Skeletal muscle metabolic adaptations in response to an acute high fat diet

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ABSTRACT

Macronutrient metabolism plays an essential role in the overall health of an individual. Depending on a number of variables, for example, diet, fitness level, or metabolic disease state, protein, carbohydrate and fat have varying capacities to be oxidized and balanced. Further, when analyzing the oxidation of carbohydrate and fat in the skeletal muscle specifically, carbohydrate balance happens quite rapidly, while fat balance does not. The ability of skeletal muscle to adapt and respond to various nutrient states is critical to maintaining healthy metabolic function. Habitual high fat intake has been associated with reduced oxidative capacity, insulin resistance, increased gut permeability, inflammation, and other risk factors often preceding metabolic disease states. The disruption of gut function leads to gut permeability and increases endotoxins released into circulation. Endotoxins have been shown to play an important role in obesity-related whole body and tissue specific metabolic perturbations. Each of these disrupted metabolic processes is known to associate with obesity, metabolic syndrome and diabetes. To date, limited research has investigated the role of high fat diet on skeletal muscle substrate oxidation and its relationship to gut permeability and endotoxins. The purpose of this study was to determine the effects of an acute, five-day, isocaloric high fat diet (HFD) on skeletal muscle substrate metabolism in healthy non-obese humans. An additional purpose was to determine the effects of a HFD on gut permeability and blood endotoxins on healthy, non-obese, sedentary humans. Thirteen college age

males were fed a control diet for two weeks, followed by five days of an isocaloric HFD. To assess the effects of a HFD on skeletal muscle metabolic adaptability and postprandial endotoxin levels, subjects underwent a high fat meal challenge before and after a HFD. Muscle biopsies were obtained; blood was collected; insulin sensitivity was assessed via intravenous glucose tolerance test; and intestinal permeability was assessed via the four-sugar probe test before and after the HFD. Postprandial glucose oxidation and fatty acid oxidation in skeletal muscle increased before the HFD intervention but was decreased after. Skeletal muscle in vitro assay of metabolic flexibility was significantly blunted following the HFD. Insulin sensitivity and intestinal permeability were not affected by HFD, but fasting endotoxin was significantly higher following the HFD. These findings demonstrate that in young, healthy males, following five days of an isocaloric high fat diet, skeletal muscle metabolic adaptation is robust. Additionally, increased fasting endotoxin independent of gut permeability changes are potentially a contributor to the inflammatory state that disrupts substrate oxidation. These findings suggest that even short-term changes in dietary fat consumption have profound effects on skeletal muscle substrate metabolism and fasting endotoxin levels, independent of positive energy balance and whole-body insulin sensitivity.

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GENERAL ABSTRACT

Macronutrients, namely carbohydrates, fats and protein, and the way they are utilized play an important role in the overall health of an individual. Many variables come into play when considering the oxidization (or utilization) of each macronutrient, including, but not limited to diet, fitness level, and metabolic disease state. Skeletal muscle and its role in these processes is of special interest as it is the largest insulin sensitive organ in the body. Its ability to adapt and respond to various nutrient states is critical to maintaining healthy metabolic function. Habitual high fat intake has been associated with insulin resistance, increased gut permeability (increasing endotoxins, which are toxins released into circulation from the intestines), reduced oxidative capacity (ability to utilize macronutrients for energy), and inflammation, all of which are risk factors that precede metabolic disease states. To date, limited research has investigated the role of high fat diet on skeletal muscle oxidation of macronutrients and its relationship to what is going on in the gut, or intestines. The purpose of the study was to determine the effects of a short term high fat diet (five days) on skeletal muscle in healthy, non-obese humans, and to determine the effects of this diet on gut permeability and endotoxins. Thirteen college-age males were fed a control diet for two weeks followed by five days of a high fat diet. Each diet had the same caloric content. Subjects underwent a high fat meal challenge before and after the diet to assess the effects of the diet on skeletal muscle adaptability and post meal endotoxin levels. Before and after the

high fat diet, muscle biopsies were obtained, blood was collected, insulin sensitivity was assessed and gut permeability was measured. We found that skeletal muscle metabolic adaptation is robust. Additionally, increased fasting endotoxin changes are a possible contributor to the inflammatory state that disrupts macronutrient oxidation. Therefore, even short-term changes in dietary fat consumption have profound effects on skeletal muscle metabolism and fasting endotoxin levels, independent of positive energy balance and whole-body insulin sensitivity.

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ATTRIBUTIONS

CHAPTER 2: LITERATURE REVIEW

Matthew Hulver, PhD Madlyn Frisard, PhD and Suzanne Bowser conceived and designed the review; Ms. Bowser wrote the review; Dr. Hulver and Dr. Frisard edited the document.

CHAPTER 5: SKELETAL MUSCLE METABOLIC ADAPTATIONS IN REPONSE TO AN ACUTE HIGH FAT DIET

Matthew Hulver, PhD was the principal investigator on the grant that funded the research. He oversaw the entire study. Kevin Davy, PhD, a co-investigator on the project, was responsible for day to day operations in the clinical laboratory. Brenda Davy, PhD, RDN, a co-investigator on the project, was responsible for all aspects of dietary control. Andrew Nielson, PhD a co-investigator on the project, was responsible for measures of gut permeability. Ryan McMillan, PhD, the study coordinator, managed all aspects of scheduling, testing, sampling, and data collection and oversaw all aspects of measurement of skeletal muscle. Madlyn Frisard, PhD and the above mentioned personnel contributed to the design of the study and will co-author the manuscript. Suzanne Bowser wrote the manuscript and assisted Dr. McMillan in scheduling, testing, sampling, and data collection as well as in measurements of skeletal muscle.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2: LITERATURE REVIEW

CHAPTER 5: SKELETAL MUSCLE METABOLIC ADAPTATIONS IN RESPONSE TO AN ACUTE HIGH FAT DIET

LIST OF TABLES

CHAPTER 5: SKELETAL MUSCLE METABOLIC ADAPTATIONS IN RESPONSE TO AN ACUTE HIGH FAT DIET

CHAPTER 1: INTRODUCTION

Obesity and other metabolic diseases are major contributors to serious health conditions among Americans. The prevalence of obesity in the United States and globally has grown rapidly in the last three decades. In 2014 more than one-third (27.9%) of US adults met the definition of obesity (Body Mass Index of greater than 30kg/m^2)[1]. Likewise, according to the 2014 National Diabetes Statistics Report, the prevalence of Type 2 Diabetes mellitus (T2DM) is on the rise. In 2012, 9.3% of the population had T2DM, accounting for 29.1 million people. The prevalence for adults age 20 and older in 2012 was 12.3%. Diabetes is the 7th leading cause of death within the United States in 2013[2]. In order to better understand T2DM, obesity and other metabolic diseases, research into the mechanisms contributing to or priming the body for these conditions is imperative.

While overall health is multi-factorial, a number of characteristics of metabolic health and likewise, metabolic disease, have been elucidated. Below are two simplified diagrams illustrating in Figure 1, a metabolically healthy individual and in Figure 2, a metabolically diseased individual. These are certainly not exhaustive in nature; however provide an exemplary of disturbances that occur as a result of consuming a habitual high fat diet.

Figure 1. Schematic of Metabolically Healthy Individual

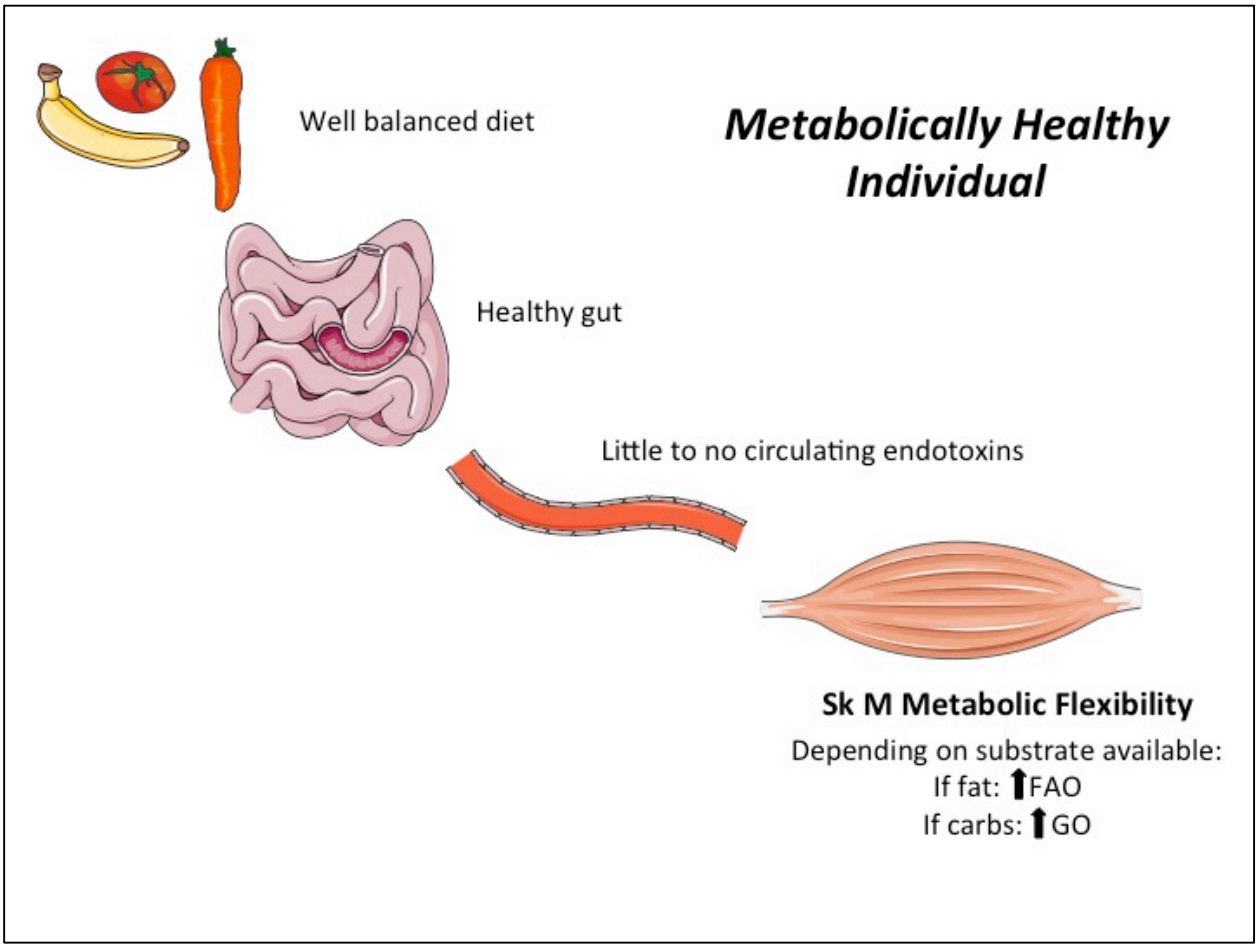
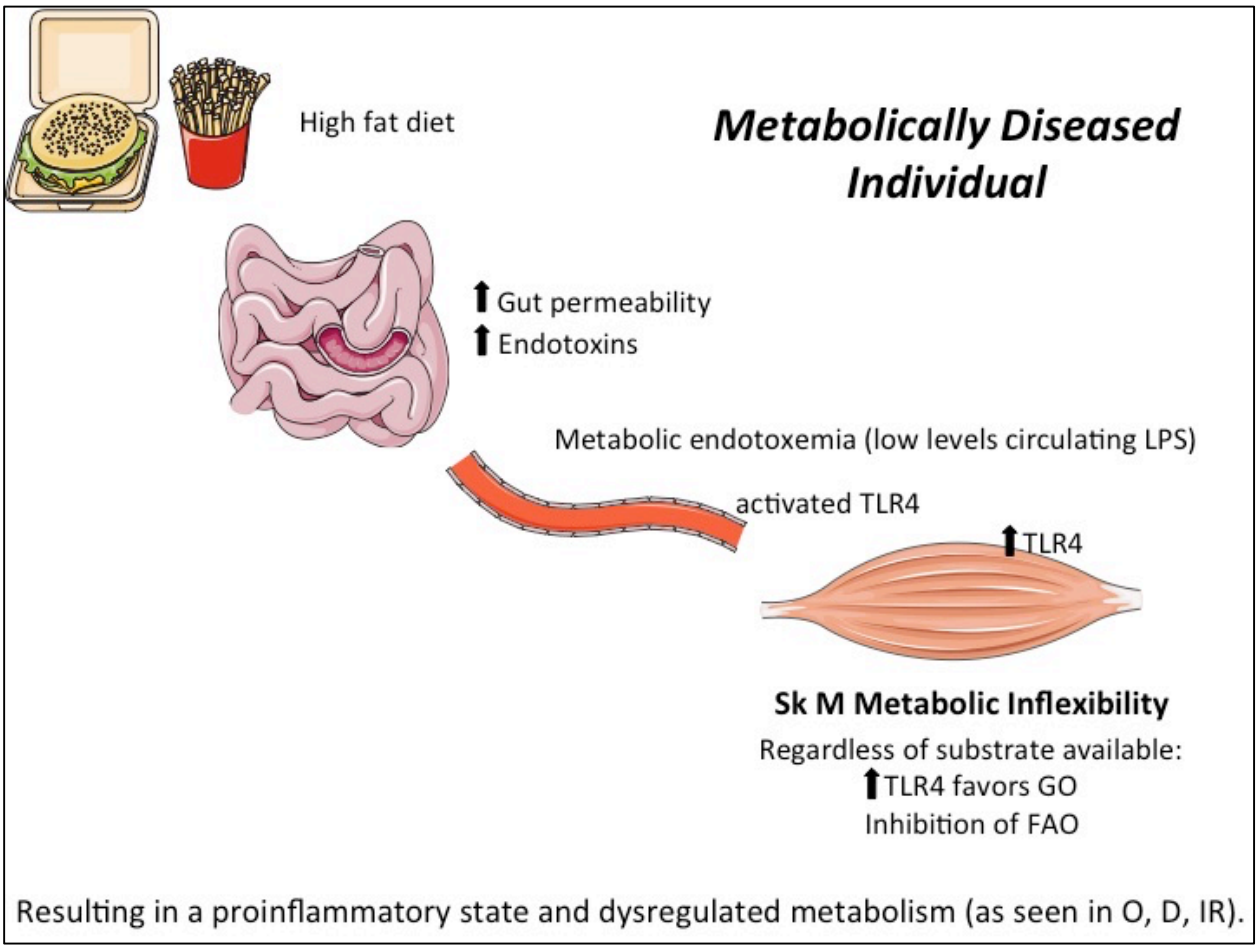


Figure 1, depicts the processes in a metabolically healthy individual. When consuming a well-balanced diet, the gut maintains integrity and proper function of its barrier, releasing little to no endotoxins into circulation. The skeletal muscle responds to substrates available and oxidation of the most predominant macronutrient is upregulated. Skeletal muscle is metabolically flexible, and the processes are highly functioning.

However, in figure 2, which depicts a metabolically diseased individual, these processes are disrupted. A high fat diet disrupts gut barrier function, increasing gut permeability, leading to endotoxins being released into circulation. Low-grade elevation of plasma endotoxins, metabolic endotoxemia, activates toll-like receptor-4 (TLR4), which in

turn causes an increase in TLR4 expression in skeletal muscle. An increased TLR4 presence in skeletal muscle favors glucose oxidation (GO) regardless of the substrate that is available. Likewise, this favoring of GO, inhibits fatty acid oxidation (FAO). These disrupted processes lead to a proinflammatory state and dysregulated metabolism as seen in obesity, Type 2 Diabetes and insulin resistance.

Figure 2. Schematic of Metabolically Diseased Individual



The complexity of substrate oxidation in the presence of different dietary compositions has been connected to metabolic disease states including obesity, T2DM and

metabolic syndrome[3–7]. While protein oxidation remains relatively stable regardless of the composition of the meal, carbohydrate and fat oxidation are shown to fluctuate given different percentages of macronutrients in the diet[8]. The consequences of the alterable oxidation and utilization of these substrates has been a subject of research as the growing epidemic of obesity and T2DM continues to plague people of the world.

Skeletal muscle is not only a primary site of glucose oxidation[9], but also makes substantial contributions to whole body fat oxidation[10]. Habitual as well as acute diet are associated with varying degrees of glucose and fat oxidation within the skeletal muscle. The ability of skeletal muscle to utilize and adapt to available substrates is termed metabolic flexibility[11]. Linked to the variable oxidation rates among different diet compositions, metabolic flexibility (or inflexibility) in the skeletal muscle has been associated with disease states, such as insulin resistance and obesity[12]. What is unknown is if metabolic inflexibility in skeletal muscle precedes disease states or if disease states cause metabolic inflexibility. Further research is needed to further elucidate this question and to understand disruptions in substrate oxidation and metabolic inflexibility when participants are subjected to a high fat diet.

Gut permeability, which is the control of substances passing through the intestinal wall, has been associated with disease states mentioned above. Dietetic factors have been shown to increase gut permeability[13]. Diet has also been linked to an increased presence of endotoxins in the blood[14]. The association of high fat diet and endotoxemia originating from the gut has been a topic of great interest. Further research is needed in order to understand the contributing factors of metabolic endotoxemia.

A variety of factors must be considered when determining substrate metabolism in skeletal muscle and its association to disease states. An additional tool that can prove valuable is categorizing metabolic phenotypes by classifying groups of adapters versus non-adapters; adaptation to which variable depends on the research question to be answered. For example, when analyzing fatty acid oxidation, the adapters are in reference to those who adapted to high fat feeding by increasing fatty acid oxidation, whereas the non-adapters are those who did not. By characterizing, we may be able to potentially identify factors that contribute to the onset and/or progression of metabolic disease in the context of high fat feeding.

CHAPTER 2: LITERATURE REVIEW

INTRODUCTION

Only within the last 60 years has obesity become a widespread issue of public concern. While there are historic artifacts of Stone Age Venus and paintings of Chinese emperors who would be considered obese, and ancient scholars and doctors who tied obesity to health (or lack thereof), the widespread prevalence and resulting epidemic of obesity is fairly recent. According to the most recent (2011-2014) United States National Health and Nutrition Examination Survey (NHANES) data, nearly 40% of Americans are obese (BMI greater than or equal to 30kg/m^2)[1], spanning across socioeconomic classes, age, race, and gender. Annually, the estimated medical costs of obesity are nearly $150 billion[15]. Because of the considerable effect of obesity on chronic disease, an immense amount of research has gone into understanding its impact. Research shows that life expectancy can decrease anywhere from 3 to 14 years for obese individuals, noting that as BMI increases, relative risk of mortality increases[16,17]. Trends show the potential for children born in this generation to have a shorter life expectancy than those of their parents; the first time this effect is realized[18]. Risk of T2DM, cardiovascular disease, cancer, becoming and remaining disabled, and psychological disorders each have a positive correlation with obesity[19–21]. Obesity is a risk factor for 7 of the 10 leading causes of death in the United States[22]. Obesity has not only become medicalized itself, but its close association with other risk factors and chronic diseases make it a significant issue of public concern.

Although earlier research exists on obesity and its relationship to the development of chronic disease, in the 1960's and 70's, there began to be a concentrated effort to define

the causes, risks, mechanisms and anything more that could be a contributor to obesity. Much of the research was focused on determining body weight regulation and its connection to the development of chronic disease. Macronutrients have been a primary focus of this discussion.

An extensive amount of resources have been committed to understanding obesity and chronic disease, but what do we really know about the effects of macronutrient metabolism on health? Research is prevalent, but a concrete understanding and comprehensive knowledge is lacking in many areas of this important issue. There are many schools of thought in the highly debated and controversial topic of the primary dietary factors affecting cardiovascular disease, T2DM and obesity. However, in the 1950s-1960s, there were two main areas of focus, 1) fat was the main dietary influence of coronary heart disease (CHD) or 2) sugar was a more significant contributor to the associated risks of CHD.

Studies examining the role of fat oxidation and balance on metabolism and the regulation of body weight are interspersed in the literature, but due to observed associations between sugar intake and the rise in obesity, the study on CHO load and its effects on obesity has been quite popular. Recommendations from the United States Department of Agriculture, as early as the 1980s, were made to decrease fat consumption, which resulted in an unintended increased refined sugar and CHO consumption[23]. The guidelines, even from 1980, suggest an increase in *complex* carbohydrates, meaning vegetables, fruits and whole grains. However, the food industry's marketing response was the low fat craze, which incidentally increased intake of refined sugar and simple carbohydrates. Body weight, T2DM, and other chronic diseases among Americans continued to rise.

This review is intended to examine what is known about macronutrient metabolism and its effects on health. The Randle cycle and substrate metabolism and its in relation to obesity and chronic disease with a concentration on skeletal muscle will be discussed. More specifically, whole body and skeletal muscle metabolic flexibility, in the context of high fat feeding, will be examined, further exploring fat balance and fat oxidation in skeletal muscle.

BACKGROUND

The glucose fatty acid cycle, or Randle cycle, named for Sir Philip Randle (1963), is foundational to our understanding of macronutrient metabolism and energy homeostasis. In his work, he and his colleagues detailed the mechanisms behind the ability of cardiac and skeletal muscle to shift between carbohydrate (CHO) and fat use and storage, depending on substrate availability. As the theory was conceived, Randle and his group used the long-standing ideas that substrates compete for respiration. For example, early research in the 1930s indicated competition between amino acids and glucose when the deamination of amino acids in kidney tissue was inhibited by oxidizable substrates[24], and in the perfusate of dog heart-lung preparation, the presence of carbohydrates inhibit ketone utilization[25]. Further work in the early 1960s reported inhibition of glucose utilization and oxidation by acetoacetate and palmitate[26,27]. These and other studies led Randle and his group to devise the theory of the glucose fatty acid cycle. The theory included a few key components; the first of those components, simply stated, is that the relationship of glucose and fatty acid metabolism is reciprocal, and not dependent, meaning that elevated glucose concentrations stimulate insulin secretion and suppress fatty acid release from adipose tissue. Secondly, fatty acids and ketone bodies that are released into

circulation in times of disease or starvation inhibit the breakdown of glucose in muscle. Elevated fatty acid concentrations in circulation are usually indicative of low glucose and insulin, thereby becoming the primary fuel source of skeletal muscle, which reduces glucose uptake and oxidation. The purpose of the glucose-fatty acid cycle theory, which is not a "cycle" at all, was to explain the biochemical mechanism of the competition/interaction of glucose and fatty acid oxidation.

Researchers have continually investigated the Randle cycle and its constituents to further understand mechanisms responsible for the development of insulin resistance, T2DM, and obesity, which are clearly associated with altered macronutrient metabolism. In order to obtain a clearer understanding of their mechanisms of action, methods of measuring macronutrients and specific hormones, such as insulin, have been developed, improved and reinvented. Reubin Andres and his group were first to describe the methods of the hyperglycemic and euglycemic clamps and their use for measuring glucose and insulin sensitivity[28]. The use of these methods improved the assessment of 2 variables: beta-cell response to glucose and sensitivity of body tissues to insulin. Previously, ratios of insulin and glucose concentrations were used to calculate these variables, however, the results were often inaccurate given neither value stays constant, and the relationship is not linear. Additionally, the hyperglycemic portion of the method quantifies the time course of the amount of glucose metabolized. The euglycemic portion alleviates the neuroendocrine response of hypoglycemia and the potential hazard of hypoglycemic reactions that the insulin tolerance test induced[28].

Ravussin and Bogardus's work of putting together the methods for the use of the euglycemic clamp and indirect calorimetry was monumental in our further understanding

of the fates of glucose and fatty acids[29,30]. Combining the data for these two testing methods has enabled scientists to not only have a clearer picture of macronutrient metabolism, but also a more dependable measure. Previous estimations were calculated by ratios and other equations and were inconsistent. Additionally, Ravussin's group did early research on use of the human respiratory chamber for determining metabolic rate which enabled them to identify physiological determinants of energy metabolism in humans[31]. Use of the chamber is still a gold standard in measuring metabolic rate. Study of respiratory exchange ratio (RER) which is the ratio of carbon dioxide produced to oxygen consumed, continues to reveal factors other than diet composition that contribute to the fat to CHO oxidation ratio. Factors worth mentioning (that contribute to macronutrient metabolism) are gender[32,33], family membership[33], total energy expenditure[34], muscle fiber type[35], muscle mass[36], training status[4,37], habitual physical activity level[37], lean or obese body composition[32,37,38], and of course, the presence of insulin resistance/T2DM[39–41].

In a review written in 1998, Randle acknowledged new developments on the effects of fatty acid oxidation on glucose metabolism, citing work from a number of scientists over the period of 35 years, recognizing the importance of ongoing research and the complexity of these metabolic processes[42]. One of the main conclusions drawn from this review involved the more extensive role of fatty acids in glucose metabolism. A few examples include fatty acid oxidation's inhibition of glucose catabolism and stimulation of gluconeogenesis, the role of fatty acids in the insulin secretory response of islet beta cells to glucose, fatty acid oxidation impairment of glucose oxidation in disease states such as T2DM, and elevated serum fatty acids inhibiting glycogen synthesis. Many foundational principles are accepted, but researchers are constantly challenging them further in order to

better understand disease states that are affecting people all over the world (obesity, insulin resistance, T2DM).

MACRONUTRIENT METABOLISM

CHO balance is tightly regulated, substantially more than fat balance, due to its limited storage capacity and the body's obligatory use of glucose as a fuel source[3–8]. Protein offers a very small and constant supply of energy, therefore the intake and utilization of CHO and fat are of primary interest when determining macronutrient metabolism.

Protein balance and oxidation

Protein intake, as long as it is adequate, has little to no bearing on protein balance. The healthy body instinctively maintains a protein balance by adjusting amino acid oxidation to amino acid intake. Recent research has shown in an insulin resistant state, increased serum BCAA concentration detrimentally affects mitochondrial function[43,44]. Additional research is needed to further understand the role of BCAAs in insulin resistance. Positive energy balance, a condition often associated with metabolic disease states is related to a disruption in the efficiency of protein degradation and storage[45]. Also, high protein intake shows a reduced energy efficiency[46]. However, in comparison to CHO and fat, the fraction of dietary energy from protein is relatively small. Therefore, regulation of body weight, when a diet has sufficient amounts of protein, is not determinant on protein balance[5,7,8]. Proteolysis is essential, however, during the beginning stages of starvation (24-48 hours). After liver glycogen is depleted, blood glucose homeostasis is maintained through gluconeogenesis. Proteolysis is the primary source of energy until ketone

production, after ~ 48 hours, becomes the main energy source in order to preserve protein[47]. In the postprandial period (1-5 hours), protein balance is affected very little by protein intake.

CHO balance and oxidation

Postprandially, CHO oxidation happens within minutes, and balance within hours. Glucose oxidation in the postprandial state happens at the rate of ~10g/hr[5]. To put that amount into context, a 500-calorie meal that is 50% CHO would contain about 65 grams of carbohydrates. Some of the ingested CHO (glucose) is converted into glycogen, the storage form of glucose, primarily in the skeletal muscle and the liver. The body's glycogen stores are fairly small (approximately 120g in the liver and 200-500g in skeletal muscle)[5,6,48] compared to the daily CHO turnover, so glucose oxidation and storage must be fine-tuned to match intake. In an effort to maintain blood glucose concentrations within a specific range, the hormones insulin, in the event of hyperglycemia, and glucagon, in the event of hypoglycemia are released. These hormones either promote storage of glucose (insulin) or elicit a breakdown of glycogen to glucose (glucagon). These processes are tightly controlled in order to maintain CHO balance, in turn facilitating physiological homeostasis in the context of blood glucose.

Fat balance and oxidation

Fat does not have the direct regulatory interactions in response to diet composition that is found in protein and CHO metabolism. Fat balance can take up to several days, if it balances at all – considering disease states and habitual diet[4,32,49]. The ingestion of fat does not automatically stimulate fatty acid oxidation[49], unlike the presence of CHO stimulating

glucose oxidation. It has been suggested that the correlation of intake to fat balance is more pertinent to the amount of CHO intake rather than fat intake[3–5]. To expand on this idea, some researchers suggest that fat oxidation occurs after CHO oxidation, not only because of the longer time period needed for fat to be digested, but also due to the high priority given to CHO balance. It has also been suggested that when glycogen stores are low and a high fat diet is consumed, the body tends to oxidize fat in order to preserve glycogen[7,8].

Flatt and his group found that fat oxidation did not change when comparing a low fat meal to a meal supplemented with long-chain triglycerides (LCT) or medium-chain triglycerides (MCT)[6]. Respiratory exchange measurements were taken using a ventilated hood system (indirect calorimetry) after participants ate one of the three meals. Carbohydrate, protein, and fat oxidation were calculated using the respiratory quotient (RQ), and no differences in oxidation were found across the meals. While the oxidation was not different, the changes of RQ over time were different, showing that participants' fat balance was negative after being fed a low fat meal, suggesting importance of fat intake to short term energy balance. In addition, the participant's energy balance was essentially equal to their fat balance. This suggests that when determining energy balance, importance must be placed on fat intake, even though fat content in a meal does not influence CHO or fat oxidation.

When blood glucose concentrations rise, insulin secretion is stimulated, which in turn, increases carbohydrate oxidation, and decreases fat oxidation[5]. Glycogen stores are also a determinant of fat oxidation. When glycogen stores are depleted, and the meal is high in fat, postprandially, the body is primed to first utilize the CHO available in the meal, but between meals, due to the low glycogen available, fat oxidation will be increased. This was

observed in human subjects who consumed MCT as part of a mixed meal in comparison to those who consumed LCT or a low fat meal[6]. The ingestion of MCT promoted fat oxidation in the postprandial period, therefore more glycogen was spared, evidenced by the RQ staying higher after the MCT meal compared to after the other two meals. Conversely, if glycogen stores are at maximum capacity, dietary fat is often converted to chylomicrons in the gut and targeted for lipogenesis.

In 1996, Sidossis, et al. found that glucose and/or insulin determines the rate of fat oxidation and termed it the "Randle cycle reversed"[50]. The ratio of fat to CHO oxidation determines the RQ. High RQ indicates more CHO oxidation, and less fat oxidation, whereas low RQ is less CHO oxidation and more fat oxidation. This value ranges from 0.70, which is considered to be primarily fat oxidation, to 1.0, which is considered to be primarily CHO oxidation. Fat and carbohydrate oxidation rates are dependent on a number of variables, as mentioned previously. No matter the composition of the mixed meal, if CHO is present, CHO oxidation will be a part of the postprandial period (1-5 hours post meal) due to the tight regulation of this substrate; however, fat oxidation may not be as actively engaged. Fat oxidation occurs after the amino acid and CHO oxidation rates adjust themselves to the amount consumed in the meal[5,7].

Ingestion of food at levels sufficient enough to maintain glycogen stores may cause fat accumulation in adipose tissue, which can store an enormous amount of fat energy. However, the process of lipolysis is complex. Even though fat storage capacity may be much greater than that of CHO storage (CHO storage is ~5% of fat storage.), fat energy stores may not be as readily available or accessible. Endocrine hormones, such as catecholamines and glucagon in addition to other proteins throughout the gastrointestinal

tract, blood, adipose tissue and skeletal muscle (adipose triglyceride lipase – ATGL, monoacylglycerol lipase – MGL, hormone sensitive lipase – HSL), work together to promote mobilization of fat as an energy source when needed.

The process of lipolysis, briefly, involves catecholamines and/or glucagon signaling the need for energy from triglycerides. Hydrolysis of triglycerides releases fatty acids and glycerol into the circulation to be used as energy. The process involves ATGL, MGL and HSL, lipases that hydrolyze triglycerides, diacylglycerol (DAG) and monoacylglycerol (MAG), into free fatty acids and glycerol. Fatty acids formed from these processes can be oxidized and utilized for energy through beta-oxidation. The amount of activity (or amount of energy needed from lipolysis) is determined by the allosteric or covalent modifications of specific steps in the process. When a sufficient amount of free fatty acids have met the energy demand, insulin increases or catecholamine and glucagon decrease, which inhibits lipolysis. An exception to this is found in states of fasting, starvation or extended exercise, when lipolysis is active.

Oxidation rates are highly variable. Even in a case of energy balance, the oxidation of a substrate for one individual does not necessarily equal the oxidation rate of another, given the same meal or diet. Adaptations in macronutrient metabolism are extensive in different conditions. Differences in fat oxidation are observed during exercise between endurance trained and untrained individuals, regardless of the composition of the pre exercise meal[51]. Further evaluation showed endurance trained individuals have a higher rate of fat oxidation at a higher exercise intensity when compared to untrained individuals, likely due to differences in intramuscular triacylglycerol stores, potentially greater oxidative capacity, and recent research shows increased vasculature in skeletal muscle of

trained individuals[52]. In another study, highly trained individuals had a higher gene expression of specific fatty acid binding proteins, which is observed in conditions of increased fatty acid utilization[53]. A group at the National Institutes of Health used mice deficient in myostatin, and therefore with greater skeletal muscle hypertrophy than wild type mice, to show that animals with more lean mass can oxidize fat at a rate similar to that of CHO[36]. In obese mice, fast/glycolytic muscle fiber type was associated with improvements in fatty acid oxidation[54]. Familial membership and gender was found to be associated with metabolic differences, specifically lower fat utilization, in female Pima Indians[33]. These and other characteristics show individual variance in energy balance and oxidations of substrates.

Because fat oxidation and balance is dependent on so many different variables, improving our understanding of the effects of diet composition, disease states, and the myriad of inter-participant differences remains an important aspect of developing expertise in the metabolic perturbations associated with lipids contributing to disease.

Fat oxidation and fat balance in skeletal muscle

Fatty acid oxidation affects glucose metabolism not only at the whole body level, but also within muscle[50,55–57]. A once highly debated topic, de novo lipogenesis (DNL) or the enzymatic pathway responsible for turning dietary carbohydrates into fat[58], is still under review and is far from understood. Research minimally shows that it is functionally important[58–60]. Additionally, its relation to CHO and fat intake affects metabolic homeostasis[61,62]. DNL occurs primarily in hepatic tissue, especially after a high CHO load when glycogen stores are full and excess CHO is converted to fatty acids and triacylglycerols (TAG)[58]. However, DNL, in the muscle, is a contributing factor to insulin

sensitivity and muscular strength[63] as well as a potential marker for disease[64]. An example of its impact was an investigation done in rodents, which revealed skeletal muscle specific inactivation of fatty acid synthase protected mice from insulin resistance, but induced muscle weakness[63]. Further research is needed to understand de novo lipogenesis and its impact on fat metabolism, specifically.

A common theme in the literature addressing fat oxidation and balance is the altered fat metabolism that occurs in skeletal muscle in the presence of insulin resistance and/or T2DM[12,65–71]. It is well-known that insulin resistant muscle has an impaired ability to oxidize fat during conditions of increased fatty acid supply, such as in times of fasting or exercise[68,72]. Additionally, fat oxidation has been shown to be impaired in the postprandial state in T2D and obesity[68,73]. Research has also shown through study of in vitro myotubes that when extracellular fatty acids are elevated, fatty acid oxidation is also elevated, which in turn suppresses the oxidation of intramyocellular lipids[74]. They also found that the oxidation rate of these lipids were dependent upon mitochondrial function, rather than mass, observed through the staining and live cell imaging of mitochondria. The accumulation of intramuscular triglycerides over time is associated with reduced oxidative capacity[68,75] and development of insulin resistance[76]. Mechanisms are not clearly defined, but mitochondrial function is a likely contributor. Clearly, fat oxidation and balance at the level of the skeletal muscle plays a critical role in health of the whole body.

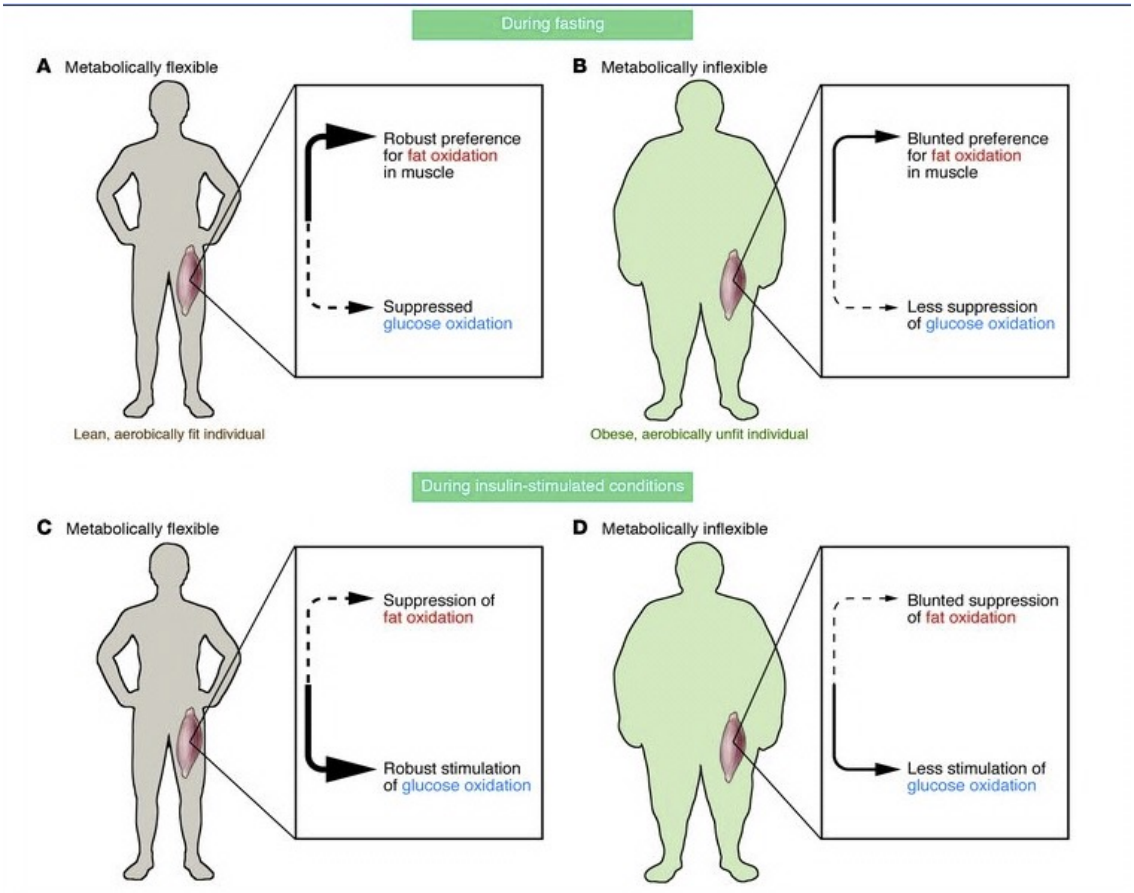
Further investigation of fat oxidation and balance, especially in skeletal muscle, will lead to more answers about what may be happening before the onset of obesity, insulin resistance, or T2DM. In fact, in 2008, Galgani, Moro, and Ravussin recognized the lack of studies investigating skeletal muscle response to high fat diets[77]. Recently, Saponaro, et al.

concluded their review focused on lipolysis and lipogenesis, with a call to identify early biomarkers of cardio-metabolic disease[62]. In the author's view, analysis of the mechanisms behind change in fat oxidation (and likewise metabolic flexibility) in high fat feeding studies may offer further understanding in those areas.

METABOLIC FLEXIBILITY

The ability of the body to utilize and adapt to the fuel sources available is metabolic flexibility, a term introduced by Kelley and Mandarino[11]. When exploring the capacity of whole body or skeletal muscle to switch between substrates, CHO and fat oxidation and uptake are analyzed. In lean, healthy models (animal and human), glucose uptake and oxidation is the primary source of energy until the fasted state, at which time fatty acid oxidation ramps up in order to preserve glucose. A dysfunction in these processes is termed metabolic inflexibility, occurring when either substrate is inefficiently oxidized while it's the primary fuel source, as seen in Figure 3. The complexity of this inflexibility is seen in metabolic disease states[12].

Figure 3: Metabolic Flexibility from Kelley, J Clin Invest. 2005;115(7):1934-1931.



Metabolic flexibility has primarily been measured and analyzed at the whole body level. Well-known, established approaches of measuring metabolic flexibility at the whole body level include different types of methods of indirect calorimetry and rarely used direct calorimetry. Using a hood system or metabolic cart, indirect calorimetry measures the amount of heat by gathering the oxygen consumption to carbon dioxide production ratio and calculating the RQ during a certain time period. The excretion of CO_2 is used to determine the dominant fuel that was utilized during the set time period. Metabolic flexibility is typically evaluated by the change in RQ (insulin-stimulated RQ – fasting RQ). Using O_2 and CO_2, indirect calorimetry can also be used to measure RQ in a metabolic chamber and across the arterial and venous blood across extremities. On rare occasion, direct calorimetry gathers the same information, oxygen and carbon dioxide, but uses heat

production from the individual to determine substrate utilization in a metabolic chamber. Additionally, the hyperglycemic, euglycemic, and hyperinsulinemic clamp methods have been used to quantify the change in RQ in response to infusions of glucose and insulin.

Substrate utilization can also be measured in skeletal muscle. Specific skeletal muscle analysis is valuable because it is the tissue responsible for the majority of insulin stimulated glucose uptake in the body. Free fatty acid activity in skeletal muscle can be measured by the leg balance technique. Blood sampling is done before and after a substrate is infused to determine the activity of the radiolabeled substrate[78,79]. Glucose and lipid metabolism are then estimated by leg indirect calorimetry; from the blood, RQ can be analyzed. Frequently, this method is accompanied by muscle biopsies, often for purposes of determining pyruvate dehydrogenase and citrate synthase activity. The most widely used method to obtain skeletal muscle is the modified Bergström biopsy method[80]. Muscle is obtained and prepared according to the protocol utilized to determine substrate oxidation. An array of metabolomics can be analyzed in these samples using mass spectrometry[81]. Metabolic flexibility can also be measured using radiolabeled substrates - the ratio of radiolabeled pyruvate oxidation to pyruvate oxidation and palmitate (methods not yet published, Matthew Hulver laboratory, Virginia Tech).

Another recently examined method of determining metabolic flexibility is in peripheral blood mononuclear cells. Recently, Baig et al. showed that obesity related metabolic inflexibility can be seen in mononuclear cells, after a high CHO meal, by measuring post prandial expression of various genes in fatty acid and glucose metabolic pathways[82]. Evidence may not be strong enough to support using gene expression alone, but this group found the evidence compelling when compared to RQ data and suggested

this method as an alternative to skeletal muscle biopsies. However, they did not compare the data to skeletal muscle biopsies to determine if the information is directly translatable or specific to skeletal muscle metabolic flexibility.

Limited research has explored skeletal muscle metabolic flexibility. An increased understanding may further elucidate differences in individuals with and without metabolic diseases. Exploration of the variables that contribute to inflexibility, likewise, can be beneficial. Metabolic flexibility is impaired in disease states[68,77,82,83] and after high CHO or high fat meals[71,84–86]. While some of this research is specific to skeletal muscle, the largest body of research has been done analyzing whole body metabolic flexibility.

Galgani, Moro and Ravussin reviewed metabolic flexibility and insulin resistance and determined that with the research available, impaired metabolic flexibility was not responsible for insulin resistance and impaired intramyocellular lipid[77]. Differences seen in metabolic flexibility during the clamp is a consequence of glucose disposal rate, and when corrected, metabolic flexibility is not impaired[87]. In regards to lipids and metabolic flexibility, they pointed out that much of the research is done using RQ under fasting and resting conditions, which are not ideal because fat oxidation is unlikely to show a defect in those conditions. Due to the variable time for fat balance as discussed previously, the authors add emphasis on the importance of understanding the adaptations in fat oxidation, pointing out that the time to adaptation is relevant to fat gain. Skeletal muscle mitochondrial characteristics, such as size, activity, and number offer a potential reason for the variations in metabolic flexibility to lipids[88].

Research in 2011 by Chomentowski, et al., found that lower mitochondrial content in skeletal muscle of insulin resistant individuals is associated with altered patterns of fuel

oxidation (metabolic inflexibility). They suggested the lower mitochondrial content may be associated with intramyocellular lipid overload and associated mitochondrial adaptations[89]. Likewise, Boushel, et al. found mitochondrial function in T2DM patients is normal but suggested lower mitochondrial content may be the reason for the blunted oxidative phosphorylation and electron transport capacity[90]. In 2013, van de Weijer, et al. concluded from their investigation of T2DM patients, that defects in skeletal muscle mitochondrial function are only reflected in basal substrate handling[85]. These findings suggest mitochondria as a potential target in diseased models, but in order to further understand if mitochondria number, function, size or a combination of these, effects metabolic flexibility, skeletal muscle metabolism must be more thoroughly examined.

The effect of diet on skeletal muscle substrate oxidation and metabolic flexibility in lean, healthy human participants is lacking, at best. Research efforts have been made in a variety of diseased conditions and even healthy skeletal muscle cells. However, controlled feeding examining a healthy population's skeletal muscle response to a meal and an acute diet, to our knowledge, has not been done. This research will broaden our understanding of the effects of diet, specifically a high fat diet, on what? before other complications are seen at the whole body level. Are there changes in flexibility at the skeletal muscle level prior to insulin resistance or body weight change? And if so, are these changes priming the body for metabolic disease? Investigating disruptions in skeletal muscle metabolism in response to a meal, and further, a high fat diet will help us to understand baseline characteristics of disease states.

GUT PERMEABILITY

The effect of gut microbiota on obesity, T2DM and other disease states has been a subject of great interest in the past several years. Diet directly plays a role in gut microbiota, which directly influences metabolism. Several bodies of research have investigated diet and its role in the health of the microbiota, but fewer have extended that research to include its influence on metabolic perturbations.

How does metabolism relate to gut microbiota? First, we must understand the role endotoxins play. Endotoxins, complex lipopolysaccharides (LPS), are potentially toxic compounds caused by gram-negative bacteria in the gut. When these endotoxins are found in higher levels than normal in the blood, causing endotoxemia, a malfunction in the gut is evident. This malfunction is due to gut permeability being compromised by lifestyle factors (or other trauma unrelated to lifestyle) such as diet and exercise. Gut permeability is defined as a functional feature of the intestinal barrier. The interaction and proper function of the external, physical barrier and the inner, functional barrier of the intestinal wall enables equilibrium to be maintained. Disruptions in this equilibrium and consequently, its dysfunction, leads to a loss of intestinal function, homeostasis, and can lead to disease[91,92]. When the gut is unhealthy, including the physical barriers, features and active cultures that dwell there or any components of these, the control of substances passing through is compromised, leading to toxicity in the blood and inflammatory response from other organs[91].

Metabolic endotoxemia, as described by Cani, et al, is a two to three times chronic increase in plasma LPS concentration, a systemic low-level elevation. This elevation is said to contribute to the low-grade inflammation seen in obesity and cardio-metabolic disease from obesity[14]. Additionally, metabolic endotoxemia has been tied to disrupted substrate

oxidation, leading to decreased metabolic flexibility. It is well-known that dietary factors contribute to weight gain seen in obesity and other metabolic diseases; these investigations add to the body of literature dedicated to the cause of obesity and other metabolic disease suggesting that altered gut microbiota is a contributor to these diseases. The specific mechanisms need further research, but literature supports this thought.

Several studies have associated high fat diets with gut microbiota alteration, gut permeability and metabolic endotoxemia[13,14,93,94]. In one study over a one-month period, researchers found higher endotoxin levels in the western style diet (40% fat, 40% carbohydrates) than in the "prudent" diet (20% fat, 60% carbohydrate), concluding that a higher fat diet may contribute to endotoxemia[93]. A high fat diet has also been shown to induce changes in the gut microbiota, and the ratio of gram-negative and gram-positive bacteria, therefore causing a detrimental increase in gut permeability[13]. Through a series of mouse and human studies on metabolic endotoxemia, another group found evidence that plasma LPS concentrations may trigger high-fat diet induced metabolic diseases[14].

The role that the detrimental effects of increased gut permeability have on metabolism needs further research, but the indications for unintentional metabolic consequences of an unhealthy gut are far-reaching. Further research is needed, especially in humans exposed to varying dietary compositions, to more clearly understand not only the influence of the gut microbiota on metabolism, but also the inter-relationship of the diet and plasma endotoxin levels.

CONCLUSION

Further investigation about how substrate oxidation in skeletal muscle is affected by a high fat feeding, and further, how it is affected by a short term high fat diet will improve our limited understanding of its effects on metabolic heath. Adding gut permeability research to the body of literature in the context of a high fat feeding may also prove beneficial to understanding the role of the gut-endotoxin-metabolic disease relationship. Combining these variables and using a healthy, non-obese human model may improve our understanding of when metabolic inflexibility can be detected – prior to diagnosed disease or as a result of disease states. Lastly, phenotyping individuals depending on their response to specific variables may inform researchers and health professionals of characteristics that precede, prime the body for, or influence progression of disease states. This will add to the body of literature by advancing our knowledge of skeletal muscle metabolism and gut permeability and their influence on disease states.

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CHAPTER 3: SPECIFIC AIMS

SPECIFIC AIM 1: Test the hypothesis that acute high fat feeding disrupts metabolic adaptation in skeletal muscle of healthy, non-obese, sedentary humans.

Preliminary evidence shows a disruption in the adaptive response in skeletal muscle to a meal at the level of transcription and substrate metabolism. Studies are proposed using whole muscle homogenates and isolated mitochondria to assess substrate handling, metabolic flexibility, and bioenergetics.

Hypothesis: Substrate oxidation will be suppressed in response to the high fat meal challenge after the high fat diet.

Objective: Determination of fasting and postprandial metabolic adaptation in skeletal muscle in response to a high fat meal challenge before and after a high fat diet.

SPECIFIC AIM 2: Test the hypothesis that acute high fat feeding results in increased gut permeability and blood endotoxin levels in healthy, non-obese, sedentary humans.

Preliminary evidence shows a significant increase in fasting blood endotoxin levels after 5 days of high fat feeding in healthy humans. As increased gut permeability is a likely mechanism for blood endotoxin, studies are proposed to assess intestinal and colonic permeability. Serum endotoxin will be assessed under fasting and fed conditions.

Hypothesis: Gut permeability and serum endotoxin will be increased in response to five days of high fat feeding. These changes will be closely related to skeletal muscle pro-inflammatory signaling and decreased metabolic adaptability.

Objective 1: Determination of change in gut permeability before and after a high fat diet.

Objective 2: Determination of blood endotoxin levels at fasting and during the postprandial response before and after the high fat diet.

CHAPTER 4: RESEARCH DESIGN

The design of the study will be a controlled feeding where the participants will serve as their own controls. We will recruit 24 young males who are healthy but sedentary. Our exclusion criteria will include a BMI greater than 25, family history of T2DM, any known cardiovascular condition, smokers, moderate to heavy drinkers and those with a high fat habitual diet (determined by dietary food records). Each morning the participants will report to the metabolic kitchen where they will weigh in, have breakfast and take the remainder of their meals for the day. All meals will be prepared in the metabolic kitchen and daily measurements will be kept to ensure weight maintenance as well as adherence to the diet(s). The participants will undergo a two-week lead-in period where they will consume a normal, healthy control diet. Energy needs will be calculated for each individual using the Institute of Medicine estimated energy requirements equation. After this lead-in period, the participants will come to the lab fasted for a pre HFD muscle biopsy/ meal challenge day. The Bergström biopsy method will be used to obtain muscle from the vastus lateralis. After the first biopsy, they will be fed the meal and four hours later, the second biopsy will be obtained from the opposite leg. The participants will then be placed on the five-day high fat diet, which will be isocaloric to the control diet – remaining in energy balance throughout the entire study. After the five days of high fat feeding, they will repeat the biopsy/meal challenge day.

SPECIFIC AIM 1: Test the hypothesis that acute high fat feeding disrupts the metabolic adaptation in skeletal muscle of healthy, non-obese, sedentary humans.

Objective: Determination of metabolic adaptation in skeletal muscle in response to a high fat meal challenge before and after a high fat diet.

Experimental Strategy:

Skeletal muscle substrate metabolism will be assessed through the analysis of glucose, fatty acid and pyruvate oxidation in whole muscle homogenates that will be prepared immediately after sample collection. Additional measures of the enzyme kinetics of citrate synthase, malate dehydrogenase, and beta hydroxyl acyl-CoA will be performed to further understand influence of TCA cycle, beta oxidation and electron transport chain in the adaptations of substrate oxidation. Transcription of proteins important to metabolic regulation will be assessed using qRT-PCR and western blotting. These measures will be performed in isolated mRNA and/or protein extracted from samples that were flash frozen at time of collection.

SPECIFIC AIM 2: Test the hypothesis that acute high fat feeding results in increased gut permeability and blood endotoxin levels in healthy, non-obese, sedentary humans.

Objective 1: Determination of change in gut permeability before and after a high fat diet.

Experimental Strategy:

The four-sugar probe urine test will be performed to assess changes in gut permeability. This urine will be collected employed before and after the high fat feeding in order to determine differences.

Objective 2: Determination of blood endotoxin levels at fasting and during the postprandial response before and after the high fat diet.

Experimental Strategy:

Blood will be sampled during the fasted state and throughout the postprandial period to detect the change in circulating endotoxin concentrations.

CHAPTER 5: SKELETAL MUSCLE METABOLIC ADAPTATIONS IN REPONSE TO AN ACUTE HIGH FAT DIET

ABSTRACT

The ability of skeletal muscle to adapt and respond to various nutrient states is critical to maintaining healthy metabolic function. Habitual high fat intake has been associated with reduced oxidative capacity, insulin resistance, increased gut permeability, inflammation, and other risk factors often preceding metabolic disease states. To date, limited research has investigated the role of high fat diet on skeletal muscle substrate oxidation and its relationship to gut permeability and endotoxins. The purposes of this study were to determine the effects of an acute, five-day, isocaloric high fat diet (HFD) on skeletal muscle postprandial substrate metabolism in healthy non-obese, humans and to determine the relationship between metabolic adaptations, gut permeability and circulating endotoxin. Thirteen college age males were fed a control diet for two weeks, followed by five days of an isocaloric HFD. To assess the effects of a HFD on skeletal muscle metabolic adaptability and postprandial endotoxin levels, subjects underwent a high fat meal challenge before and after a HFD. After an overnight fast, muscle biopsies were obtained prior to and four hours following the meal and blood was collected prior to and every hour through four hours following the same meal. Insulin sensitivity was assessed prior to and following the HFD via intravenous glucose tolerance test. Intestinal permeability was assessed in the same manner via sugar probe test. Postprandial glucose oxidation and fatty acid oxidation in skeletal muscle increased before the HFD intervention but was decreased after. Skeletal muscle metabolic flexibility was significantly blunted following the HFD. Insulin sensitivity and intestinal permeability were not affected by HFD, but fasting endotoxin was significantly higher following the HFD. These findings demonstrate that in young, healthy

males, following five days of an isocaloric high fat diet, skeletal muscle metabolic adaptation is robust and increased fasting endotoxin independent of gut permeability changes are potentially a contributor to the inflammatory state that disrupts substrate oxidation. These findings suggest that even short-term changes in dietary fat consumption have profound effects on skeletal muscle substrate metabolism and fasting endotoxin levels, independent of positive energy balance and whole-body insulin sensitivity.

INTRODUCTION

Metabolism, the general term for the biochemical processes that contribute to the conversion of food to energy, is widely studied due to the worldwide epidemic of obesity, the consistent rise in Type 2 Diabetes and the widespread complications of cardiovascular diseases. Some conditions associated with these diseases, such as chronic inflammation, metabolic inflexibility, insulin resistance, high body mass index (BMI), and poor lifestyle behaviors, including diet and physical activity are of great interest due to their direct correlation with metabolic processes.

While overall health is multi-factorial, a number of characteristics of metabolic health and likewise, metabolic disease, have been elucidated. Metabolically healthy individuals, and those who consume a well-balanced diet have a highly functioning gut barrier. In turn, the circulating blood is without endotoxins. Skeletal muscle is metabolically flexible, oxidizing the most prominent circulating substrate, most often either fatty acids or glucose. Metabolic disease states are uncommon under these circumstances.

However, as a result of high fat diet (HFD), multiple steps important to metabolic regulation are disrupted, often resulting in metabolic disease states, such as obesity, insulin resistance, and diabetes. HFD has been shown to dysregulate not only the processes discussed here, but others throughout the body, such as adipose tissue, gut microbiota, and functions in the liver, to name a few[1–3]. Skeletal muscle and its substrate oxidation/metabolic flexibility and adaptations are of great interest, due to skeletal muscle being the largest insulin sensitive tissue in the body. Therefore, a detailed analysis of major points of dysregulation, including specific skeletal muscle examination is helpful in understanding the mechanisms contributing to metabolic disease states.

As mentioned, a HFD contributes to the detrimental changes that result in metabolic disease. Increased gut permeability caused by a HFD releases a greater number of endotoxins, which circulate in the blood, causing metabolic endotoxemia[4–6]. The effect of this specific state is yet to be fully understood, but its contribution to chronic inflammation and metabolic derangements, have been reviewed and shown to be relevant[7–11]. The disrupted processes seen as a result of HFD lead to a proinflammatory state and dysregulated metabolism seen in obesity, diabetes and insulin resistance. The purpose of the present study was to investigate the metabolic adaptations in skeletal muscle that occur as a result of an acute HFD and to examine the effects of a HFD on gut permeability and blood endotoxins on healthy, non-obese, sedentary human participants.

METHODS

Participants

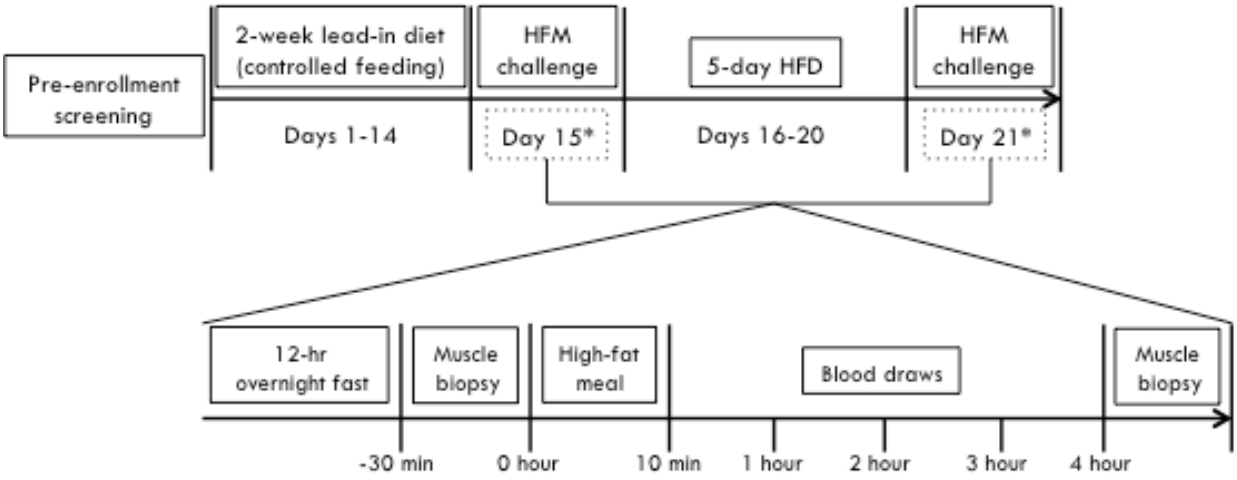
Thirteen healthy, non-obese, sedentary (≤ 2 days, 20 min/day of low-intensity physical activity) males, age 22.2 ± 1.6 years, BMI 22.3 ± 2.8 kg/m^2 served as participants for the study. Inclusion criteria included: weight stable (< ± 2.5 kg) for six months prior to enrollment, non-smokers with no history or family history of cardiometabolic disease, habitual calorie intake composed of < 40% total fat and 15% saturated fat, BMI between 20 and 25 kg/m^2, not taking medications known to affect study measures, blood pressure < 140/90 mmHg, fasting glucose < 100 mg/dL, LDL cholesterol < 130 mg/dL, total cholesterol < 200 mg/dL, and triglycerides < 250 mg/dL. The Virginia Polytechnic Institute and State University Institutional Review Board approved all study procedures. Participants were informed of all procedures, benefits and any potential risks associated with the study before written consent was obtained.

Experimental design

Following successful completion of screening procedures, participants began a two-week lead-in controlled feeding period (control diet). The prepared meals consisted of 55% CHO, 30% fat, and 15% protein. Following the control diet, participants consumed a five-day high-fat diet (HFD), isocaloric to the lead-in diet, consisting of 50% fat (45% of which was saturated fat), 35% CHO, and 15% protein. An acute HFD was employed in order to eliminate confounding factors that are often seen with longer exposure to HFDs, such as increased insulin resistance, body weight, and increased blood glucose, among others. Participants completed a high-fat meal (HFM) challenge [820 kcal (~30% kcal/d), 52g CHO

(25%), 24g protein (12%), 58g fat (63%, ~26% saturated fat)], before and after the 5-day HFD. After an overnight fast, muscle biopsies were taken immediately prior to, and four hours after the HFM for assessment of skeletal muscle metabolic response and adaptation (see Figure 1).

Figure 1: Schematic of research design



Controlled Feeding Procedures

Four-day food intake records were used to confirm that habitual diets contained less than 40% of total calories from fat. After being trained on proper reporting techniques (using food models and measurement devices) by a research dietitian, participants recorded food intake for three weekdays and one weekend day. The research dietitian using the three-pass method reviewed habitual diet records with the participant[12]. The food intake was analyzed using Nutrition Data System for Research (NDS-R) software version 2012 (University of Minnesota) by a trained diet technician. In order to estimate appropriate energy requirements for each participant, the Institute of Medicine equation was used based on height, weight, age, and activity level[13]. Both the control diet and HFD

were administered on a seven-day cycle of menus consisting of meals and snacks with two optional snack modules (± 250 kcals). Diets were planned by a registered dietitian using NDS-R software. The two-week lead-in controlled feeding and five-day HFD period required participants to consume planned meals. Diets aimed to provide 3 g of fiber per 500 kcal (± 5 g). All meals were prepared in the Dining Laboratory for Eating Behavior and Weight Management. Participants ate breakfast in the laboratory every day and carried out a cooler containing the remaining food for the day. Participants weighed in each day at the lab prior to breakfast to ensure they remained weight stable. A trend of > 1.0 kg weight loss or gain was offset by adding or subtracting 250 kcal food modules with the same macronutrient composition as the overall diet. All uneaten items and unwashed containers were returned to the metabolic kitchen where trained research staff monitored compliance. Participants were not permitted to consume any additional food, caffeine or alcohol for the duration of the study. They were also instructed to report consumption of all non-study foods.

High Fat Meal Challenge

The purpose of a HFM challenge that was performed before and after the diet was to study the fasted to fed transition period as well as post prandial response to the diet. Participants arrived at the laboratory following a 12-hour overnight fast. Upon arrival, they were interviewed to ensure protocol compliance after which their first biopsy was taken from the vastus lateralis muscle. Biopsies were taken before and four hours after a HFM. Participants were required to consume the HFM within ten minutes. Following the initial biopsy, participants were fitted with an intravenous catheter in the antecubital vein for

baseline and hourly blood sampling. Participants remained seated and awake for the duration of the meal challenge; movies, reading, and homework were the activities that were permitted. Pre- and post biopsies were taken from separate legs.

Measurements and Procedures

Body mass and composition

Body weight was measured to the nearest ± 0.1 kg on a digital scale (Model 5002, Scale-Tronix, White Plains, NY). Height was measured to the nearest ± 0.1 cm using a stadiometer (Model 5002, Scale-Tronix, White Plains, NY). Body composition (total fat and fat-free mass) was analyzed by dual-energy x-ray absorptiometry (General Electric, Lunar Digital Prodigy Advance, software version 8.10e Madison, WI).

Intravenous-glucose-tolerance test

An insulin-augmented frequently sampled intravenous-glucose-tolerance test (IVGTT) was used to assess whole-body insulin sensitivity, which was administered to subjects at baseline and after the intervention post 12h overnight fast[14]. The test was performed while the subjects were in a seated position, after a 30-min relaxation period. An intravenous catheter was placed in each antecubital vein, one for the administration of insulin and glucose and one for collecting blood samples. Blood samples for the measurement of baseline insulin and glucose concentrations was obtained ten minutes and then again five minutes before the infusion of a bolus of glucose (0.3 g/kg in a 50% dextrose solution infused over 90 s). Twenty minutes after the glucose infusion, a bolus of

insulin (0.03 U/kg) was infused. Blood samples were obtained 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180 min after the initial glucose infusion. They were then centrifuged at 4 °C for 20 min at 2500 × g and analyzed for glucose concentrations with the glucose oxidase method by using a glucose autoanalyzer (Yellow Springs Instruments, Yellow Springs, OH). A sample of serum was stored at −20 °C for later measurement of insulin concentrations by the immunoassay analyzer, Immulite 1000 (Siemens Corporation, Washington, D.C.). Insulin and glucose values from the IVGTT were entered into the MINMOD Millennial Software program (version 3.0; R. Bergman, University of Southern California) for determination of insulin sensitivity (S_I), acute insulin response to glucose (AIR_G), and glucose effectiveness (S_G). This model used measurements of plasma glucose and insulin concentrations over a 3-h period to derive in vivo whole-body S_I.

Intestinal permeability, clinical procedure

Four sugar probes were employed to assess gut permeability[15]. Sucrose is rapidly degraded by epithelial sucrose-isomaltase activity upon entering the duodenum, and is an ideal probe of gastro-duodenal permeability only[15]. Lactulose and mannitol are metabolized by the colonic microflora and are suitable as probes of small intestinal permeability[16]. Sucralose is accumulated in the colon but resists microbial degradation, and is an ideal probe of colonic permeability[17]. Therefore, this probe system was employed to assess permeability in all regions of the gut. For permeability assessment, subjects fasted overnight (12 h) with only water allowed. Subjects evacuated their bladders prior to beginning the test, followed immediately by consumption of USP-grade saccharide probes

40 g sucrose, 1 g mannitol, 1 g sucralose (Spectrum Chemicals, New Brunswick, NJ) and 5 g lactulose (The Coghlan Group, St. Paul, MN) in 250 mL bottled water[18–20]. Subjects then consumed 500 mL water within 30 min to stimulate urine production. Urine was collected in two pooled samples: a 0-5 h sample representative of gastric and small intestinal permeability (collected during the visit), and a 6-24 h sample representative of colonic permeability (collected after the visit)[18,21]. Urine was collected in 24 h collection containers with 5 mL 10% thymol in methanol (w/v) to inhibit bacterial growth.

Intestinal permeability calculations

Urine sugar concentrations were converted to total sugar excreted using urine volume. Excretion was calculated as a % of total sugar dose recovered in urine for 0-5 and 6-24 h samples. The lactulose/mannitol ratio (LMR) was calculated for both 0-5 and 6-24 h samples as the ratio of lactulose excretion to mannitol excretion[22], as mannitol a constant measure of epithelial surface area[15]. Gastro-duodenal permeability was defined as % sucrose excretion as well as sucrose/mannitol ratio (SMR) (0-5 h)[19,23]. Small intestinal permeability was defined as the 0-5 h and 6-24 h LMRs, and colonic permeability was defined as 6-24 h sucralose excretion and sucralose/mannitol ratio (SMR)[17,23]. For extraction and quantification of sugar probe, total urine volume was measured, and aliquots were frozen at −80°C. Urinary sugars were measured as described by Camilleri et al[22]. 50 µL urine was combined with 50 µL internal standard [20 mg/mL $^{13}C_6$-glucose in water/acetonitrile (98:2)], diluted to 4 mL with water and vortexed with 4 mL dichloromethane. Following 30 min incubation and centrifugation (10 min, 3500 x g), 100 µL supernatant was diluted with 900 µL acetonitrile/water (85:15) and analyzed by UPLC-

MS/MS. UPLC separation was performed on a Waters Acquity H-class (Milford, MA) equipped with an Acquity UPLC BEH Amide column (2.1 mm × 50 mm, 1.7 μm particle size). Isocratic elution was performed at 0.7 mL/min using acetonitrile:water (65:35) with 0.2% v/v triethylamine (TEA). Column and sample temperatures were 35 and 10°C, respectively. Detection by MS/MS was performed on a Waters Acquity Triple Quadrupole Detector (TQD). Negative-mode electrospray ionization [(−)-ESI] was performed with capillary voltage of −4 kV, and source and desolvation temperatures of 150 and 450°C, respectively. Desolvation and cone gasses were N_2 at flow rates of 900 and 1 L/hr, respectively. For MS/MS, the collision gas was Ar. The cone voltages, collision energy, and Multiple Reaction Monitoring (MRM) transitions for each compound are listed in Table 1. Peak widths were ~4 s, and AutoDwell was employed with required points-per-peak set at 12. The interscan delay time was 0.02 s. Data acquisition, processing, and quantification was performed using Waters MassLynx v4.1 software.

Table 1. MS/MS transitions for detection of sugar probes

compound	retention time (min)	MW (g mol^{-1})	parent [M–H]$^-$ (m/z)	daughter (m/z)	cone voltage (V)	collison energy (eV)
sucralose	0.24	396.238	395.238	358.9705	42	10
mannitol	0.38	182.1748	181.1748	88.8979	28	14
surose	0.43	342.3319	341.3319	178.959	38	12
lactulose	0.46	342.3319	341.3319	160.934	12	8
$^{13}C_6$-glucose	0.39	186.2596	185.2596	91.8909	18	8

Blood measures

Serum free fatty acid concentrations were determined using the Free fatty acids half-micro test assay (Roche Diagnostics, Penzberg, Germany). Serum triglyceride concentrations were determined using the Triglyceride-GPO reagent set assay (Teco Diagnostics, Anaheim, CA) per the manufacturer's instructions. Serum endotoxin

concentrations were determined using the PyroGene Recombinant Factor C endotoxin detection assay (Lonza, Basel, Switzerland) per the manufacturer's instructions.

Muscle biopsies

Biopsies were taken from the vastus lateralis muscle using a suction-modified Bergström-type needle (Cadence, Staunton, VA) technique[24,25]. An area of skin in the region of the vastus lateralis was shaven and cleansed with a povidine-iodine solution. The skin, adipose tissue and skeletal muscle fascia was anesthetized using 10mL lidocaine (1%). An incision (0.75 cm) was made in the skin with a #10 scalpel, and the fascia fibers were separated with the blunt edge of the scalpel. The Bergström needle (5 mm) was inserted into the vastus lateralis and suction applied. The muscle tissue was pulled into the needle, snipped and extracted. Tissue samples were immediately placed in ice cold PBS to remove blood and connective tissue. Muscle tissue used to assess substrate oxidation was immediately placed in 200uL of SET buffer (0.25 M Sucrose, 1 mM EDTA, 0.01 M Tris-HCl and 2 mM ATP) and stored on ice until homogenization (~25 min). Muscle tissue used to assess mitochondrial function were immediately placed in ice cold buffer 1 for mitochondrial isolation (IBM1) (67 mM sucrose, 50 nM Tris/HCl, 50 mM Kcl, 10 mM EDT/Tris and 0.2% BSA) and stored until isolation (~25 min). Muscle tissue used for western blotting was placed in ice-cold cell lysis buffer (50 mM Tris-HCl, EDTA 1 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5%, igepel Ca 630 1%, pH 7.5) with halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Pittsburgh, PA), then snap-frozen in liquid nitrogen. Samples collected for western blotting were stored at -80ºC for later analysis.

Muscle homogenization

Muscle samples for substrate oxidation (~ 75mg) were collected and minced with scissors followed by the addition of SET Buffer to produce a final 20-fold dilution (wt:vol), as previously described[26]. The samples were then homogenized in a Potter-Elvehjem glass homogenizer (Thomas Scientific, Swedesboro, NJ) at ten passes across 30 seconds at 150 RPM with a motor-driven Teflon pestle.

Substrate Metabolism

As previously described[26], substrate oxidation in vastus lateralis muscle was measured using radio-labeled fatty acid ([1-^{14}C]- palmitic acid) from Perkin Elmer (Waltham, MA), specifically measuring $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites (ASM). Samples were incubated in 0.5 μCi/mL of [1-^{14}C]-palmitic acid for one hour after which the media was acidified with 200 μL 45% perchloric acid for one hour to liberate $^{14}CO_2$. The $^{14}CO_2$ was trapped in a tube containing 1 M NaOH, and the sample was then placed into a scintillation vial with 5 mL scintillation fluid. The vial's ^{14}C concentrations were measured on a 4500 Beckman Coulter scintillation counter (Indianapolis, IN). ASM were determined by collecting the acidified media and measuring ^{14}C levels. Glucose oxidation (GO) and pyruvate oxidation (PO) were measured with methods similar to that of fatty acid oxidation (FAO) with the exception of a substitution of [U-^{14}C]-glucose and [1-^{14}C]-pyruvate for [1-^{14}C]- palmitic acid, respectively. Metabolic flexibility was assessed by measuring [1-^{14}C]-PO in the presence or absence of non-labeled BSA (0.5%) bound-palmitic acid. Metabolic flexibility is denoted by the percentage decrease in PO in the presence of free fatty acid and is expressed as the ratio of CO_2

production with labeled pyruvate over CO_2 production with labeled pyruvate in the presence of palmitate. Oxidative efficiency is denoted by using the ratio of CO_2/ASM, which represents complete and incomplete products of fatty acid oxidation.

Citrate Synthase (CS) activity was assessed by measuring the reduction of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) from the formation of Coenzyme A (CoASH) over time. Briefly, ten microliters of a 1:5 diluted muscle homogenate was added, in duplicate, to 170µl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a two-minute background reading, the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) was calibrated and 30µl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured at 405nm at 37C every 12 seconds for seven minutes. Maximum CS activity was calculated and reported as µmol/min/mg.

Malate Dehydrogense (MDH) activity was measured spectrophotometrically at 340nm at 37°C. Briefly, ten microliters of sample was pipetted in triplicate in wells. Then, 290ul of reaction media (0.1 M potassium phosphate buffer, pH 7.4 plus 0.006 M oxaloacetic acid, prepared in potassium phosphate buffer plus 0.00375 M NADH, prepared in potassium phosphate buffer) was added to the wells and samples were read for five minutes at 340nm. The rate of disappearance of NADH was analyzed and expressed relative to protein content. Data is expressed as means ± SEM.

For the determination of beta-hydroxyacylcoA dehydrogenase (BHAD), oxidation of NADH to NAD was measured. In triplicate, 35 µl of whole muscle homogenate was added to 190µl of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular

Devices Corporation, Sunnyvale California) was calibrated and 15µl of 2mM acetoacetyl CoA added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for six minutes at 37C. Maximum BHAD activity was calculated and reported as µmol/min/mg.

Western blot analysis

Frozen muscle tissue samples were homogenized in ice-cold lysis buffer in a Bullet Blender Homogenizer (Next Advance, NY) using 1.0mm Zirconium Oxide beads (Next Advance). Samples were centrifuged at 14,000 *g* for 15 min at 4°C to remove insoluble components. Supernatant protein concentrations were determined spectrophotometrically using the bicinchoninic acid assay (BCA) (Thermo Scientific). Lysis buffer was added to samples for adjustment to equal concentrations and combined with equal volumes 2 x Laemelli buffer and heated for five minutes at 95°C. Equal amounts of protein were separated on poured SDS-PAGE gels (TGX Fast Cast Acrylamide Solutions Kit, Bio-Rad, Hercules, CA), which were activated via ultra violet light exposure (ChemiDoc Touch Imaging System, Bio-Rad) prior to transfer. Proteins were transferred to PVDF membranes using a Trans-Blot Turbo Transfer System (Bio-Rad), which were then imaged (Bio-Rad) for quantification of total lane protein. PVDF membranes were blocked for one hour at room temperature in 5% non-fat dry milk or 5% bovine serum albumin prior to overnight incubation at 4°C with primary antibodies. Membranes were probed with primary antibodies against pyruvate dehydrogenase phosphate (PDPc; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), pyruvate dehydrogenase kinase 4 (PDK4; 1:500; Santa Cruz), p38 MAP kinase (1:1,000; Cell Signaling Technology, Danvers, MA), phosphorylated

p38 MAP kinase (1:1,000; Cell Signaling). Following primary antibody incubation, membranes were incubated for one hour at room temperature with HRP-conjugated anti-rabbit, anti-mouse (1:10,000; Jackson Immuno Research Laboratories, West Grove, PA), or anti-goat (1:2,000; Santa Cruz) secondary antibodies. Proteins were visualized via chemiluminescence (Clarity Western ECL Substrate, Bio-Rad, or SuperSignal West Femto, Thermo Scientific), quantified using Image Lab Software (v5.2.1, BioRad) and normalized to total lane protein content. Molecular weight was determined by Precision Plus Protein Unstained Standards (Bio-Rad).

Statistics

Two-way repeated measures analysis of variance was used to determine differences in meal responses pre and post-HFD. Multiple comparisons were performed using a Tukey post-hoc analysis. Independent t-tests were used to compare percent change in protein levels between pre and post-meal time points, before and after a HFD. Correlations were examined via multivariate analysis. Data that did not follow a normal distribution was logged base 10, or square root transformed. All data is expressed as means ± standard error of the mean (SEM). The significance level is set *a priori* at $\alpha = .05$.

RESULTS

Participant characteristics

Participant characteristics are shown in Table 2. Thirteen participants completed the study. There were no differences in weight or BMI after the HFD when compared to baseline ($p > 0.05$). This analysis included lean body mass, fat mass and body fat percentage, none of which were different pre to post HFD.

Table 2: Participant characteristics

Variable (n=13)	Pre HFD	Post HFD
Age (yrs)	22.2 ± 0.4	--
Height (m)	1.77 ± 0.02	--
Weight (kg)	72.09 ± 3.2	71.98 ± 2.9
BMI (kg/m^2)	23.1 ± 0.9	23.0 ± 0.8
Body Fat Mass (kg)	16.57 ± 2.1	16.28 ± 2.0
Body Fat (%)	22.03 ± 1.7	21.44 ± 1.7
Lean Mass (kg)	54.15 ± 1.7	54.51 ± 1.9

All data are expressed as mean ± SEM.

Diet

The mean energy and macronutrient content of the HFM challenge and each diet is presented in Table 3. Manipulation of the carbohydrate and fat content was the differing factor in the two diets (Table 3). The HFM challenge was ~ 30% of daily energy intake at 820 kcals/meal.

Table 3: Diet mean energy and macronutrient content

Diet Condition	Energy (kcal/day)	Protein (%)	CHO (%)	Fat (%)	SFA (%kcal)
Habitual	2318 ± 104	16.9	44.3	35.9	13.1
2-wk lead-in (control)	2768 ± 66	15.2	53.9	30.9	9.4
High Fat	2735 ± 73	15.3	30.9	53.9	24.5
HF meal challenge	*30%/day 820 kcal/meal*	*12% 24g/meal*	*25% 52g/meal*	*63% 58g/meal*	*26% kcal 24g/meal*

All data are expressed as mean ± SEM.

Whole body measurements

Fasting insulin sensitivity, fasting glucose and fasting insulin did not change in response to the HFD (Table 4, p> 0.05). No differences were found in fasting free fatty acids between pre and post HFD measures as seen in Table 3. Fasting triglycerides and fasting endotoxins were both found to be significantly different after the HFD (Table 4, p<0.001 and p=0.03 respectively). Triglycerides decreased from 75.4 ± 10.2 mg/dL to 47.2 ± 6.0 mg/dL and endotoxins nearly doubled after the HFD from 1.2 ± 0.1 EU/mL to 2.3 ± 0.4 EU/mL. Gut (gastroduodenal, intestinal, colonic) permeability did not change pre to post HFD (Table 4, p>0.05).

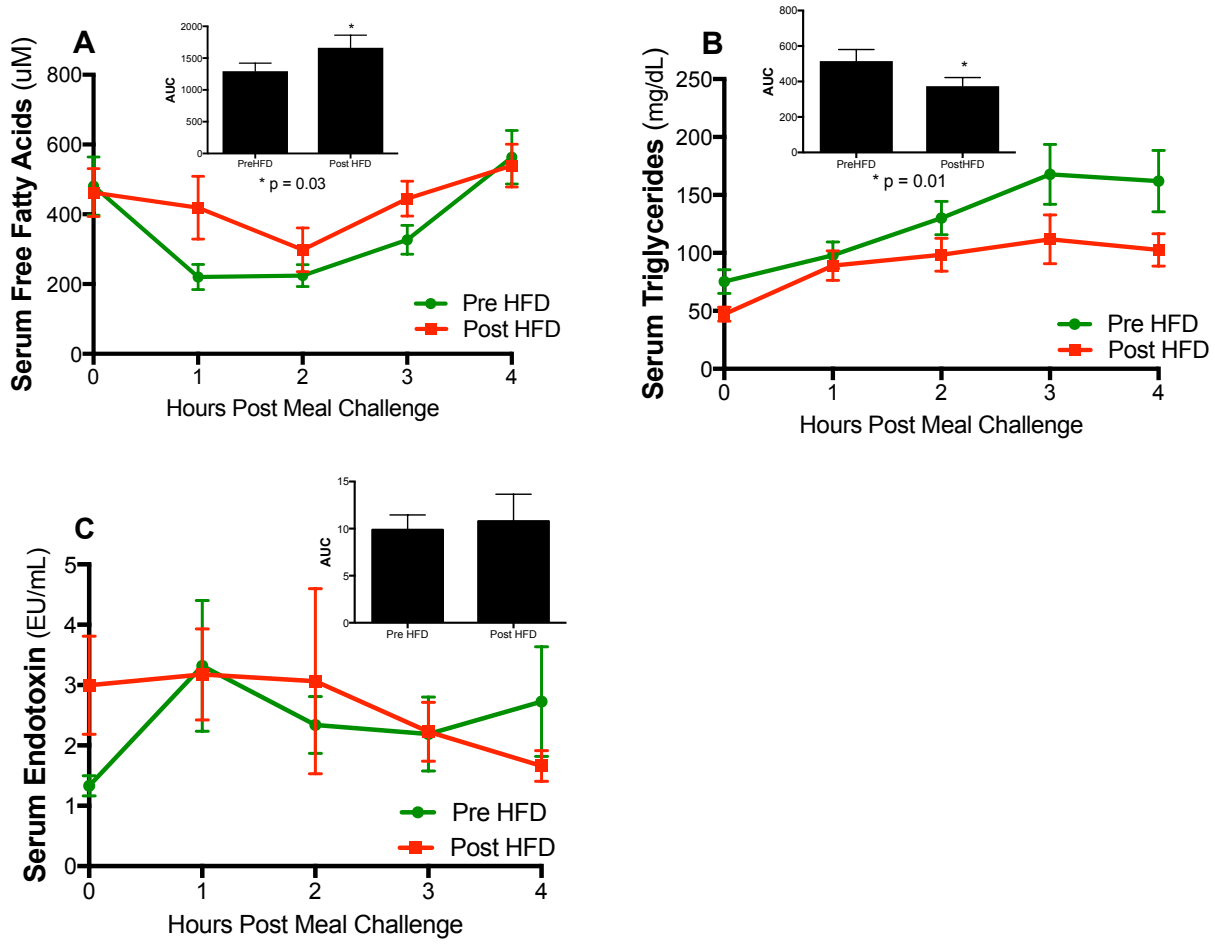
Table 4: Whole body fasting measures

Fasting Measures (n=13)		Pre HFD	Post HFD
S_i ([mU/L]/min)		5.6 ± 0.7	4.78 ± 0.6
Glucose (mmol/L)		82.1 ± 2.7	81.9 ± 2.7
Insulin (uIU/ml)		6.3 ± 2.7	6.5 ± 2.5
Free Fatty Acids (uM)		480.9 ± 83.6	462.1 ± 69.1
**** Triglycerides (mg/dL)***		***75.4 ± 10.2***	***47.2 ± 6.0***
# Endotoxin (EU/mL)		***1.2 ± 0.1***	***2.3 ± 0.4***
Gastroduodenal Permeability			
(excretion ratio)	0-5 hrs	0.07 ± 0.01	0.08 ± 0.02
Intestinal Permeability	0-5hrs	0.03 ± 0.01	0.04 ± .01
(excretion ratio)	6-24hrs	0.13 ± 0.02	0.10 ± 0.01
Colonic Permeability	0-5hrs	0.21 ± 0.07	0.28 ± 0.08
(excretion ratio)	6-24hrs	0.55 ± 0.09	0.36 ± 0.08

**** p < 0.001, # p = 0.03; All data are expressed as mean ± SEM.***

Post-HFD serum free fatty acids area under the curve was significantly higher than pre-HFD measures (Figure 2A, p = 0.03). Serum triglycerides were significantly lower after the HFD in response to the meal (Figure 2B, p = 0.01, pre-HFD= 514.7 mg/dL/hr, post-HFD = 374.0 mg/dL/hr). Serum endotoxins showed no significant difference pre to post HFD in response to the meal (Figure 2C, p > 0.05).

Figure 2: Meal challenge blood measures



Substrate metabolism

There was a significant HFD x HFM interaction for skeletal muscle GO (p=0.002), FAO (p=0.01), and metabolic flexibility (p=0.03). After controlled, lead-in feeding conditions, postprandial FAO, GO, and metabolic flexibility increased, but after the HFD, these measures were blunted (Table 5). Percent change in GO (p=0.003), FAO (p=0.04), PO (p=0.09) and metabolic flexibility (p=0.01) is presented in Figure 3.

There was a significant HFD x HFM interaction for CS and MDH activity (p=0.04) as shown in Table 5. Both CS and MDH activity increased postprandially before the HFD, but

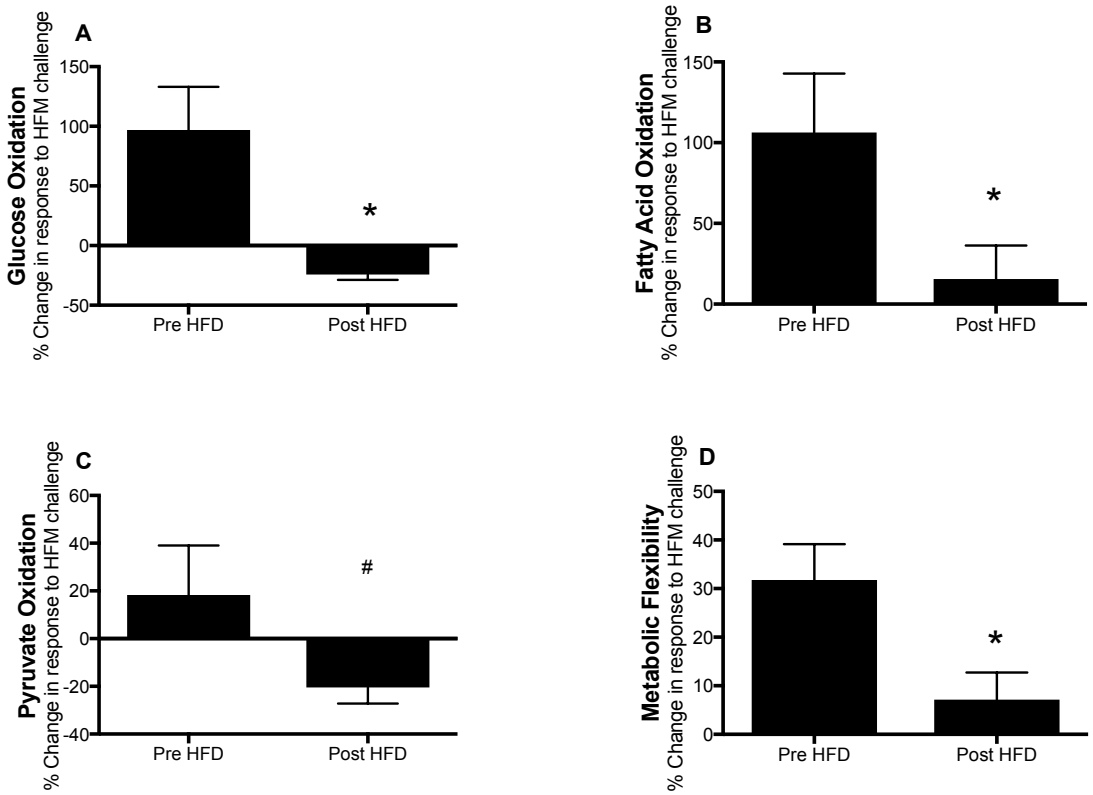
their activity decreased following the HFD. No interaction or difference was found for BHAD.

TABLE 5: Substrate Metabolism

	Pre HFD Fasted	**Pre HFD Fed**	**Post HFD Fasted**	**Post HFD Fed**
*Glucose Oxidation (nmol/mg protein/hr)	4.5 ± 0.7	7.3 ± 1.1	6.2 ± 0.7	4.6 ± 0.5
*Fatty Acid Oxidation (nmol/mg protein/hr)	7.4 ± 1.0	10.3 ± 1.4	10.7 ± 1.1	8.4 ± 1.1
Pyruvate Oxidation (nmol/mg protein/hr)	427.6 ± 33.4	444.1 ± 36.4	386.9 ± 35.5	289.8 ± 21.2
*Metabolic Flexibility (ratio of pyruvate oxidation ± *FFA*)	1.4 ± 0.1	1.8 ± 0.2	1.5 ± 0.1	1.6 ± 0.1
*CS (umol/mg protein/min)	105.6 ± 14.0	143.3 ± 20.2	104.3 ± 12.9	81.7 ± 13.1
*MDH (umol/mg protein/min)	1760.9 ± 144.0	2004.1 ± 89.3	1589.9 ± 154.0	1440.1 ± 93.4
BHAD (umol/mg protein/min)	53.9 ± 6.0	47.8 ± 7.7	52.9 ± 6.7	35.3 ± 4.0

Significant difference found ($p < 0.05$); all data are expressed as mean ± SEM.

Figure 3: Substrate oxidation

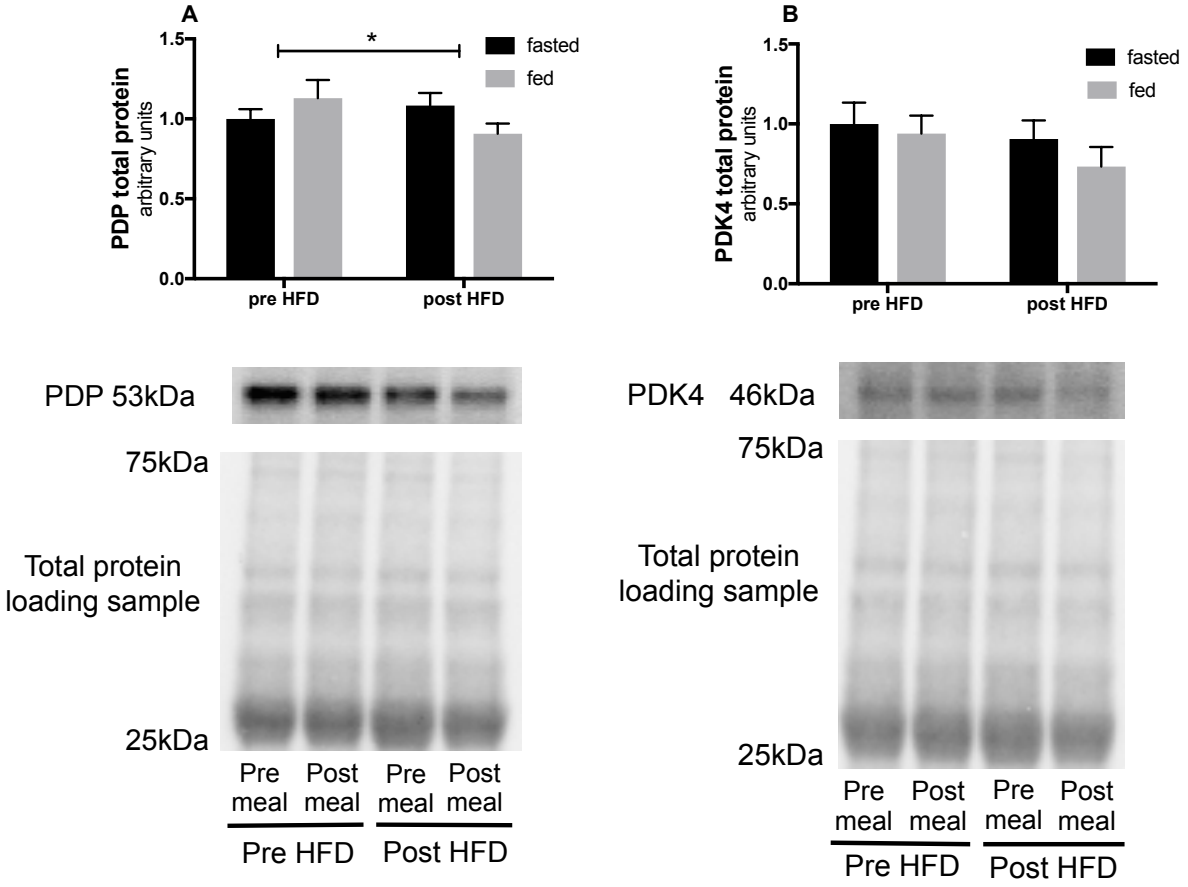


Significant difference found (p<0.05)
 # (p=0.09)

Pyruvate dehydrogenase complex

Increased PDK4 expression suppresses the pyruvate dehydrogenase complex, and conversely, PDP activates, or increases the activity of the complex. As measured by the protein content visualized in western blots, there was a significant HFM x HFD interaction for PDP (Figure 4A, p=0.02). In response to a meal, PDP was blunted after the HFD (p=0.02). In response to the meal, PDK4 shows a slight decreased expression, however it is not significant (Figure 4B, p=0.5).

Figure 4: Pyruvate dehydrogenase complex



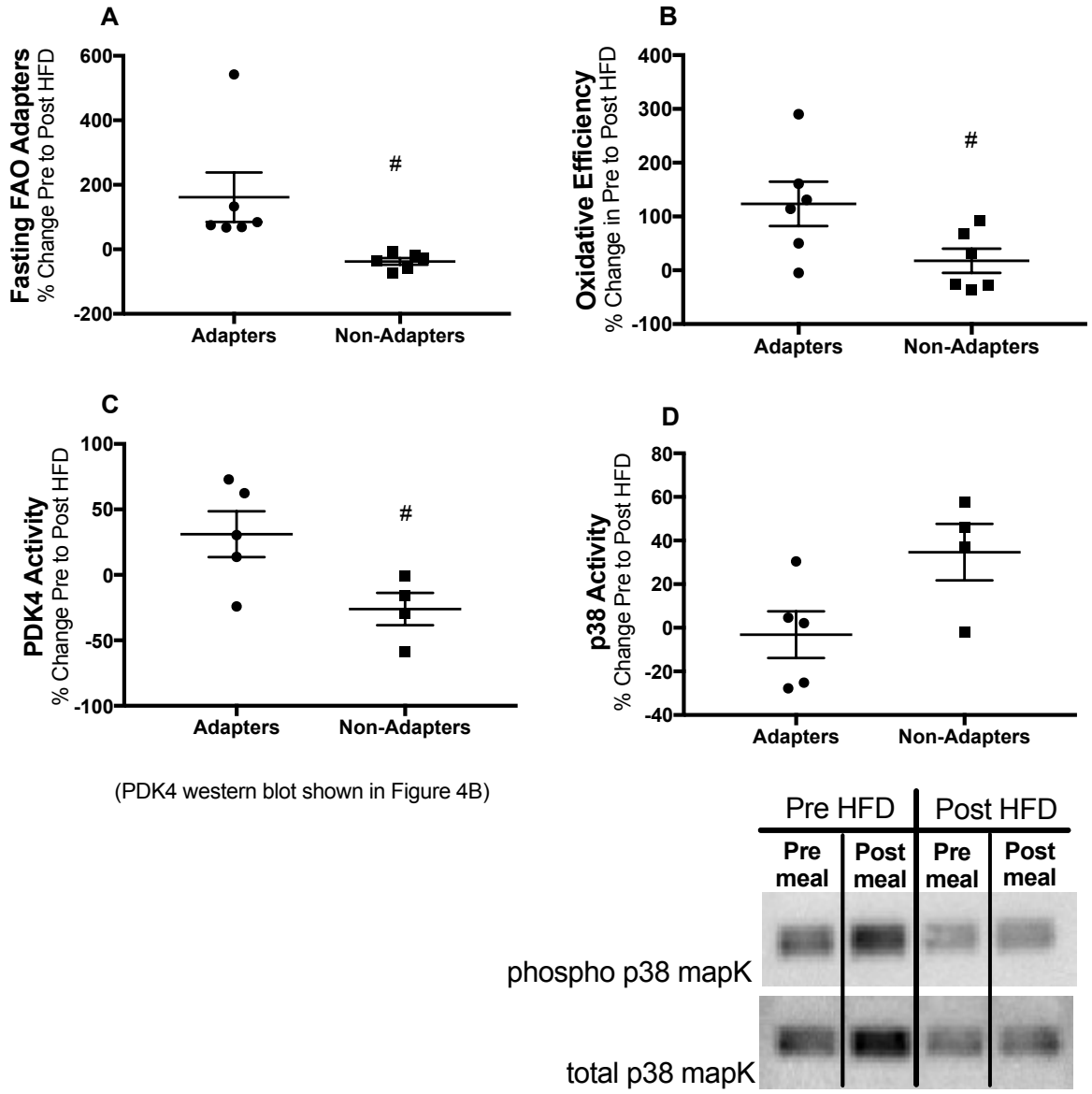
**** Significant difference found (p<0.05).***

Adapters and Non-Adapters in FAO and GO

To better understand contributors to FAO adaptation, a median split of fasting FAO percent change from pre- to post- HFD was examined (Figure 5A, p = 0.03). Those participants' who increased skeletal muscle FAO above the median split, in response to HFD, were classified as adapters and those who fell below the median split were classified as non-adapters. Oxidative efficiency, which is the ratio of complete/incomplete fatty acid oxidation, was significantly higher in adapters following a HFD when compared to non-adapters (Figure 5B, p = 0.05). PDK4 protein content was higher among adapters following a HFD when compared to non-adapters (Figure 5C, p = 0.04), suggesting a greater

inhibition of pyruvate dehydrogenase complex in the adapters. P38 activity trended higher among non-adapters, although significance was not reached (Figure 5D, p = 0.06).

Figure 5: Fatty Acid Oxidation Adaptation

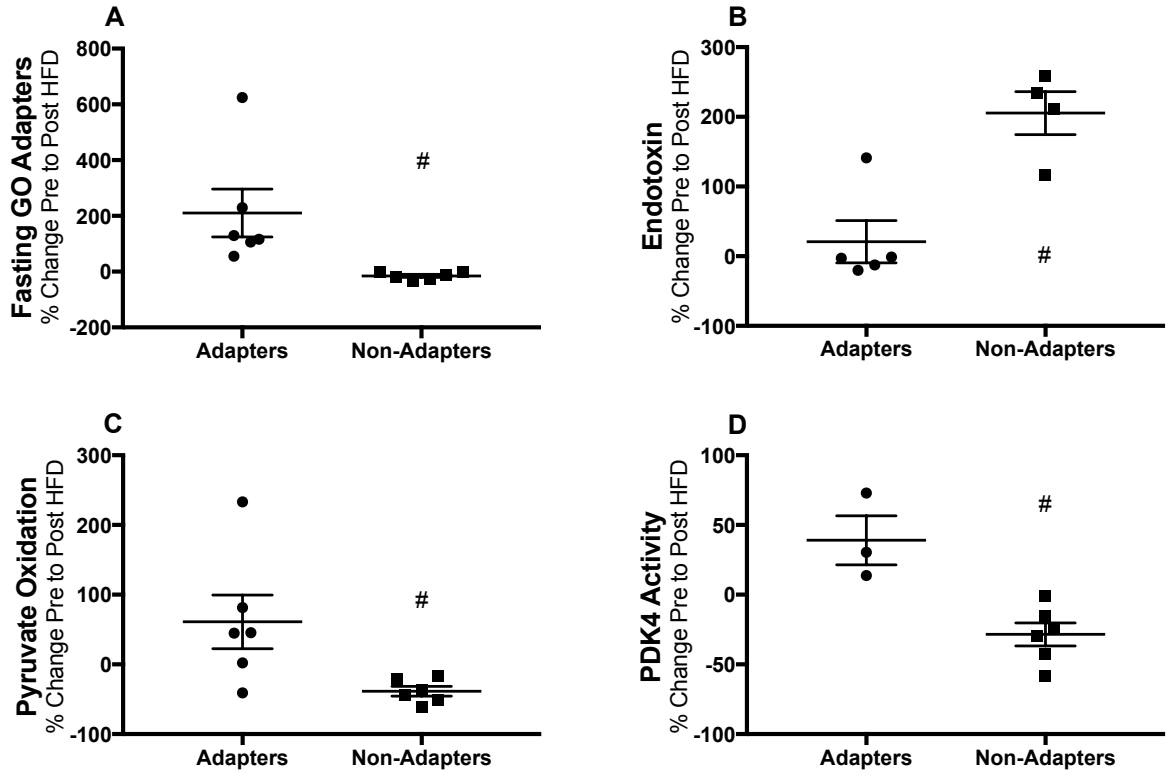


(PDK4 western blot shown in Figure 4B)

 # Significant difference found (p<0.05).

Similarly, a median split of GO percent change from pre- to post- HFD was conducted to better understand contributors to GO adaptation (Figure 6A, p = 0.03). Those participants who increased skeletal muscle GO above the median split in response to HFD were classified as adapters and those who fell below the median split were classified as non-adapters. Endotoxins were significantly higher among non-adapters following a HFD when compared to adapters (Figure 6B, p = 0.004). Pyruvate oxidation, which based on our measure, reflects PDH activity, was lower among non-adapters following a HFD when compared to adapters (Figure 6C, p = 0.03). PDK4 activity was lower among non-adapters (Figure 6D, p=0.01).

Figure 6: Glucose Oxidation Adaptation



 # Significant difference found (p<0.05).

DISCUSSION

The purpose of this study was to investigate the postprandial metabolic adaptations that occur as a result of an acute HFD and to examine the effects of a HFD on gut permeability and blood endotoxins on healthy, non-obese, sedentary human participants. In the present study, five days of HFD in healthy participants produces a significant postprandial metabolic adaptation in skeletal muscle without a change in insulin sensitivity, body weight, or gut permeability. FAO, GO and metabolic flexibility were blunted during the fasted to fed transition after the HFD. The lack of change in insulin sensitivity, body weight or gut permeability indicates that these adaptations are presenting at the skeletal muscle level before they are being detected at the whole body level.

Important to note is that the participants in the current study are healthy. Adaptations observed may not indicate a detrimental change, but instead, a necessary, and likely normal, metabolic response to the HFD (and HF meals). It is difficult to determine if the healthy participants would return to baseline if they began consuming the lead in diet after the conclusion of the HFD; perhaps they may adjust and adapt further if they remained on the HFD, or potentially, the fat balance would be unattainable. When exposed to a HFD, fat balance can take several days, with many contributing factors associated[27–29]. Perhaps the majority of the participants were in the process of finding that fat balance, which would in turn, affect the oxidation status. In the present study, GO and FAO were blunted in response to the meal after the HFD, but this observation is most likely a beneficial adaptation.

Metabolic flexibility, or switching, is defined as the preferential oxidation of the substrate that is more available. A dysfunction in these processes is termed metabolic inflexibility, occurring when either substrate is inefficiently oxidized while it is the primary fuel source. Very little of the metabolic flexibility research has been done at the skeletal muscle level, most having looked more broadly at whole body flexibility. Metabolic flexibility observed in this study may have been blunted according to this definition; however, as with substrate oxidation, the changes are likely a beneficial response as the participants adapt to better utilize available substrates. Their ability to switch between substrates may have become different, but should not be necessarily classified as inflexibility. The present study adds to the body of literature by showing that previous to whole body changes, adaptations at the skeletal muscle are occurring, and that perhaps acute metabolic "inflexibility" observed in a healthy population when challenged with a HFD or HF meal is not detrimental, but a natural, beneficial response.

A number of measures were analyzed to understand the underlying mechanisms of these changes in skeletal muscle. CS is one of the key regulatory enzymes in the energy producing metabolic pathway, forming citrate needed for the tricarboxylic acid cycle (TCA). In the present study, there was a significant HFD x HFM interaction; before the HFD, in response to the HFM, CS activity increased, however, after the HFD, in response to the meal CS activity decreased. In obese individuals and those with Type 2 Diabetes, skeletal muscle citrate synthase activity is attenuated[30,31]. The subjects in our study are lean and healthy, therefore this adaptation may suggest a mechanism behind the decreased oxidation seen previous to weight gain or insulin resistance. Additionally, MDH, another important enzyme to the TCA cycle, catalyzing the conversion to oxaloacetate, had a significant HFD x

HFM interaction, similar to CS. These results indicate an adaptation present in the regulatory steps of oxidation that is comparable to the adaptation observed in GO and FAO, and likely is one of the underlying mechanisms for the changes seen.

Fasting endotoxins nearly doubled after the HFD. The increased endotoxins seen in this study add to other notable research in animal models as well as human participants which demonstrate after a HFD, endotoxins were significantly higher in comparison to the control, which also confirms our previous findings[6–11,26,32]. Endotoxin circulation leads to dysregulated signals in skeletal muscle that contribute to impaired metabolic switching whereby regardless of substrates available, GO is increased and FAO is suppressed. This is detrimental due to the HFD yielding fat as the predominant substrate available. Increased gut permeability has been linked to elevated circulating endotoxins. The present study did not show a change in gut permeability, potentially because a longer amount of time is needed for healthy participants to see a difference in gastrointestinal permeability as a result of a HFD. The assay used to determine gut permeability (sugar probe urine test) is typically used to detect irritable bowel syndrome, a chronic condition, and therefore may not be sensitive enough to track small changes that may have occurred in the acute time frame of five days[23,33–35]. Future studies may want to employ a measure of plasma levels of glucagon-like peptide-2 (GLP-2) which has been shown to detect gut barrier function[36].

The postprandial AUC measurements of serum free fatty acids were elevated after the HFD. It is important to note that high serum free fatty acids are associated with metabolic syndrome – the elevated serum free fatty acids seen in the current study can be considered a marker of perturbations prior to whole body disease states. Similar results were observed in a rat model prone to obesity where fasting serum free fatty acids were

not different, but in the fed state, they were elevated[37]. This effect may be associated with a compromised ability of insulin in the fed state to inhibit lipolysis efficiently, which increases circulating free fatty acids.

The pyruvate dehydrogenase (PDH) complex is a major control point for determination of substrate oxidation. Referring to Figure 4, two proteins, pyruvate dehydrogenase kinase 4 (PDK4) and pyruvate dehydrogenase phosphatase (PDP) were analyzed to further understand the HFD effect on this complex. An increase in PDK4 activity suppresses glycolysis and enhances FAO, inhibiting the use of glucose. An increase in PDP activity utilizes glucose, promoting GO. In the present study, the postprandial PDP activity was blunted after the diet, indicating disruptions in activating the cycle as efficiently as previous to the HFD. We would expect PDK4 to be up-regulated, enhancing FAO, due to the high volume of fat in the diet and meal challenge. However this is not observed in the present study, which indicates an overall decreased functionality of the PDH complex after the HFD. While we did not see statistical significance likely due to low sample size, pyruvate oxidation suppression after the HFD is trending (Figure 3C). Our measure of pyruvate oxidation reflects PDH activity. The PDH complex is a control point used to drive ATP synthesis via oxidative phosphorylation. When not functioning properly, the interconnection of glycolysis or FAO to the TCA cycle is compromised, affecting the utilization of substrates.

To further understand adaptations in the present study, a median split of the percent change from pre to post HFD was calculated in both fasting FAO and fasting GO measures. Fatty acids and glucose are the primary substrates that have been shown to fluctuate with changes in diet. As with most human responses to an intervention, results

are quite variable. However, the adaptations were further understood by analyzing the groups of participants who fell above and below the median split.

Fasting FAO non-adapters had diminished oxidative efficiency (Figure 5B) and PDK4 activity (Figure 5C). Oxidative efficiency, as measured by CO2/ASM ratio of FAO, is indicative of the body's capacity to completely oxidize fatty acids to CO2. As expected, FAO non-adapters' capacity to do so was significantly blunted after the HFD, characterizing those participants with an inability to adapt to the HFD. Incomplete oxidation of fatty acids leads to activation of pro-inflammatory pathways[38,39], which could be a contributor to the chronic low-grade inflammation observed in metabolic disease states.

We also observed a trend where FAO non-adapters had an increased p38 activity, which is in line with previous research from our lab[26]. The lack of significance ($p=0.06$) is likely due to the small sample size. Due to our other findings of circulating inflammatory markers, a discussion of p38 in this study is warranted. P38 has three isoforms, α, β, and γ, all of which were captured in our assay. We do not know which isoforms are changing, but to understand further, p38α is found globally and one of its functions is to regulate production of inflammatory mediators[40,41]; p38β, also found globally, but more concentrated in the brain and lungs, similarly contributes to inflammatory mediator synthesis[42]; p38γ is most significantly found in skeletal muscle and is essential for promoting mitochondrial biogenesis[26,43]. In the current study, we can 1) speculate that the increased p38 activity observed is regulating production of inflammatory mediators indicating that those responding poorly to FAO may have an increased inflammatory response and/or perhaps less likely 2) attribute the p38 increase to the γ isoform, indicating that p38 is promoting mitochondrial biogenesis, an adaptation that may be

necessary to compensate for the decreased FAO and GO observed. Low-grade inflammation is often associated with metabolic diseases, therefore the findings of the first scenario enhance the body of literature by adding that FAO non-adapters, those who do not adapt as well to the HFD, have an increased inflammatory response after the HFD. The second scenario may be explained by the knowledge that in obese and insulin resistant individuals, mitochondrial function, size and morphology are impaired[31], and that increased mitochondrial biogenesis has been suggested to prevent obesity and glucose intolerance in a rodent model[44]. The latter would suggest adaptation of the FAO non-adapters that may not be necessary in those who adapted and therefore have adequate fatty acid oxidation.

Fasting GO non-adapters had elevated endotoxins after the HFD (Figure 6B), and decreased PDH activity, as measured by pyruvate oxidation and PDK4 protein content (Figures 6C and D respectively). Endotoxins are responsible for activating an immune response, often associated with low levels of inflammation and contributing to metabolic disorders by way of metabolic endotoxemia. An elevated level of endotoxins in the non-adapters after just five days of the HFD is indicative of the effect of HFD on the gut and its contribution to overall health. In the present study, the GO non-adapters significantly decreased PDH activity and PDK4 protein content after the HFD whereas those who responded well did not. These results may be the driving factor behind the significance found in Figure 4, where PDP was significantly blunted after the HFD, disrupting the PDH complex. This analysis, and the practice of metabolically phenotyping individuals can drive further questions and research to better determine the effects of diet on substrate metabolism.

Further directions

Types of fats were not manipulated in the present study. Saturated fat was the most abundant fat in the diet, as the intention was to examine a typical high fat western diet, which is high in saturated fat. Manipulation of other types of fats in the HFD may have different outcomes than the present study. Also, we looked at the level of the skeletal muscle but what this means exactly for substrate oxidation and metabolic flexibility at the whole body level is yet to be determined. Finally, metabolic phenotyping should be an area of further exploration, characterizing participants and their metabolic adaptations to different interventions. This information will contribute to the body of literature and inform scientists who are dedicated to understanding metabolic disease states and finding solutions to obesity, diabetes and insulin resistance.

Conclusion

In conclusion, the present study demonstrated that after five days of a HFD, adaptations in both GO and FAO in skeletal muscle of healthy participants are observed. Mechanisms such as increased fasting endotoxins, dysregulation of the PDH complex, enzymatic disruption, and key protein modulators have been shown to contribute to the adaptations observed. Metabolically phenotyping by participants' adaptations to substrate oxidation revealed valuable insight to be used to further the study of individual changes and metabolic disease.

FIGURE LEGENDS

Figure 2: Meal challenge blood measures
Blood was taken at baseline and every hour for four hours after the meal challenge and was analyzed by assay kits to determine differences pre and post HFD. **(A)** Serum free fatty acids (FFA) trend similarly after the HFD in response to the meal, although significantly higher (p=0.03). **(B)** Serum triglycerides (Tg) are significantly lower after the HFD in response to a meal (p=0.01). **(C)** Serum endotoxins show some variation before and after the HFD, although not significant. All data are expressed as mean ± SEM.

Figure 3: Substrate oxidation
Substrate oxidation was measured using radiolabeled substrates in muscle homogenates. Five days of isocaloric HFD disrupted postprandial GO in skeletal muscle. **(A)** GO increased in response to a meal before HFD (+96.9% ± 36.3) but not after (-24.3% ± 4.5, p=0.003). **(B)** Skeletal muscle increased FAO before the HFD after a meal by 106.3% ± 36.6, but after the HFD, this effect was blunted to 15.6% ± 20.8 (p=0.04). **(C)** PO meal response before the HFD was 18.3% ± 20.7 and after the HFD, it was -20.42 % ± 6.8 (p=0.09) **(D)** In response to the meal, skeletal muscle metabolic flexibility was significantly blunted following HFD (-24.7% ± 9.5, p=0.01). All data are expressed as mean ± SEM.

Figure 4: Pyruvate dehydrogenase complex The pyruvate dehydrogenase complex was analayzed by detecting pyruvate dehydrogenase phosphatase (PDP) and pyruvate dehydrogenase kinase 4 (PDK4) proteins via western blotting. **(A)** There was a significant HFM x HFD interaction for PDP (p=0.018). In response to a meal, PDP was blunted after the HFD (p=0.0241). **(B)** PDK4 shows a similar trend in response to the meal, although not significant. All data are expressed as mean ± SEM.

Figure 5: Fatty Acid Oxidation Adaptation
A median split of fasting FAO percent change from pre- to post- HFD was done to determine adapters and non-adapters **(A)**. **(B)** Oxidative efficiency was significantly lower among non-adapters following a HFD when compared to adapters (p=0.05). **(C)** PDK4 activity was lower among non-adapters following a HFD when compared to adapters (p=0.04). **(D)** p38 activity trended higher among non-adapters, although significance was not reached (p=0.06). All data are expressed as mean ± SEM.

Figure 6: Glucose Oxidation Adaptation
A median split of GO percent change from pre- to post- HFD was done to determine adapters and non-adapters **(A)**. **(B)** Endotoxin was significantly higher among non-adapters following a HFD when compared to adapters (p=0.004). **(C)** Pyruvate oxidation was lower among non-adapters following a HFD when compared to adapters (p=0.03). **(D)** PDK4 activity was lower among non-adapters (p=0.01). All data are expressed as mean ± SEM.

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CHAPTER 6: CONCLUSIONS/FUTURE DIRECTIONS

Skeletal muscle substrate metabolism and the adaptations that occur following a high fat diet was the principle objective of this project. A secondary objective was to determine the change in gut permeability and circulating endotoxins after a HFD. Adaptations were observed in substrate oxidation, metabolic flexibility, endotoxins and many mechanistic studies related to metabolic processes. These adaptations may be the normal metabolic response when healthy processes are challenged with unhealthy food intake. Further research is needed to investigate this idea. Repeating this study while adding an in vivo metabolic flexibility measure is needed in order to validate the in vitro measurement of metabolic flexibility used in this study. Extending the time line of the study and repeating collection of measurements after participants return to the normal diet would reveal further adaptations, or more likely, a return to baseline. Other future directions might also include a diet that has increased caloric intake during the HFD, or changing the high fat portion of the diet to high sugar consumption. Participants were sedentary, so adding an exercise element may change the adaptations observed. Analyzing the bacterial landscape of the gut potentially would clarify some of the metabolic perturbations observed. The observation of the adapters and non-adapters could be further analyzed characterizing different variables within the results. Ultimately, future directions in substrate oxidation and metabolism should include an intentional effort to phenotype and categorize specific populations in order to determine the differences seen in subgroups. Investigation of these differences could potentially clarify causation of many metabolic perturbations.

Substrate oxidation at the skeletal muscle level is an important aspect of understanding metabolic disease states. This project adds valuable insights about adaptations at the skeletal muscle before whole body disturbances occur. Additionally, this project adds insight to the discussion about metabolic endotoxemia and its potential contribution to disrupted substrate oxidation. Continuing investigation to determine how and why the metabolic processes are disrupted by diet is necessary.