blood samples – An IV catheter will be placed in your arm vein for blood draw purposes and will remain there throughout the testing period. Blood will be drawn at specific times. About 5 teaspoons of blood will be collected each trial and measured for glucose, insulin, and free fatty-acids. The remaining blood will be stored for this study and for future research if you consent.

- Respiratory measures - a plastic, transparent hood will be placed over your head before and periodically during the oral glucose tolerance test to measure resting energy expenditure and the type of fuel you are using (carbohydrate or fat). The hood will be attached to the metabolic cart similar to the exercise test.
- Resting muscle oxygen consumption and blood flow measurements as described during testing visit 1 will be performed before and 1 and 2 hours after starting he mixed meal tolerance test.

6- Risks/Discomforts:

VO_{2max} and exercise testing: All exercise testing is completed in accordance with the American College of Sports Medicine's Guidelines for Exercise Testing and Prescription as well as the American Heart Association. There is minimal risk of injury or a cardiovascular event during testing. We believe the use of a highly trained staff, a pretest review of participant associated risk factors including the Physical Activity Readiness Questionnaire (PAR-Q) and medical screening by your doctor, and well-defined emergency procedures minimize the risk of an event during testing. During testing you may experience temporary discomfort during blood pressure recordings due to the pressure of the blood pressure cuff on the arm. You may also experience muscle fatigue, weakness, soreness and/or muscle pulls or tears. All tests are conducted in the presence of trained staff. In addition, all staff are trained in BLS (basic life support-CPR) and/or ACLS (advanced cardiac life support). In the event of an emergency, you would be treated appropriately and transported to the nearest acute care medical-surgical facility via Emergency Medical Services.

IV procedure and blood draws: There may be minimal discomfort and bruising and/or bleeding where the needle is inserted for finger prick blood sampling. There is a possibility of pain, bruising, and/or infection at the site of the needle insertion for the IV line for mixed meal tolerance test. Aseptic (sterile) technique and trained personnel minimize this risk.

Body composition: The measurement of skinfold thickness using skinfold calipers requires a firm squeezing and subsequent measurement of the skin. This measurement may cause slight discomfort and possible bruising of the measured site. However, if undue bruising, soreness, or if more than slight swelling occurs, please notify the Principal Investigator.

Metabolic testing: It may be uncomfortable to have the plastic hood over your upper body during resting measurements if you are claustrophobic. The hood is easily removed if you experience this symptom.

Loss of Confidentiality: Completing questionnaires may result in a breach in confidentiality of personal data. Participants will be assigned ID numbers and information that could identify a participant will not appear in publications.

Muscle Blood Flow and Metabolism Measurements: Inflation of the blood pressure cuff around the leg may cause some discomfort and pain during the test. The temporary numbness and tingling are similar to the sensation of having your foot "fall asleep". If the discomfort is too severe, you may stop the test by notifying the technician to stop and the test will be immediately terminated. There is no known risk from the use of skin folds and/or ultrasound to measure your adipose tissue thickness.

CGM Sensor Insertion: Inserting the sensor and wearing the adhesive patch might cause infection, skin irritation, bleeding, or pain. Sensors can be easily removed without discomfort.

Unknown risks: In addition to the risk listed above, you may experience a previously unknown risk or side effect.

7- Benefits:

We cannot promise any benefits from your being in the study. However, possible benefits include:

- Information about your general health
- Knowledge of your cardiovascular fitness
- An understanding of your exercise performance and physical activity

8-Alternatives to Participation:

There are no alternatives to the study described in this consent. You have the choice at any time not to participate in this research study. If you choose not to participate, any benefits to which you are entitled will not be affected in any way.

9- Injury/Illness or Questions:

If you have any questions about your rights as a research volunteer, you should call Dennis Landin, Ph.D., Institutional Review Board Office at 225-578-8692. If you have any questions about the research study or think you have a research-related injury or medical illness, contact Neil Johannsen, Ph.D. at 225-578-5314 during regular working hours.

10-Privacy:

Every effort will be made to maintain the confidentiality of your study records. Results of the study may be published; however, we will keep your name and other identifying information private. Other than as set forth above, your identity will remain confidential unless disclosure is required by law.

11-Early Study Withdrawal:

Neil Johannsen, Ph.D. can withdraw you from the study for any reason or for no reason. You may withdraw from the study at any time without penalty. Possible reasons for withdrawal include injury, the presence of an old or existing injury that may be deemed risky, sufficient medical history deemed too risky for testing.

12-Additional Information:

During the course of this study there may be new findings from this or other research that may affect your willingness to continue participation. Information concerning any such new findings will be provided to you.

13-Charges for Participation:

None

14-Payments for Participation:

Participants will receive $100.00 for completion of the study.

15- Compensation for study-related injury or medical illness:

No form of compensation for medical treatment or for other damages (i.e., lost wages, time lost from work, etc.) is available from Louisiana State University. In the event of

injury or medical illness resulting from the research procedures in which you participate, you will be referred to a treatment facility. Medical treatment may be provided at your own expense or at the expense of your health care insurer (e.g., Medicare, Medicaid, Blue Cross-Blue Shield, Dental Insurer, etc.) which may or may not provide coverage.

16- Signatures:

The study has been discussed with me and all my questions have been answered. I understand that additional questions regarding the study should be directed to the study investigators. I agree with the terms above and acknowledge that I have been given a copy of the consent form.

_____

Printed Name of Volunteer

_____ _____

Signature of Volunteer Date

_____

Date of Birth of Volunteer

_____ _____

Signature of Person Administering Informed Consent Date

Neil M. Johannsen, Ph.D.

Principal Investigator

18- Tissue/Specimen Storage for Future Research or Use

Biospecimens for future research:

You are being asked to allow some of your blood to be stored and used for research at a later time. These bodily materials are called biospecimens. The donation of biospecimens in this study is optional. No matter what you decide to do, it will not affect your study participation. You will still be allowed to take part in the study even if you don't want your specimens to be collected and used for future research. Some biospecimen samples will be stored and used for the study and other biospecimen samples will be stored for future studies. The collection of samples may give scientists valuable research material that can help them to develop new diagnostic tests, new treatments, and new ways to prevent diseases. If you agree to have your samples stored, you can change your mind up until the end of the study.

The samples will be stored indefinitely. If you agree to donate your samples, they may be given to other investigators for future research as well. The future research may take place at Louisiana State University and may involve Louisiana State University Researchers in this study. The future research may not take place at Louisiana State University and may not be reviewed by Louisiana State University's Institutional Review Board. For privacy and confidentiality, your biospecimens will be labeled with a unique series of letters and numbers. Louisiana State University will store your biospecimens with this unique identifier and the minimum number of personal identifiers to meet laboratory standards. The research done with your specimens may help to develop new products in the future, or may be used to establish a cell line or test that could be patented or licensed. You will not receive any financial compensation for any patents, inventions or licenses developed from this research.

Making your choice about future research:

Please read about each biospecimen below. It is your choice which samples will be collected, stored and used for future research for this study or future studies. After reading about each below, initial next to "Yes" or "No" to show your choice about the collections for this research study and for future research studies.

Blood

If you give permission, approximately 1 tablespoon of blood will be stored by this study. Your stored samples may be tested at Louisiana State University or other locations used in future research.

Do you give permission for your blood to be collected and used in future research by this study?

Yes, I give permission _____

SIGNATURE

No, I do not give permission _____

SIGNATURE

If you decide you would like to withdraw your consent to use your samples, you will be able to do so until the study ends. After the study ends, you will not be able to withdraw your consent to use your samples because investigators will not know which one is yours. You must provide a written request to have your samples destroyed.

For destruction of your samples, you can contact the Principal Investigator at:

Neil Johannsen, PhD

Louisiana State University

112 Huey P. Long Fieldhouse

Baton Rouge, LA 70803

VITA

Timothy Allerton was born in New Orleans, LA to William and Constance Allerton. Timothy received his bachelor's degree in Kinesiology in May of 2005 from Louisiana State University. In December of 2007 Timothy received his master's degree in Clinical Exercise Physiology from Northeastern University in Boston, MA. Upon graduation, Timothy will receive the Doctor of Philosophy in Kinesiology from the Graduate School at Louisiana State University and become a postdoctoral fellow in the T32 "Training in Botanical Approaches to Combat Metabolic Syndrome" at Pennington Biomedical Research Center. Timothy currently resides in New Orleans with his wife, Kelly, and two sons, Harry and Finn.