Skeletal muscle metabolic adaption to high-fat feeding

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ABSTRACT

Skeletal muscle is highly involved in macronutrient metabolism. To maintain proper energy metabolism and physiology, skeletal muscle must adapt to nutrient supply. Thus, diet macronutrient composition is an important modulator of skeletal muscle metabolism. Evidence from rodent and human models show high-fat diets contribute to impaired insulin signaling, as well as decreased fatty acid and glucose oxidation. Utilizing proteomic analysis of metabolic proteins in humans may lead to the mechanism behind skeletal muscle adaption to macronutrient composition, potentially providing the groundwork for characterizing the etiology of high-fat feeding induced metabolic disease. The objective of this study was to compare the substrate oxidation patterns and the levels of metabolic proteins in the fasted skeletal muscle of lean, healthy males that either increased fatty acid oxidation in response to the high-fat diet, termed responders, or males that decreased fatty acid oxidation, termed non-responders. We employed a controlled feeding study design, where the participants served as their own controls. Following a 2-week control diet (30% fat, 55% carbohydrate and 15% protein), participants came to the lab fasted overnight and a muscle biopsy was taken from their vastus lateralis muscle. Participants were then placed on a 5-day high-fat diet (50% fat [45% saturated fat], 35% carbohydrate, and 15% protein). Following this diet, participants again came to the lab fasted overnight and another muscle biopsy was taken from their vastus lateralis muscle. Both the control and the high-fat diets were isocaloric to habitual diets. Muscle from the biopsies were utilized for substrate metabolism measures and mass spectrometry. We did not observe any significant differences in glucose oxidation between responders and non-responders, prior to or following the high-fat diet. Our proteomic analysis identified 81 proteins and protein subunits involved in substrate metabolism but only 6 were differentially regulated by the high-fat diet. Independent of the high-fat diet, compared to non-responders, responders contained an overall higher content of protein subunits belonging to Complex I and ATP synthase. The findings from this study suggest that adaption to high-fat feeding is individual specific and proteomic changes alone cannot explain high-fat feeding induced metabolic changes.

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GENERAL AUDIENCE ABSTRACT

Skeletal muscle is highly involved in macronutrient metabolism, which consist of the breakdown and utilization of glucose and fatty acids, thus making the foods we ingest a major modulator of skeletal muscle metabolism. Over the last few decades, Americans have increased their ingestion of foods high in saturated fats, which has coincided with the increased prevalence of obesity and type 2 diabetes. Further, evidence suggests these metabolic diseases are associated with the skeletal muscle's inability to switch between the utilization of glucose and fatty acid in response to nutrient supply. Analyzing metabolic protein content in humans may lead to the mechanism behind skeletal muscle adaption to macronutrient composition, potentially leading to the cause behind the development of high-fat feeding induced metabolic disease. The objective of our controlled feeding study was to compare the macronutrient metabolism and the content of metabolic proteins in the fasted skeletal muscle of healthy males that either increased fatty acid utilization in response to a high-fat diet, termed responders, or males that decreased fatty acid utilization, termed non-responders. Following a 2-week control diet (30% fat, 55% carbohydrate and 15% protein), participants came to the lab fasted overnight and a biopsy was taken from their thigh muscle called the vastus lateralis. Participants then began a 5-day high-fat diet (50% fat [45% saturated fat], 35% carbohydrate, and 15% protein). Following this diet, participants came to the lab fasted overnight and another biopsy was taken from their vastus lateralis muscle. Both the control and the high-fat diets were isocaloric to habitual diets. The muscle samples were used to analyze macronutrient metabolism and identify metabolic protein content. We did not observe differences in glucose utilization between responders and non-responders, prior to or following the high-fat diet. We identified 81 metabolic proteins and protein subunits but only 6 were differentially regulated by the high-fat diet. Independent of diet, responders contained higher levels of subunits from 2 proteins involved in cell energy production, Complex I and ATP synthase. Our findings suggest that adaption to high-fat feeding is individual specific and protein content changes alone cannot explain high-fat feeding induced metabolic changes.

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CHAPTER I

Introduction

Since the 1990's, the prevalence of obesity and Type 2 diabetes (T2DM) have drastically increased[1-3]. In 2014, nearly 70% of adult Americans were either overweight, categorized by a Body Mass Index (BMI) between 25 kg/m^2 and 29.9 kg/m^2, or obese, a BMI greater than 30kg/m^2[3,4]. In 2015, diabetes was the seventh leading cause of death in the United States[5]. In particular, T2DM accounted for up to 95% of those cases[5]. To date, in the United States alone, over 30 million Americans suffer from diabetes.

Obesity and T2DM have been associated with the disruption of metabolic flexibility, the ability to switch between glucose and fatty acid oxidation in order to maintain proper energy metabolism and physiology[6,7]. The skeletal muscle is highly involved in glucose and fatty acid utilization and the malfunction of this tissue has been implicated in the onset of metabolic diseases. The skeletal muscle accounts for up to 80% of insulin-stimulated glucose disposal[8-17] and evidence suggest T2DM, obesity, and insulin resistance are associated with skeletal muscle's inability to utilize free fatty acids as a fuel source as well as decreased oxidative enzymatic activity[18-21].

The increased prevalence of T2DM and obesity, coincides with society's increased ingestion of foods high in saturated fat and decreased consumption of fruits and vegetables[1,2,4,22,23]. This change in diet, increased prevalence of metabolic diseases, and the vital role skeletal muscle plays in macronutrient oxidation, leads to the question, do high-fat diets or lipid enriched environments disrupt metabolic flexibility within in these tissues? However, to answer this question, the molecular cues that regulate metabolism must be elucidated.

To date, most research examining skeletal muscle's adaption to a high-fat diet or lipid enriched environments utilize cell culture or mouse models. Analyzing the effect of high-fat feeding on

enzymes involved in fatty acid and glucose metabolism within human skeletal muscle may potentially lead to the etiology of metabolic disease in response to high fat feeding.

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CHAPTER II

Review of Literature

1. Introduction

In 1963, Randle and his colleagues proposed the Randle cycle, which describes organ fuel selection in response to nutrient availability[1]. The liver and skeletal muscle are two major metabolically active organs that have the ability to adapt to available fuel sources[1-5]. Interestingly, hepatocytes are one of the few cells types that have the ability to consume as well as export glucose based on the nutrient status of the cell[2,6-9]. Furthermore, the liver plays an important role in the breakdown, synthesis, storage, and export of nutrients[2,6,9-15]. Due to this ability, one of hepatocytes main functions is to maintain metabolic homeostasis. Hepatic glucose production is increased during a fasted state to provide energy to tissues in need[2,12]. However, when glucose is readily available, in a fed state, this production is suppressed to avoid an excess flux of glucose[2,6,7,16]. Likewise, skeletal muscle is highly involved in the oxidation of glucose and fatty acids and must adapt to nutrient availability to maintain metabolic homeostasis. For instance, the skeletal muscle accounts for up to 80% of insulin-stimulated glucose disposal[17]. Even further, skeletal muscle is thought to provide up to 90% of fasting fuel production through fatty acid oxidation[18,19]. Moreover, to stress its importance, the skeletal muscle accounts for up to 50% of the average person's body weight[20].

When the liver and skeletal muscle function properly, in a fed state glucose serves both as the main oxidative fuel source and substrate for non-oxidative disposal, such as glycogen synthesis [1,4,5,7,11,17,21]. An increase in blood glucose stimulates insulin secretion, thereby suppressing the release of fatty acids from adipose tissue[1,3,10,22,23]. In contrast, during fasting, lipolysis is initiated and fatty acids become the main fuel source[1,10,13,24-27]. The rise in plasma non-esterified fatty acids simultaneously increases fatty acid oxidation (FAO) while inhibiting

glucose uptake and oxidation[1,13,28]. This is called metabolic flexibility, the ability to switch between glucose and fat oxidation in order to maintain proper energy metabolism and physiology[4,29]. Thus, to maintain metabolic flexibility these major oxidative tissues must be able to adapt to macronutrient composition of meals ingested.

To ensure the maintenance of metabolic homeostasis, oxidative tissues contain mitochondria, energy producing organelles that serve as the primary sites of FAO and utilization, as well as continued glucose oxidation of glycolytic products[30-35]. Mitochondria contain many metabolites that function as nutritional sensors to maintain metabolic homeostasis[35-40]. One metabolite in particular is acetyl-coenzyme A (acetyl-CoA), a two-carbon molecule activated with a coenzyme A[41]. This metabolite is at the crossroads of glucose and fatty acid catabolism, suggesting its importance in metabolism. Glucose catabolism, by way of glycolysis, results in the production of pyruvate, which is transported into the mitochondria where pyruvate dehydrogenase (PDH) converts it to acetyl-CoA[42]. Furthermore, every turn of beta-oxidation, the pathway that catabolizes fatty acids, produces an acetyl-coA[42]. The acetyl-CoA produced by these pathways have the ability to be utilized in metabolic processes to produce energy. For instance, the tricarboxylic acid (TCA) cycle uses acetyl-CoA to generate NADH and $FADH_2$, which subsequently serve as electron donors to the electron transport chain (ETC), thereby supplying fuel for adenosine triphosphate (ATP) production[42]. The dependence of the liver and skeletal muscle on mitochondria for fuel oxidation and energy production demonstrates this organelle's vital role in the maintenance of metabolic flexibility.

Interestingly, in the liver and skeletal muscle, diminished mitochondrial ability to oxidize fat along with decreased activity of enzymes involved in oxidative phosphorylation, a process that produces ATP, is associated with metabolic inflexibility[29,43-46]. Additionally, metabolic inflexibility is implicated in the pathogenesis of metabolic syndrome, a collection of conditions- high blood pressure, high fasting blood sugar, high triglyceride (TAG) levels, abnormal

cholesterol levels and excess abdominal fat- that increase risk of metabolic diseases such as, cardiovascular disease and Type 2 diabetes (T2DM) [29,43,47-58]. For instance, T2DM, accompanied with insulin resistance, is associated with the skeletal muscle's inability to utilize free fatty acids as a fuel source as well as decreased oxidative enzymatic activity and ETC function[43,44,59,60]. Even further, high levels of circulating free fatty acids is associated with abdominal obesity as well as been implicated in the onset of hepatic insulin resistance, the inability of insulin to inhibit glucose production[61-63]. Additionally, diminished mitochondrial respiratory capacity is exhibited *in vitro* in obesogenic environments as well as displayed in hepatocytes harvested from obese, insulin resistant rodents[64-66]. Together, this data illustrates the importance of proper mitochondrial function to maintain metabolic homeostasis in oxidative tissues.

Recently, the prevalence of T2DM, obesity, and other diseases associated with metabolic syndrome has increased[67-69]. Interestingly, this upward trend coincides with society's decreased consumption of fruits and vegetables, but increased ingestion of saturated fats[70,71]. Due to this rise in ingestion of fat enriched diets, increased prevalence of metabolic syndrome and mitochondria's role in fat oxidation, research has investigated how high-fat diets (HFD) or lipid enriched environments *in vitro* alter mitochondrial function. This has led to discoveries such as rodents on a 12-week HFD accumulate skeletal muscle mitochondrial FAO intermediates, components resulting from incomplete beta-oxidation that contribute to impaired insulin signaling and decreased glucose oxidation in their skeletal muscle[72-76]. Furthermore, in as little as 3 days, healthy men on a HFD exhibited decreased FAO, diminished enzymatic activity of ETC proteins and decreased expression of oxidative genes and proteins[77,78]. However, the amount of studies analyzing the effect of HFDs on mitochondrial function are few and more research must be done to elucidate the mechanism by which mitochondria respond to varying macronutrient compositions.

Interestingly, evidence shows that the mitochondrial nutrient sensor, acetyl-CoA, has the ability to directly regulate protein function[79,80], localization[81,82], stability[83,84], and abundance[83,85]. This is done via lysine acetylation, a post-translational modification in which an acetyl group is added to a lysine residue[86]. For instance, *in vitro,* the acetylation of insulin receptor substrate 1 promotes its phosphorylation and thus increases its activity as well as insulin signaling[87]. This novel role of acetyl-CoA has led scientists to examine acetylation's role in the regulation of metabolism. However, most research examining the role of acetylation in maintaining metabolic homeostasis in response to a HFD or lipid enriched environments have been performed in the mouse models or cell culture, while few have been performed in humans. Additionally, most research done in mouse models focused on liver metabolism. The liver has the ability to reduce the build-up of excess fatty acids, thereby decreasing its susceptibility to lipotoxicity. Many hepatic transporters and other key enzymes that metabolize fatty acids are upregulated in times of nutrient excess or disease states[88,89]. In particular, diabetic mice have an increased expression of the hepatic fatty acid translocase, CD36, a transporter with the ability to bind and transport fatty acids into the cell[90]. An increase in CD36 promotes an increase in fatty acid oxidation, suggesting the liver can adapt to lipid enriched environments[91]. Further, Buqué et al.[88] demonstrated hepatocytes from genetically obese rodents displayed an in increase in plasmalemmal CD36 expression along with increased FAO. On the other hand, in the skeletal muscle of genetically obese rodents, CD36 resided primarily in intracellular vesicles, which was accompanied by diminished FAO[88,92,93]. However, a particular study utilized transgenic mice that overexpressed plasmalemmal CD36 to demonstrate that if skeletal muscle could mimic the livers ability to increase CD36 expression in response to an influx in fatty acids, fatty acid as well as glucose metabolism would improve in lipotoxic states[94]. This slight disadvantage of skeletal muscle exhibits the necessity to also study the effects of HFDs on this major oxidative tissue and the mechanism, if any, by which it promotes metabolic inflexibility. Thus, research on how lipid enriched environments affect the acetylation of metabolic proteins in skeletal muscle is needed. This review underlines the importance of acetylation in regulating metabolism of

glucose and fatty acids, with a particular focus on mitochondrial function in lipid enriched states, as well as highlights the need for more human studies, especially within the skeletal muscle.

2. The Acetylome

Originally, acetylation was studied in context of its role in modifying histones, proteins that condense DNA into orderly structures[86,95]. However, half a century after acetylation was recognized for this role, L'Hernault et al.[96] demonstrated that acetylation targets more than histones. It is now known that a wide range of proteins located in the nucleus[90,97-100], cytosol[90,97-102] and the mitochondria[90,97-103] can also be acetylated.

The focus on histone acetylation gave rise to research on histone acetyltransferases (HATs), enzymes that transfer an acetyl group from acetyl-CoA to a histone[104-108]. The 3 major families of HATs consist of Gcn5-related N-acetyltransferases (GNAT)[105], the p300 family[106], and the MYST family which is named for the first acetyltransferases found in this family[109]. These HATs all have a conserved region where acetyl-CoA binds[110-113]; however, subfamilies are defined by the structural[110,111,114,115] and amino acid sequence[110-112,116,117] divergences that flank this binding site. Additionally, many of these HATs have the ability to acetylate protein lysine residues[118-120]. Thus, those particular HATs can also be considered lysine acetyltransferases (KATs), which facilitate acetylation by using acetyl-CoA as a substrate for the addition of an acetyl group to a lysine residue[121]. Now over 20 KATs have been identified that acetylate not only histones but also transcription factors[97-99,118,122-124], structural proteins[96,98,99,125], and proteins that catalyze metabolic reactions[97,99,126].

To date, a KAT has not been identified as a regulator of mitochondrial proteins. Mitochondrial acetylation is thought to occur via mass action rather than enzymatically[127,128]. Meaning, the concentration of acetyl-CoA itself regulates the acetylation status of mitochondrial proteins.

Furthermore, this theory suggests the pKa of these targeted lysine residues is also a driving force behind this chemical process[129]. Lysine has a pKa of 10.4, thus it must be deprotonated to allow the attachment of the partially positive carbonyl carbon of the acetyl-CoA[130]. It is thought that some lysine residues within the active site of target proteins display a depressed pKa. The microenvironment provided by local amino acid residues destabilizes the protonated NH_3^+ group, thus allowing the lysine to be easily deprotonated. Therefore, the increased concentration of mitochondrial acetyl-CoA may allow for the acetylation of the more susceptible lysine residues. Interestingly, studies performed in mouse hepatocytes and human fibroblast suggest that the acetyl-CoA that acts as the substrate for mitochondrial acetylation is derived from FAO, suggesting acetylation is sensitive to the metabolic status of the cell[131].

To fully appreciate the function of acetylation, cellular cues that promote deacetylation must also be understood. Recently, research involving the deacetylation of metabolic proteins has focused on the lysine deacetylases called sirtuins (sirts). Humans express seven distinct sirts, sirt1-7, throughout the cell and their selective expression can be found in Table 1[80,85,132-144]. These deacetylases are thought to be nutrient sensitive due to their dependence on NAD^+, a coenzyme that serves as a metabolic nutrient sensor, for activation[145-149]. Excluding sirt5 and sirt7, research shows sirts have the ability to regulate macronutrient metabolism in the liver and skeletal muscle; however, sirt1 and sirt3 are the most heavily studied[146,150-177]. Sirt1 is localized to the nucleus and cytosol, where it primarily regulates macronutrient metabolism by targeting transcription factors such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) and forkhead box O1 (FOXO1), both of which are involved with regulating the expression of genes involved in fatty acid and glucose metabolism[80,85,132,142,145,154,157,169,178]. While the mitochondrial deacetylase, sirt3, primarily targets enzymes facilitating reactions involved in hepatic fatty acid oxidation and total oxidative metabolism in the skeletal muscle[103,138,158,160-164,168,179-185].

Sirtuin knockout (KO) models have become a useful tool to help elucidate the effect acetylation has on metabolism. The global absence of sirt3 promotes hyperacetylation of mitochondrial proteins; therefore, sirt3KO mouse models in particular, allow researchers to analyze the affect hyperacetylation has on mitochondrial proteins[138,163,166]. These models have led to the discovery of data suggesting sirt3 is protective against skeletal muscle insulin resistance during high-fat feeding and is important for the maintenance of mitochondrial respiration[158,166,168]. Utilizing permeabilized skeletal muscle fibers, Lantier found that the global absence of sirt3, and therefore mitochondrial hyperacetylation, promotes fuel switching by decreasing skeletal muscle reliance on glucose and increasing the rate of mitochondrial fatty acid oxidation[166]. Additionally, hepatocytes missing sirt3 exhibited lower ATP levels and membrane potential, suggesting hyperacetylation promotes disrupted mitochondrial respiration [158,168]. Thus, researchers can utilize these sirtKO models to study the effect acetylation, or in particular hyperacetylation, has on macronutrient oxidation, insulin action, mitochondrial respiration, and more.

3. Glucose and Fatty-Acid Metabolism

In addition to sirtuin KO studies, proteomic techniques have served an invaluable role in the study of reversible acetylation's involvement in metabolic processes. Wang et al.[186] performed gas chromatography–mass spectrometry enriched acetyl peptides from *Salmonella enterica* and found 50% of the detected acetylated proteins were involved in metabolic processes. Additionally, scientists have found that in rodents more than 40% of mitochondrial proteins are acetylated and of those proteins over 60% are involved in metabolic processes[99,103,187,188]. The available mass spectrometry data demonstrates that nearly every enzyme that participates in carbohydrate and fatty acid metabolism has the ability to be acetylated[90,97,99,101-103,186,189]. The acetylated enzymes, found to date, involved in glucose and fatty acid metabolism are shown in Table 2 as well as Figure 1. However, only a few studies have analyzed the physiological effects reversible acetylation ensues on these metabolic proteins.

3.1. Glycolysis

Carbohydrate catabolism is an important component of cellular respiration, the process of creating energy, ATP, for the cell[42]. Jing et al.[179] demonstrated that sirt3KO mice had no differences in total glycolytic rate compared to wild-type (WT) mice; however, sirt3KO had a considerable lower rate of glucose oxidation. Along with the decreased rate of glucose oxidation, sirt3KO mice accumulated pyruvate and lactate within the cytosol, suggesting that the absence of sirt3 diminishes the oxidation of glycolytic end products. These results reflect glycolytic flux as a whole; however, analyzing the effect sirt3 has on the function of individual proteins may provide insight of mechanism by which sirts regulates metabolism.

The initial step of carbohydrate catabolism is performed by hexokinase (HK), in which glucose is phosphorylated, trapping it in the cell where it can then enter glycolysis[42]. Disrupted hexokinase activity leads to impaired glucose uptake, which promotes hyperglycemia and dysfunctional hepatic glycogen synthesis, as well as is implicated in the pathogenesis of T2DM[190-193]. The liver form of HK, glucokinase (GK), is indirectly inhibited by acetylation[194,195]. *In vitro,* the acetyltransferase P300 acetylates glucokinase regulatory protein (GKRP), a protein that regulates GK[195]. When GK is bound to GKRP it is retained in the nucleus, thereby inhibiting its involvement in glycolysis[196,197]. When acetylated, GKRP is resistant to degradation via the ubiquitin-dependent proteasome pathway, suggesting continued binding to GK and decreased GK activity[195]. This data in particular suggests acetylation can decrease glucose uptake, and in turn glycolysis[179].

Furthermore, reversible acetylation targets aldolase (ALDO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphoglycerate mutase (PGAM), all of which are glycolytic proteins that also participate in gluconeogenesis[42]. ALDO catalyzes the reversible reaction of cleaving the 6-carbon sugar molecule, fructose-1,6-bisphosphate, into two 3-carbon molecules[130]. The liver isoform of ALDO, ALDOB, can be acetylated at K147, a particular

residue needed for substrate binding[187,198]. Enzymatic assays revealed that an acetyl mimetic of this lysine residue displayed depressed ALDOB enzymatic activity, indicating acetylation negatively regulates this enzyme[187]. However, this study fails to specify if acetylation of ALDOB decreases glycolytic or gluconeogenic activity.

In vitro and *in vivo* studies have attempted to elucidate the ramifications of the reversible acetylation of GAPDH, the glycolytic enzyme that produces the electron carrier, NADH[130,186,199,200]. Prokaryote and mouse models revealed the acetylated form of GAPDH stimulated glycolysis, while conversely inhibiting gluconeogenesis[186,199,200]. A study from Li et al.[199] performed in human embryonic kidney (HEK) cells, went one step further and discovered GAPDH is targeted by the acetyltransferase p300/(CREB-binding protein)-associated factor (PCAF) and is deacetylated by histone deacetylase (HDAC) 5. Li et al.[199] also analyzed the effect of nutrient cues on the acetylation status of GAPDH. The addition of glucose to the cell culture media promoted acetylation, and in turn increased GAPDH's glycolytic activity, suggesting reversible acetylation is dependent on the metabolic state of the cell[199]. However, Bond et al.[200] found that addition of key hormones that regulate metabolism, such as insulin and glucagon had no effect on the acetylation status of hepatic GAPDH in mice. These differences in GAPDH acetylation status in response to nutrient cues could be due to study design, since, *in vitro* studies are more isolated and easier to control, the data produced does not always representative of the *in vivo* environment.

Phosphoglycerate mutase (PGAM) 1, the enzyme that catalyzes the reversible isomerization of 3-phosphoglycerate to 2- phosphoglycerate, is regulated by reversible acetylation based on carbon flux[130,201,202]. Acetylation positively regulates PGAM1 activity and is suggested to increase glycolytic flux[201,202]. Interestingly, PGAM1 can be deacetylated by both sirt1 and sirt2[201,202]. In HCT cancer cell culture, PGAM1 interacts more strongly with sirt2, compared to

its strong interaction sirt1 in HEK cells[201,202]. Despite the difference in deacetylase association, these studies both suggest acetylation promotes PGAM1 activty[201,202]. Enzymatic activity assays performed in the HCT cell model revealed the presence of sirt2 downregulated PGAM activity[202]. However, it is unknown if suppressed PGAM1 activity dampens glycolysis or gluconeogenesis. On the other hand, in HEK cells, an acetyl mimetic of PGAM1 consumed more glucose compared to wild-type PGAM1. Based on these results, the authors suggest acetylation of PGAM1 increases glycolytic flux[201].

Pyruvate kinase (PK) is also glycolytic enzyme that is regulated by acetylation[126,186,203]. This enzyme catalyzes the irreversible last step of glycolysis, in which a phosphate is transferred from phosphoenolpyruvate (PEP) to ADP, thus forming pyruvate and ATP[42]. Multiple forms of PK exist; however, the researchers studied PKM2, which is found in most tumor cells[203-205]. Even though, this review does not focus on cancer, the cell model utilized by Lv et al.[203] to study PKM2 provides valuable information that serves as a foundation for future research. Lv et al.[203] found that PKM2 protein level was inversely correlated with its acetylation status. Enzymatic assays and kinetic analyses revealed an acetyl mimetic of PKM2 displayed depressed activity and a low affinity for its substrate PEP[203]. Interestingly, PKM2 contains a motif that allows it to interact with heat shock cognate protein 70 (HSC70), a protein that recruits proteins to lysosomes[203]. The PKM2 acetyl mimetic strongly interacted with HSC70, suggesting that acetylation targets PKM2 to the lysosome for degradation. Furthermore, the addition of a lysosomal protease inhibitor to the cells elicited an accumulation of both the unaltered and acetylated form of PKM2, providing more evidence that acetylation targets PKM2 to the lysosome and negatively regulates glycolysis[203]. This demonstrates that acetylation also has the ability to regulate the protein abundance of metabolic proteins.

These studies on glycolytic proteins provide invaluable information on how acetylation regulates glucose catabolism. Research suggests acetylation mainly plays an inhibitory role in glycolysis, particularly by negatively regulating the irreversible glycolytic steps catalyzed by of hexokinase and pyruvate kinase[195,203]. Furthermore, phosphofructokinase (PFK), the enzyme that catalyzes the rate limiting step of glycolysis, can also be acetylated; however, to date no studies have demonstrated the physiological effect acetylation elicits[42,126]. This exhibits there is still knowledge to be gained from examining how acetylation regulates glycolysis.

3.2. Gluconeogenesis

In an energy deficient state, gluconeogenesis is initiated to produce glucose from non-carbohydrate sources by the liver, and to a lesser degree the kidney[42]. Of note, gluconeogenesis accounts for over 90% of glucose production during fasting[206]. The dysregulation of gluconeogenesis is implicated in many diseases, including T2DM, demonstrating its importance in whole body metabolism as well as the importance of its regulation[192,193,207,208].

Researchers have attempted to elucidate the physiological ramifications acetylation has on phosphoenolpyruvate carboxykinase 1 (PEPCK1), the gluconeogenic enzyme that catalyzes the irreversible conversion of oxaloacetate to phosphoenolpyruvate[126,130,209,210]. Studies suggest acetylation downregulates this gluconeogenic protein[126,209,210]. Lin et al.[210] performed a study using human hepatocellular carcinoma (HepG2) cells and found that the addition of histone deacetylases led to a decrease in PEPCK protein expression and activity. Years later, Jiang et al.[209] performed a study to find the mechanism by which acetylation regulates PEPCK1 and in turn gluconeogenesis. In mouse hepatocytes and HEK cells when coexpressed with P300, PEPCK1 acetylation increased [209]. In a P300 knockout model PEPCK1 acetylation decreased and in contrast glucose production increased, providing more evidence that PEPCK1 is targeted by this acetyltransferase[209]. Additionally, PEPCK1 was found to strongly associate with Sirt2 and this association evoked a decrease in acetylation and an increase in stability as well as

activity of PEPCK1[209]. This data suggests an increase in PEPCK1 acetylation leads to a decrease in its activity. The acetylation of PEPCK1 was also found to promote its interaction with E3 ubiquitin-protein ligase (UBR5), a protein in the ubiquitin-proteasome pathway[209]. Overexpressing UBR5 elicited a decrease in PEPCK1 protein level as well as a decrease in glucose production[209]. This study suggest acetylation negatively regulates gluconeogenesis by promoting PEPCK1 ubiquitination and degradation by the proteasome. Additionally, it was found that a high concentration of glucose increased PEPCK acetylation and decreased the production of glucose[126,210]. Taken together these studies provide evidence that acetylation regulates gluconeogenesis in response to nutrient availability, specifically by down regulating PEPCK1.

3.3. Glycogen Metabolism

Glycogenolysis, the breakdown of glycogen, is another process that is important during a nutrient deficient state. Glycogen serves as an energy reserve that primarily resides in the liver and skeletal muscle. When blood glucose is low, glycogen is broken down and converted to glucose for tissues in need[42]. Impaired glycogen metabolism has been associated with metabolic states, including hyperglycemia and insulin resistance[211,212]. The enzyme glycogen phosphorylase (GP) catalyzes the rate-limiting step of glycogenolysis, in which glucose-1-phosphate is cleaved from glycogen to later be converted to glucose[42]. *In vitro*, the addition of deacetylase inhibitors induced an increase in GP acetylation, but a decrease in activity, suggesting GP is negatively regulated by acetylation. To confirm acetylation was the source of decreased activity, a GP acetyl mimetic was engineered and, expectedly, also displayed a depressed enzymatic activity. Acetylation of GP increased with the addition of glucose and insulin, thereby decreasing the production of glucose, suggesting acetylation is nutrient sensitive[213,214]. This study also demonstrates the specificity of acetylation due to insulin selectively increasing acetylation in GP but not in enzymes such as GAPDH[200,214]. Interestingly, the acetylation of GP has an inverse relationship with its phosphorylation[214]. Phosphorylation is

one of the most studied post-translational modification and affects many cellular processes, including gene and protein expression, mitogenesis, and metabolic signaling pathways[215-217]. Deacetylase inhibitors decreased GP phosphorylation; however, when coexpressed with sirt1 and sirt2, GP phosphorylation as well as activity increased[214]. To elucidate this mechanism, researchers analyzed protein phosphatase 1α (PP1α), the protein that binds and inactivates GP by promoting dephosphorylation[214,218]. The presence of deacetylase inhibitors increased the GP-PP1α interaction, while sirt2 decreased the interaction and increased GP's activity[214]. Evidence suggests acetylation promotes this interaction by recruiting a substrate-targeting subunit of PP1α, G_L, to GP[214]. Of note, the GP- G_L interaction was also increased with the addition of glucose, illustrating how acetylation follows the same trend as the normal glucose response[214]. This suggests that acetylation responds to nutrient cues and negatively regulates GP activity, while promoting its dephosphorylation and inactivation. The revelation of acetylation's ability to interact with other post-translational modifications, like phosphorylation , provides more insight into the mechanism by which acetylation regulates metabolism[214]. This information may provide the ground work of research elucidating the mechanisms by which post-translational modifications cooperate to maintain metabolic homeostasis.

3.4. Fatty-acid Metabolism

Fatty acids are one of the most important fuel sources during fasting[1,10,13,24-27]. Once in the mitochondria, fatty acids are activated with the addition of a CoA molecule by fatty acyl-CoA synthetase (FAS) and then continue through several cycles of beta oxidation[130,219]. In the postabsorptive state, FAO accounts for up to 90% of skeletal muscles oxygen consumption[18,19]. The activation of mitochondrial beta-oxidation inhibits glycolysis to spare glucose for tissues that solely use glucose as a fuel[4,220,221]. Abnormal lipid metabolism, in which there is a decrease in utilization of fatty acids in the fasted state, has been associated with insulin resistance accompanied both with and without in T2DM[43,59-61,63,222].

Sirt3KO models have served as the basis of research assessing the involvement of acetylation in the regulation in FA metabolism. The addition of palmitate to mice hepatocytes was shown to increase cell death and the absence of sirt3 aggravated these effects[158]. Further, during fasting, compared to WT mice, sirt3KO mice hepatocytes accumulated long-chain acylcarnitines and triacylglycerides (TAG), suggesting an increased rate of incomplete FAO[162]. This disrupted lipid metabolism is present without a change in citrate synthase activity, mitochondrial morphology, lipogenesis, FA uptake, or very-low-density lipoprotein export, suggesting an unknown mechanism at work[160,162]. When stimulated with insulin, hemidiaphragms, skeletal muscle composed mostly of oxidative muscle fibers, from sirt3KO mice exhibited higher rates of palmitate oxidation compared to control mice, providing more evidence the absence of sirt3 promotes a fuel preference switch towards fatty acids in the fed or insulin stimulate state[179]. These studies suggest the absence of sirt3 promotes abnormal fatty acid utilization as well as promote metabolic inflexibility by altering fuel preference[160,162,179]. However, these changes are present without disruption of other components of lipid metabolism like lipogenesis or FA uptake, suggesting sirt3 is selective in its regulation of lipid metabolism[162].

Scientist have found the presence of deacetylase inhibitors in *Salmonella enterica*, *Escherichia coli*, and cos-7 cells cell culture diminished the activity of AceCS2, the mitochondrial enzyme that produces acetyl-CoA from acetate, suggesting that acetylation negatively regulates this enzyme's activity, thereby decreasing the production of acetyl-CoA that could be used in processes like the TCA cycle or de novo lipogenesis[223-225]. Research shows that the AceCS2 knockout mice have diminished levels of ATP during fasting, suggesting the importance of this enzyme for energy production[226]. Even more, hyperacetylated human AceCS2 expressed in E. Coli displayed a depressed catalytic activity[227], while the deacetylation by sirt3 lead to increased activity, providing more evidence that acetylation downregulates AceCS2 activity[227]. Hallows et al.[142] exhibited acetylation rendered AceCS2 inactive; however, when deacetylated its activity

was restored. Together, these studies suggest that acetylation downregulates AceCS2's activity, providing more evidence that acetylation regulates energy production. However, the effect acetylation has on AceCS2 in mammals is still unknown.

Long-chain acyl-CoA dehydrogenase (LCAD), which catalyzes the first step of beta oxidation of long-chain acyl-CoAs, is also a major target of sirt3[130,162,163,181]. Knockout LCAD mouse models develop hepatic insulin resistance as well as a build-up of lipids, suggesting the importance of this enzyme in lipid metabolism[228]. Diminished expression of sirt3 leads to the hyperacetylation LCAD, which is shown to decrease its activity and in turn decreases FAO[162,163,181]. Decreased rates of FAO are shown to promote insulin resistance, suggesting a link between hyperacetylation and metabolic dysfunction[72-76]. It is suggested that acetylation of certain lysine residues near LCAD's active site promotes a conformational change, suggesting another mode of regulation by acetylation [181]. It is possible acetylation negatively regulates LCAD's enzymatic activity by decreasing access of its substrate, fatty acyl-CoAs, to its active site.

Zhao et al.[126] found the addition of fatty acids increased the acetylation of enoyl–coenzyme A hydratase/3-hydroxyacyl–coenzyme A dehydrogenase (EHHADH), the multi-purpose enzyme that catalyzes both a hydration and oxidation reaction, resulting in the production of NADH and ketoacyl-CoA[130]. Enzymatic assays revealed that the acetylated form of EHHADH exhibits a higher activity than the non-acetylated form[126]. This is interesting because the other available studies analyzing the effect of acetylation on enzymes involved in beta oxidation suggest acetylation has an inhibitory effect on fatty acid oxidation[126,142,162,163,181,223,227,229].

Sirt3 also targets the last enzyme in beta-oxidation, acetyl-coenzyme A acetyltransferase 1 (ACAT1), which catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA[130,229]. Enzymatic assays provided evidence that sirt3 deacetylates ACAT1, as well

as increases the activity of this metabolic protein[229]. Utilizing bioinformatics, Still et al.[229] found that the acetylation of a lysine residue in the active site of ACAT1 neutralizes its charge. This neutralization is likely to decrease the protein's electrostatic interaction with its substrate, decreasing the likelihood of reaction completion[229]. Overall, studies analyzing the physiological ramifications of acetylation on enzymes involved in fatty acid oxidation suggest that acetylation plays an inhibitory role on beta oxidation. However, there is still a need to elucidate the mechanism by which acetylation selectively regulates metabolic proteins negatively or positively.

3.5. Tricarboxylic Acid Cycle

The TCA cycle is an aerobic pathway that can oxidize the end products of glycolysis and beta-oxidation[130]. Acetyl-CoA produced from every turn of beta-oxidation or the glycolytic end product, pyruvate, can participate in the TCA cycle to produce electron carriers that supply electrons to the ETC[130]. Thus, it can be said that the TCA cycle is the bridge between ADP phosphorylation from glycolysis, fatty acid beta-oxidation and oxidative phosphorylation[130]. Of note, all enzymes in the TCA cycle can be acetylated, suggesting the tight regulation of this pathway[100,126].

The enzyme PDH, specifically, is suggested to serve as the link between glucose catabolism and oxidative phosphorylation. This enzyme is an important component of a complex that converts the resulting pyruvate from glycolysis to acetyl-CoA, which subsequently enters the TCA cycle[130]. Decreased activity of PDH may lead to the build-up of pyruvate, as well as lactate, potentially forcing skeletal muscle to rely on FAO for energy[4]. This increase in FAO could potentially promote reactive oxidation species production and insulin resistance[230,231]. To better understand the regulation of energy production it is important to elucidate the mechanism by which PDH is regulated. The knockout of sirt3 is shown to lead to the hyperacetylation of PDH and even further shows that acetylation decreases PDH activity, suggesting sirt3 is crucial for

regulating pyruvate oxidaiton[179]. Additionally, it provides more evidence that acetylation is involved in the regulation of glucose metabolism by specifically regulating PDH acetyl occupancy.

Mass spectrometry revealed sirt3 targets isocitrate dehydrogenase (IDH) 2, suggesting this TCA enzyme is regulated by reversible acetylation[99,103,141,185,189]. IDH2 is one of the TCA's rate limiting steps and is positively regulated by ADP, while negatively regulated by ATP and NADH, demonstrating how in tune this enzyme is to metabolic fluxes[130]. Disrupted catalytic activity of this enzyme would halt the TCA cycle, leading to the build-up of citrate, which can exit the mitochondria and participate in lipogenesis[232]. Enzymatic activity assays showed sirt3 promoted an increase in IDH2 activity, suggesting acetylation serves as a negative regulator of this TCA enzyme[141]. Additionally, an acetyl mimetic of IDH2 portrayed lower activity compared to the wild-type protein, providing further evidence corroborating acetylation as a negative regulator[185].

Succinate dehydrogenase (SDH), also referred to as Complex II, is a metabolic enzyme that participates in both the TCA cycle and the electron transport chain (ETC)[130,182,233]. The subunit involved in TCA, SDHA, catalyzes the oxidation of succinate to fumarate and produces the electron transporter $FADH_2$[130]. The electrons from $FADH_2$ can then be donated to the complex of SDH that participates in the ETC[130]. Using a combination of immunoprecipitation and mass spectrometry techniques, Cimen et al.[233] revealed that SDHA, the subunit of SDH involved in the TCA cycle, is a target of sirt3. Interestingly, only the subunit of SDH involved in the TCA is acetylated, the component of SDH involved in ETC has no known acetylation site [158,182,233]. To examine the effect of acetylation on SDHA activity and in turn in TCA cycle, hepatic sirt3KO mouse models were developed[182,233]. These studies revealed that compared to WT hepatic SDHA, sirt3KO SDHA possessed higher levels of acetylation, while in contrast exhibited

diminished activity [182,233]. This suggest that acetylation negatively regulates SDHA and sirt3 combats these inhibitory effects. However, these studies are only performed in mouse liver tissue, providing more evidence research needs to be performed in humans as well as other metabolically active tissues, such as skeletal muscle.

In human liver tissue a high concentration of glucose increased the acetylation of malate dehydrogenase (MDH) 2, the enzyme that facilitates the transformation of malate to oxaloacetate, producing the electron carrier NADH as a byproduct[126,130]. The loss of hepatic sirt3 in mice induced a dramatic increase in acetylation of MDH2[188]. One particular lysine residue located near MDH2's substrate binding site became hyperacetylated in the absence of sirt3[188]. Enzymatic activity assays revealed the acetylation of this residue drastically decreased MDH2 activity[188]. This data suggests this lysine residue is important for the stability of this protein and acetylation promotes instability thereby decreasing the likelihood of substrate binding. In contrast, in human liver tissue, acetylation of MDH2 increased its activity. Furthermore, the acetylation of MDH2 was found to be dependent on the nutrient availability, as acetylation increased in response to glucose addition[126]. The differing data again could be due to specie differences, due to this mouse model not translating to humans or study design differences. The human study performed by Zhao et al.[126] focused on global deacetylase inhibitors instead of specifically knocking out sirt3. Different deacetylases may target different lysine residues, thereby eliciting different functional responses, which could account for the discrepancies. In spite of the differing data, the human study still suggests that acetylation is nutrient dependent due to its response to glucose[126]. In general, acetylation is shown to play an inhibitory role on TCA flux.

3.6. Electron Transport Chain

The ETC concludes aerobic cellular respiration[130]. The electrons transferred from the TCA cycle enter the ETC and drive the synthesis of ATP via ATP synthase (ATPase)[130]. The final electron

acceptor of the aerobic respiration is oxygen; therefore, oxygen consumption can be used as an indicator of respiration[130,234,235].

Acetylation is also found to regulate the ETC[158,165,166]. Diminished sirt3 expression has also been shown to compromise proper mitochondrial respiration[158,165,166]. Both human and mice hepatocytes lacking sirt3 exhibited depressed oxygen consumption, attributed to the reduced activity of Complex I, IV and V[158,165]. However, oxygen consumption due to Complex II activity was similar to control cells[158]. This altered mitochondrial respiration could be attributed to the specificity of sirt3 activity. Complex I has a confirmed subunit, NDUFA9, that is regulated by sirt3[233]. While in contrast, Complex II has no known subunit that is targeted by sirt3 and localized to the ETC. [158,182,233]. The absence of sirt3 promoted an increased in the acetylation of ATPase subunits alpha and oligomycin sensitivity-conferring protein (OSCP). This increase in acetylation correlated to a decrease in activity[184].

In summary, these functional studies on proteins involved in fatty acid and glucose metabolism suggest that acetylation regulates proteins' localization, abundance, and activity in a nutrient dependent way. Overall, acetylation is found to play an inhibitory role on glycolysis, pyruvate oxidation, glycogenolysis, FA oxidation, TCA cycle flux and ETC flux, while it plays a stimulatory role on gluconeogenesis. This suggest acetylation inhibits the macronutrient catabolism but stimulates anabolism.

4. Fed Versus Fasted State

When examining the role acetylation plays in regulating metabolic flexibility, it is necessary to analyze the differences in acetylation status of different nutritional states such as the fed and fasted states. Research illustrated hepatocytes harvested from mice fasted for 16 hours exhibited a 3-fold increase in global protein acetylation[131]. This drastic change is further evidence of

acetylation's sensitivity to the nutritional status of the cell and stresses the importance of more research in this area.

Mitochondria's role in maintaining metabolic homeostasis has promoted research examining the changes in acetyl occupancy of mitochondrial proteins as well as sirt3 function between the fed and fasted state. Following a 24 hour fast, mouse hepatocytes exhibit a significant increase in sirt3 expression which corresponded to a decrease mitochondrial protein acetylation[162]. While in contrast, after a 24 hour fast, in mouse skeletal muscle, Sirt3 protein expression lowest, suggesting mitochondrial proteins are hypoacetylated in the fed state[164,179]. These trends exemplify the specificity of acetylation's response to the nutrient status of the cell and, even further, the importance of the tight regulation of mitochondrial proteins. However, in human skeletal muscle mitochondria, Edgett et al.[236] found sirt3 gene, but not protein, expression changes between fed and fasted states, suggesting specie differences.

Yang et al.[101] analyzed whole cell lysates and found that in mice , non-insulin sensitive tissues such as the brain had a lower amount of acetylation in the fasted state compared to the fed state. In insulin sensitive tissues, such as skeletal muscle and liver, a majority of proteins acetyl occupancy was found to be higher after an 18 hour fast compared to 5 hours after a meal[101]. After a meal, skeletal muscle and liver respond to the increase in glucose concentration, stimulating the deacetylation of a majority of the proteins in the whole cell lysate. This coincides with the research suggesting some glycolytic enzymes such as HK and PK are hypoacetylated when active, suggesting reversible acetylation is sensitive to nutrient cues to help maintain metabolic homeostasis[166,195,203]. These results suggest that hepatic global cell acetylation decreases in a fed state, while Hirschey et al.[162] presented evidence suggesting hepatic mitochondrial acetylation is high in the fed state. An explanation of these differences could be that global acetylation takes into account all sirts and not just the mitochondrial deacetylase, sirt3. None the less, these conflicting results provide more evidence that acetylation is specific in its regulation of metabolism.

In mouse liver, 62% of acetylated proteins found in the mitochondria were acetylated in both the fed state and after a 12 hour fast[99]. Of those acetylated proteins 14% were specific to fed mice and 24% were specific to fasted mice[99]. This data illustrates some differences in acetylation status between these metabolic states. However, it is unknown the magnitude of the acetylation status of these proteins in both the fed and fasted state and thus research analyzing particular proteins within these states are beneficial. Still et al.[229] analyzed the extent of changes in acetylation status of hepatic mitochondrial acetyl isoforms, variants of acetylated proteins, from mice being fed compared to after a 16 hour fast. Acetyl isoforms were particularly scrutinized due to post-translational modification isoforms having the ability to perform different tasks[102,237-240]. The study revealed that about 10% of the acetyl isoforms exhibited a statistically significant change in acetylation status from the fed to the fasted state[229]. However, research needs to be done to study these isoforms and elucidate first their importance in metabolism and second the physiological ramifications they ensue.

Additionally, PDH has particularly examined under both fed and fasted states. Following a 24 hour fast, the E1-α subunit of mice skeletal muscle PDH in mice is hyperacetylated and even further acetylated in sirt3KO fasted mice[179]. This increase in acetylation corresponded to a decrease in PDH activity[179]. These data suggest acetylation down regulates PDH catalytic activity in the fasted state, which corresponds to data showing an inactive PDH and the shutdown of the unnecessary production of acetyl-CoA from pyruvate during this physiological state[241,242].

Furthermore, hepatic upregulation of sirt3 in response to fasting coincides with the hypoacetylated LCAD found in mice hepatocytes following a 24 hour food withdrawal[162]. Research shows that acetylation negatively regulates this enzyme; therefore, it seems logical for LCAD to be hypoacetylated, thereby more active, in the fasted state to promote FAO for energy

production[162,163,181]. Sirt3KO mice have diminished FAO in the fasted state, promoted the hyperacetylation of LCAD[162]. This provides more evidence of acetylation's negative regulation on LCAD and the importance of acetylation's response to nutrient cues to maintain FAO for energy production during fasting.

Due to its importance in aerobic ATP production, researchers have also analyzed how the change between feeding and fasting alter ATPase acetyl occupancy. In mouse skeletal muscle, Yang et al.[101] illustrated that the ATPase coupling factor 6 (F6) subunit was hyperacetylated in the fed state compared to the fasted. The authors speculate that in this instance acetylation is positively regulating skeletal muscle ATPase, due to the need to increase energy production in the fed state. On the other hand, research has shown that acetylation of OSCP in skeletal muscle decreases ATPase activity[168]. These disparities may be due to the location of these subunits. For instance, F6 resides in the external stalk of ATPase. This stalk stabilizes the core of ATPase, which serves as the catalytic domain. OSCP in particular is located right on top of the this central domain[243]. These different locations may serve different functions or possibly the acetylation of these subunits may promote different conformational changes. Even though the actual reasoning behind these discrepancies are unknown, these studies show the specificity of acetylation's regulation. After either an 18 or a 26 hour fast, hepatic OSCP in mice was found to be hypoacetylated, corresponding to diminished fasting sirt3 levels[101,162,168]. Research shows acetylation negatively regulates hepatic ATPase, suggesting that ATPase is more active in the fasted state, when an energy demand is present[168,184]. The results of studies analyzing acetyl occupancy of metabolic proteins during fed and fasted states demonstrate the sensitivity of reversible acetylation to the nutritional status as well as its crucial role in maintaining metabolic homeostasis.

5. High Fat Diet and Disease

Due to acetylation's sensitivity to the nutrient status of the cell, researchers have also examined how diet macronutrient composition influences the reversible acetylation of metabolic proteins.

The evidence linking fat enriched diets to the onset of metabolic syndrome, prompted scientist to examine the effect HFDs, have on reversible acetylation, particularly mitochondrial proteins due to their pertinent role in fatty acid oxidation[70-72,77,78]. Accordingly, sirt3's role in the maintenance of metabolic homeostasis in response to a HFD has also been examined, due to its robust mitochondrial deacetylase activity[132,135,136,138]. WT mice on a HFD develop obesity, hyperlipidemia, type 2 diabetes, insulin resistance, fatty liver, and the loss of sirt3 exacerbates these effects, illustrating the importance of this deacetylase[163].

Mice on a chronic HFD of 13 weeks or more, but not an acute HFD of a week or less, can decrease sirt3 protein expression and activty[163,165,167]. This decreased expression of sirt3 promotes an increase in mitochondrial protein acetylation[158,163,165,167]. Compared to mice on a standard diet (SD), mice on a 12-day HFD exhibited hyperacetylated mitochondrial proteins within their skeletal muscle and an even greater state of hyperacetylation was observed in sirt3KO mice on the HFD[166]. This indicates that a HFD promotes a hyperacetylated state within the mitochondria and the loss of sirt3 exacerbates this effect. These studies demonstrate sirt3's role in regulating mitochondrial proteins in response to a HFD; and even further, has allowed researchers to utilize sirt3KO mouse models to elucidate the mechanism by which HFD affects the acetylation status of mitochondrial proteins.

Only a few studies have analyzed how HFDs, as well as metabolic diseases, affect the acetylation of individual metabolic proteins. Researchers utilized sirt3KO mouse models to study the effect of a 12-day HFD on the acetylation status of the skeletal muscle isoform of HK, HKII[166]. When active, HKII is bound to the mitochondria via forming a complex with voltage-dependent anion channel (VDAC) and adenine nucleotide translocator (ANT)[244-247]. After a 12 day-HFD, sirt3KO mice displayed decreased HKII-VDAC-ANT complex formation compared to WT littermates, suggesting lower activity of HKII as well as decreased glucose uptake[166]. Even though the mechanism is unknown, this data suggests that HFD induced acetylation leads to

decreased HKII activity by inhibiting complex formation with VDAC and ANT. The data alludes to a link between HFDs and the onset of metabolic diseases associated with decreased glucose uptake, such as T2DM.

In rodents, a 16-week HFD influenced the acetylation status of pyruvate carboxylase (PC), a sirt3 target that catalyzes the first step of gluconeogenesis, in which pyruvate is converted into oxaloacetate[130,165,248]. After 16 weeks of high fat feeding, PC was hyperacetylated compared to mice on a SD[165]. High-fat fed sirt3KO mice exhibited an even further increase in acetylation of PC, suggesting sirt3 plays a role in combating hyperacetylation during a HFD.[165] However, it is not known if hyperacetylation of PC promotes or decreases its activity due to the lack of functional studies. Of note, research shows fasted rats display up to a 3-fold increase in PC activity, corroborating the evidence that gluconeogenesis is stimulated in nutrient deficient states[249]. Even further, obesity and diabetes are associated with increased PC activity, correlating to the inability to suppress gluconeogenesis even in the presence of glucose in these metabolically inflexible states [250,251]. Thus, functional studies are needed in order to elucidate the relationship, if any, between the physiological ramifications of a HFD induced hyperacetylation of PC and the pathogenesis of metabolic syndrome. It would prove interesting if found that hyperacetylation increases PC enzymatic activity, thus providing more evidence linking HFD to metabolic syndrome due to the increased PC activity shown in metabolically inflexible states[251].

WT mice on a 13 week HFD displayed increased acetylation of LCAD when compared to WT mice on a SD, providing more evidence that HFDs promote hyperacetylation of mitochondrial proteins[163]. Sirt3KO on this HFD exhibited an even further hyperacetylated LCAD[163]. Accordingly, the downregulation of LCAD function by acetylation suggests a HFD leads to diminished LCAD enzymatic activity, potentially leading to impaired lipid metabolism.

In the liver of genetically obese mice, LCAD is found to have decreased acetylation compared to WT mice, indicating its increased activity as well as increased hepatic FAO[162,163,181,229]. Both genetically obese mice as well as mice obese due to 25-week HFD diet exhibit higher rates of hepatic FAO[252-254]. Some studies done in obese humans with fatty liver disease also exhibited increased rates of hepatic FAO[255,256]. Therefore, the more active, hypoacetylated LCAD is in concert with data presenting increased rates of hepatic FAO in these obese states.

However, the results of these studies analyzing reversible acetylation of LCAD lead to the question, why would LCAD be hyperacetylated during a HFD but hypoacetylated in obese states? A possible answer could stem from the state of metabolic flexibility of these different animals. Obesity is associated with a state of metabolic inflexibility, in which there is the loss of the ability to switch to glucose even in a fed state[29,53]. It is possible that during this dysfunctional state, even in the presence of glucose excess, FA may serve as the primary source for energy production, making it necessary to upregulate enzymes involved in FAO. In contrast, an organism on a HFD, still attempting to maintain metabolic homeostasis, would react to the influx of FA by attempting to store the fat, downregulate FAO enzymes and continue to utilize glucose as the primary fuel source[59,77,252].

A comparative acetylomic study was performed on WT and diabetes/diabetes (Db/Db) mouse strains[90]. Db/Db contains a leptin receptor mutation that leads to the development of insulin resistance, early onset of obesity, hepatic steatosis, and hyperglycemia that progresses into diabetes[257-259]. This study demonstrated Db/Db mice have increased acetylation of a subunit of the enzyme, adenine nucleotide translocator 1 (ANT1), a transporter of ATP from the mitochondrial matrix to the cytosol [90,260,261]. However, functional studies were not performed; therefore, more research needs to be done to discover if acetylation negatively or positively regulates this protein. This is of importance because research shows decreased expression of ANT1 is linked to metabolic dysfunction. Decreased activity of ANT1 promotes the build-up of

ADP[262,263]. To maintain energy production in a state such as this, cells use two molecules of ADP to produce ATP and AMP [264,265]. AMP can be converted to nucleoside adenosine (ADO) and evidence suggest a buildup of ADO is associated with insulin resistance[265-269]. Moreover, decreased activity of ANT1 has been seen to disrupt mitochondrial membrane potential, thus leading to the increased production of reactive oxygen species and intensifying mitochondrial stress[270].

The acetylation status of hepatic GAPDH has also been studied in the Db/Db mouse strain. Bond et al.[200] found that these Db/Db mice have reduced GAPDH acetylation. This suggests that in states of diabetes and obesity GAPDH is hypoacetylated, which promotes gluconeogenesis[186,199,200]. This theory is in line with the data showing that type 2 diabetics and obese individuals have an increased rate of gluconeogenesis[192,193,207,208]. This provides more evidence that suggests hepatic reversible acetylation has a role in the pathophysiological onset of diabetes within an obesogenic state.

Even further, sirt3 plays a role combating the negative effects of HFD on mitochondrial respiration[158,164,166,168,180]. Lantier et al.[166] examined how the presence or absence of sirt3 influenced mitochondrial respiration in the skeletal muscle of mice either on a SD or a 12 week HFD. Both sirt3KO mice on either a SD or HFD exhibited decreased basal respiration in the presence of malate-glutamate (MG) as well as decreased stimulated respiration in the presence of MG with ADP[166]. This suggests that the absence of sirt3 disrupts respiration from Complex I of the ETC. In contrast to sirt3KO mice on a SD, sirt3KO mice on a HFD exhibited and increase in respiration in the presence of fatty acids[164]. This study suggests a HFD promotes a switch in proper fuel source utilization by increasing reliance on fatty acids and thus contributing to metabolic inflexibility.

6. Conclusion

Acetylation has the ability to coordinate macronutrient metabolism within the liver and skeletal muscle by regulating processes including glycolysis, gluconeogenesis, glycogen metabolism, fatty acid metabolism, the TCA cycle and the ETC. To date, most research involving the regulation of mitochondrial metabolism via acetylation is examined in mice and cell culture. These models cannot truly represent the molecular environments of the human body; therefore, it is highly stressed that more human research be performed. The available data particularly focuses on sirt3KO models and not solely on how HFDs themselves affect acetylation patterns or promote metabolic diseases. However, these models do serve as a good resource due to research suggesting that a decrease in sirt3 expression, and following increase in mitochondrial acetylation is associated with the changes in metabolism[158,159,162-166,179,271]. Lastly, most research examining the role acetylation plays in maintaining metabolic homeostasis is performed in the liver. To fully understand glucose and fatty acid metabolism other metabolically active tissues, particularly the skeletal muscle, needs to be examined. The liver is a tissue that can both utilize and produce energy, while skeletal muscle is solely energy consuming[2,4-9,17,272]. The differing metabolic activities of these tissues may affect how acetylation regulates their metabolism. This review recapitulates the current knowledge on acetylation's role in regulating glucose and fatty acid metabolism and stresses the importance of human research particularly in skeletal muscle mitochondria.

Tables and Figures

Table 1: Cellular Sirtuin Location

Sirtuin	Localization
Sirt1	Nucleus[80,85,132], Cytosol[132,142]
Sirt2	nucleus[143,144], Cytosol[133]
Sirt3	Mitochondria[132,135,136,138,142]
Sirt4	Mitochondria[132,140,273]
Sirt5	Mitochondria[132,141]
Sirt6	Nucleus[132,137,139]
Sirt7	Nucleolus[132,134]

Table 2: Regulation of Glucose and Fatty Acid Metabolic Proteins by Acetylation

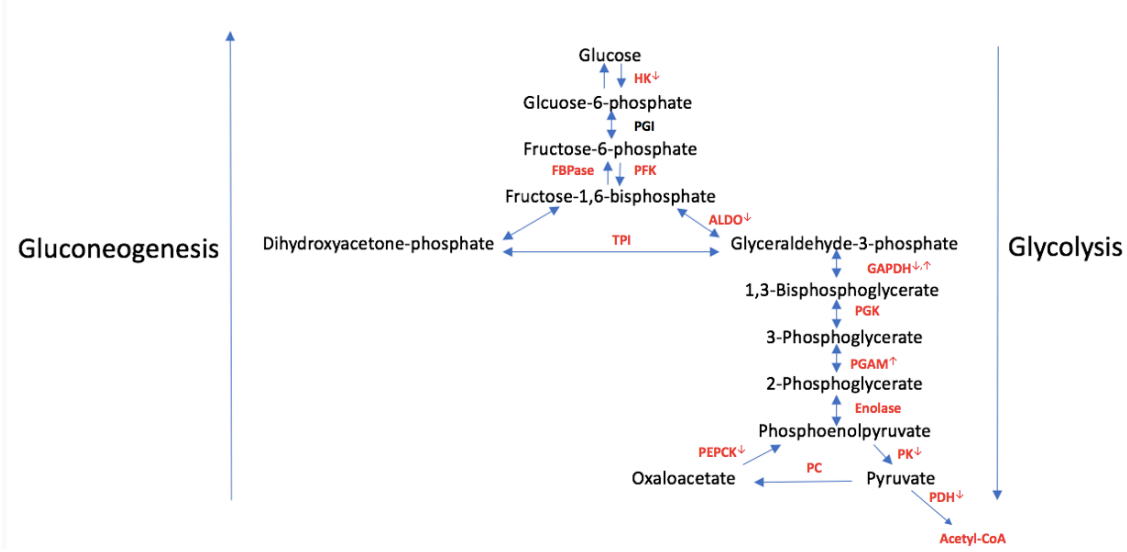
Pathway	Enzyme		Organism	Model	Ramification
Glycolysis	Hexokinase	Hexokinase II	Mouse	Skeletal muscle Tissue	↓[166]
		Glucokinase	Hela cells	Cell culture	↓[195]
	Glucokinase Regulatory Protein (GKRP)		Hela cells	Cell culture	↑[195]
	Phosphofructokinase		Human	Liver Tissue	Unknown[126]
	Aldolase		Rat	Liver Tissue	↓[187]
	Triosephosphate Isomerase		Rat	Kidney Tissue	Unknown[100]
	Glyceraldehyde-3-Phosphate Dehydrogenase		*Salmonella enterica*	Cell culture	↑[186]
			HEK cells	Cell culture	↑[199]
			Mouse	Liver Tissue	↓[200]
	Phosphoglycerate Kinase		Human	Liver Tissue	Unknown[126]
	Phosphoglycerate Mutase		HEK cells	Cell culture	↑[201]
			HCT cells	Cell culture	↑[202]
	Enolase		Mouse	Liver Tissue	Unknown[103]
	Pyruvate Kinase		HEK cells	Cell culture	↓[203]
	Pyruvate Dehydrogenase		Mouse	Skeletal muscle Tissue	↓[179]
Gluconeogenesis	Pyruvate Carboxylase		Mouse	Liver Tissue	Unknown[103]
			Mouse	Liver Tissue	Unknown[165]
	Phosphoenolpyruvate Carboxykinase (PEPCK)		*Saccharomyces cerevisiae*	Cell culture	↓[210]
			HEK cells	Cell culture	↓[209]
			Human	Liver Tissue	↓[126]
	Enolase		Mouse	Liver Tissue	Unknown[103]
	Phosphoglycerate Mutase		HCT cells	Cell culture	↑[202]
	Phosphoglycerate Kinase		Human	Liver Tissue	Unknown[126]
	Glyceraldehyde-3-Phosphate Dehydrogenase		Mouse	Liver Tissue	↑[200]
	Triosephosphate Isomerase		Rat	Kidney Tissue	Unknown[100]
	Aldolase		Rat	Liver Tissue	↓[187]
	Fructose 1,6-Bisphosphatase		Human	Liver Tissue	Unknown[126]
	Glycogen synthase		Human	Liver Tissue	Unknown[126]

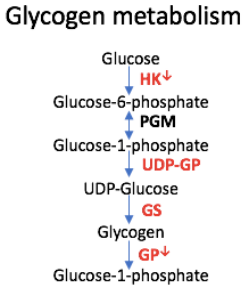
Glycogen Metabolism	UDP-Glucose Pyrophoshorylase	Human	Liver Tissue	Unknown[126]
	Glycogen Phosphorylase	Cheng liver cells	Cell culture	↓[214]
Fatty acid Metabolism	Acetyl-CoA Carboxylase	Human	Liver Tissue	Unknown[126]
	Fatty Acid Synthase	Human	Liver Tissue	Unknown[126]
	Acetyl-Coenzyme A Synthetase 2	*Salmonella enterica*	Cell culture	↓[223]
		Escherichia coli	Cell culture	↓[227]
		Cos-7 cells	Cell culture	↓[142]
	Carnitine-Acylcarnitine Translocase	Mouse	Liver Tissue	Unknown[103]
	Carnitine Palmitoyltransferase I	Mouse	Liver Tissue	Unknown[103]
	Carnitine Palmitoyltransferase II	Mouse	Liver Tissue	Unknown[103]
	Long-Chain Acyl-CoA Dehydrogenase (LCAD)	Mouse	Recombinant protein (assay)	↓[181]
		Mouse	Liver Tissue	↓[162]
		Mouse	Liver Tissue	↓[163]
	Enoyl–Coenzyme A Hydratase/3-Hydroxyacyl–Coenzyme A Dehydrogenase (EHHADH)	Human	Liver Tissue	↑[126]
	Hydroxyacyl-CoA Dehydrogenase, beta subunit (HADHA)	Mouse	Liver Tissue	Unknown[103]
	Acetyl-coenzyme A acetyltransferase 1	Mouse	Liver Tissue	↓[229]
	Propionyl-CoA carboxylase	Human	Liver Tissue	Unknown[126]
	Methylmalonyl-CoA mutase	Mouse	Skeletal muscle Tissue	↓[166]
Tricarboxylic acid Cycle	Citrate Synthase	Mouse	Liver Tissue	Unknown[103]
	Aconitase	Mouse	Liver Tissue	Unknown[103]
	Isocitrate Dehydrogenase	*Saccharomyces cerevisiae*	Cell culture	↓[141]
		HEK cells	Cell culture	↓[185]
	α-Ketoglutarate Dehydrogenase	Mouse	Liver Tissue	Unknown[103]
	Succinyl-CoA Synthetase	Mouse	Liver Tissue	Unknown[103]
	Succinate Dehydrogenase	Mouse	Liver Tissue	↓[233]
		Mouse	Liver Tissue	↓[182]
	Fumarase	Mouse	Liver Tissue	Unknown[103]
	Malate Dehydrogenase 2	Human	Liver Tissue	↑[126]
		Mouse	Liver Tissue	↓[188]

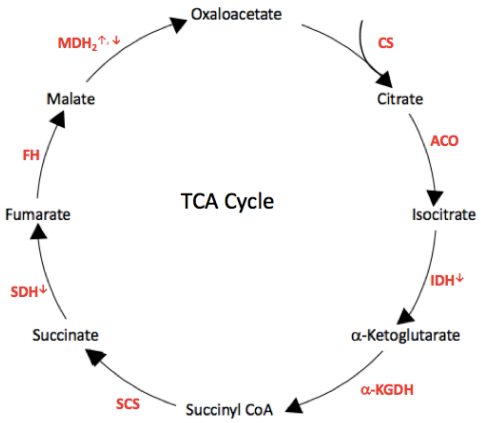
Oxidative Phosphorylation	Complex I	Mouse	Skeletal muscle Tissue	↓[166]
		Mouse	Liver Tissue	↓[165]
		HepG2 cells	Cell culture	↓[158]
	Complex III	Mouse	Liver Tissue	↓[165]
		HepG2 cells	Cell culture	↓[158]
	ATP Synthase	HepG2 cells	Cell culture	↓[158]
		Human osteosarcoma cells	Cell culture	↓[184]

*Correlation +Abundance #Inadvertent effect

HEK, Human embryonic kidney cells; HCT, Human colon carcinoma cells; Hep, Human liver cancer cells; Cos-7, Fibroblast-like monkey kidney cells







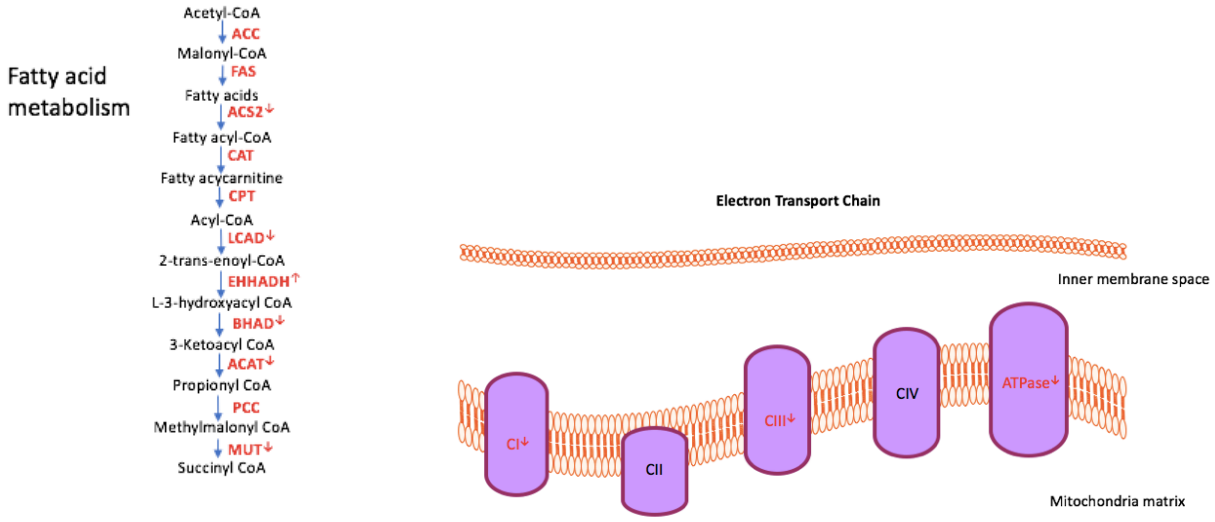


Figure 1: Regulation of Glucose and Fatty Acid Metabolic Proteins by Acetylation

Integral pathways of fatty acid and glucose metabolism including: glycolysis, gluconeogenesis, fatty acid oxidation, the TCA cycle, and the ETC. Enzymes colored in red are enzymes known to be acetylated. The arrows, facing up or down, indicate if acetylation has either a stimulatory or inhibitory effect, respectively. No arrows next to the enzyme indicates no known functional studies have been done. The particular studies that reference these biological ramifications can be found in Table 2.

HK, hexokinase; PGI, Phosphoglucose isomerase; PFK, Phosphofructokinase; TPI, Triosephosphate isomerase; ALDO, Aldolase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PGK, Phosphoglycerate kinase; PGAM, Phosphoglycerate mutase; PK, Pyruvate kinase; PDH, Pyruvate dehydrogenase; PC, Pyruvate carboxylase; PEPCK, Phosphoenolpyruvate carboxykinase; FBPase, Fructose-1,6-bisphosphate; PGM, Phosphoglucomutase; UDP-GP, UDP-Glucose pyrophosphorylase; GS, Glycogen synthase; GP, Glycogen phosphorylase; ACC, Acetyl-CoA carboxylase; FAS, Fatty acid synthase; ACS, Acetyl-Coenzyme A synthetase; CAT, Carnitine-acylcarnitine translocase; CPT, Carnitine palmitoyltransferase; LCAD, Long-chain acyl-CoA dehydrogenase; EHHADH, Enoyl–coenzyme A hydratase/3-hydroxyacyl–coenzyme A dehydrogenase; HADHA, Hydroxyacyl-CoA dehydrogenase, beta subunit (also known as BHAD); ACAT, Acetyl-coenzyme A acetyltransferase; PCC, Propionyl-CoA carboxylase; MUT, Methylmalonyl-CoA mutase; CS, Citrate synthase; ACO, Aconitase; IDH, Isocitrate dehydrogenase; ✔-KGDH, ✔-Ketoglutarate dehydrogenase; SCS, Succinyl-CoA synthetase; SDH, Succinate dehydrogenase; FH, Fumarase; MDH. Malate dehydrogenase; CI, Complex I; CII, Complex II; CIII, Complex III; CIV, Complex IV; ATPase, ATP Synthase.

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CHAPTER III

Specific Aims

We utilized the percent change in fasting skeletal muscle fatty acid oxidation in response to the high-fat diet as a variable to characterize metabolic adaptation to a high-fat diet in humans. Participants that increased fatty acid oxidation in response to the high-fat diet were termed responders, while the participants that decreased fatty acid oxidation were termed non-responders.

Aim 1: Assess fasting skeletal muscle substrate metabolism in responders vs non-responders in response to a 5-day high-fat diet, under controlled feeding conditions.

Hypothesis 1: We hypothesize that prior to and following the high-fat diet, responders will exhibit lower levels of carbohydrate oxidation, which will be associated with higher enzymatic activity of the key oxidative proteins, citrate synthase, malate dehydrogenase, and β-hydroxyacyl-CoA dehydrogenase, compared to non-responders.

Hypothesis 2: We hypothesize the high-fat diet will decrease levels of carbohydrate oxidation but increase enzymatic activity of the key oxidative proteins, citrate synthase, malate dehydrogenase, and β-hydroxyacyl-CoA dehydrogenase in responders, while having an inverse effect on non-responders.

Aim 2: Assess fasting skeletal muscle proteomic analysis of metabolic proteins in responders vs non-responders in response to a 5-day high-fat diet, under controlled feeding conditions.

Hypothesis 1: We hypothesize that prior to and following the high-fat diet, responders will contain lower levels of glycolytic proteins but higher levels of proteins involved in beta oxidation and the electron transport chain, compared to non-responders.

Hypothesis 2: We hypothesize the high-fat diet will decrease the levels of glycolytic proteins but increase the levels of proteins involved in beta oxidation and the electron transport chain proteins, while having an inverse effect on non-responders.

CHAPTER IV

Skeletal muscle metabolic adaption to high-fat feeding

Abstract

Skeletal muscle accounts for up to 50% of the average person's body weight and is highly involved in macronutrient metabolism. To maintain proper energy metabolism and physiology, skeletal muscle must to adapt to available fuel sources, thus making the diet an important modulator of skeletal muscle metabolism. In the last few decades, our society has moved towards consuming more processed foods and diets high in saturated fatty acids. Evidence from rodent and human models show high-fat diets contribute to impaired insulin signaling, as well as abnormal fatty acid and glucose oxidation. The mechanism of how skeletal muscle adapts to high-fat feeding in humans is not yet known.

We sought to elucidate the mechanism of metabolic adaption to short term high-fat feeding and why individuals differentially respond to these diets. We employed a controlled feeding study consisting of a 12-day lead-in diet (30% fat, 55% carbohydrate and 15% protein) followed by a 5-day high-fat diet (HFD, 50% fat, 35% carbohydrate, and 15% protein). Both diets were isocaloric to habitual diets. Muscle biopsies were obtained from the vastus lateralis muscle under fasting conditions prior to and following the HFD, and measures of substrate metabolism (glucose, pyruvate, and fatty acid oxidation) and the skeletal muscle proteome were performed.

We observed differential fatty acid oxidation (FAO) patterns in response to the HFD. Participants that increased FAO in response to the HFD were termed responders, while the participants that decreased FAO were termed non-responders. To elucidate the reason for this differential response to high-fat feeding, we compared responders and non-responders' fasting substrate metabolism and protein content of enzymes involved macronutrient oxidation, prior to and following the HFD. Following the HFD, responders had a significantly higher oxidative efficiency, defined as the ratio of complete to incomplete FAO, than non-responders. However, we did not observe any significant group differences in glucose, prior to or following the HFD. We observed no group differences in malate dehydrogenase or citrate synthase enzymatic

activity; however, responders had an overall lower enzymatic activity of β-hydroxyacyl-coenzyme A dehydrogenase (BHAD), compared to non-responders.

Our proteomic analysis identified 81 proteins and protein subunits involved in substrate metabolism. Independent of the HFD, compared to non-responders, responders contained an overall higher content of protein subunits involved in Complex I (CI) and ATP synthase (ATPase), but lower content of enzymes involved in glycolysis and beta-oxidation. From the subunits identified from our proteomic analysis, 6 were differentially regulated by the HFD. In response to the HFD, responders decreased a subunit of Complex IV (CIV) and ATPase. While non-responders decreased a subunit of isocitrate dehydrogenase (IDH) and increased a subunit content of CI. Surprisingly, non-responders simultaneously decreased and increased two subunits of CIV. The major findings from this study suggest that adaption to high-fat feeding is individual specific. Further, our results suggest that proteomic changes alone cannot explain high-fat feeding induced metabolic changes.

Introduction

Skeletal muscle contributes up to 50% of the average person's body weight[1]. Further, skeletal muscle accounts for up to 95% of insulin-stimulated glucose disposal[2] and under fasting conditions utilizes fatty acids to provide up to 90% of oxidative fuel production[3,4]. Thus, this highly oxidative tissue is essential to whole-body metabolic homeostasis.

To sustain proper energy metabolism and physiology, skeletal muscle must maintain metabolic flexibility, the ability to switch between glucose and fatty acid oxidation in response to nutrient availability[5,6]. Thus, studies on the effect of diet macronutrient composition, such as high fat diets (HFD), on skeletal muscle metabolism are needed. Koves et. al observed that rodents on a 12-week HFD accumulate skeletal muscle mitochondrial fatty acid oxidation (FAO) intermediates, components resulting from incomplete beta-oxidation that contribute to impaired insulin signaling and decreased glucose oxidation in their skeletal muscle[7-11]. Furthermore, in as

little as 3 days, healthy men on a HFD exhibited decreased FAO, diminished enzymatic activity of electron transport chain (ETC) proteins, and decreased expression of oxidative genes and proteins[12,13]. However, the human study that found a decrease in protein content utilized an overfeeding protocol[12]. The positive energy balance in this study inhibits the ability to elucidate how high-fat feeding alone affects metabolism.

To understand how skeletal muscle adapts to altered dietary macronutrient composition we must elucidate how skeletal muscle is regulated. Deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins are all major metabolic regulatory targets. Regulatory steps in the cell cycle controls DNA expression[14], while regulation of transcription, the process that produces RNA from DNA, modulates RNA expression[14]. Regulation of protein expression can occur during translation, the process by which protein is synthesized from RNA[14], as well as post-translationally[14]. Many techniques, such as mass spectrometry have allowed researchers to study how skeletal muscle proteins, in particular metabolic proteins, are regulated. For instance, Kim et al.[15] found that rats fed a HFD for 8 weeks compared to mice fed a low-fat diet had no differences in glycolytic protein levels but had differences in structural and contractile protein levels. However, the physiological ramifications of dietary composition on skeletal muscle metabolism is not fully understood. The goal of this study is to understand and characterize metabolic adaption to high-fat feeding in human skeletal muscle. To this end, we assessed functional measures of substrate metabolism paired with proteomic analyses of skeletal muscle samples collected from participants under controlled feeding conditions.

Material and Methods

Study participants

Thirteen non-obese (body mass index [BMI], 18 - 30 kg/m^2) males (18-40 years old) served as participants for the study. Participants were sedentary ($\leq$ 2 days, 20 min/day of low-intensity physical activity) and weight stable ($< \pm$ 2.5 kg) for 6 months prior to initiating the study. All participants were normotensive (blood pressure < 140/90 mmHg), normoglycemic (fasting

glucose < 100 mg/dL), and normolipidemic (low-density lipoprotein [LDL] cholesterol < 130 mg/dL, total cholesterol < 200 mg/dL, and triglycerides < 250 mg/dL). The participants were not under the influence of any medications known to affect study measures. Participant habitual caloric intake was composed of < 40% fat, and of that fat content < 15% saturated fat. Our exclusion criteria included family history of type 2 diabetes mellitus (T2DM), any known cardiometabolic condition, smokers, moderate to heavy drinkers and those with a high fat habitual diet (determined by dietary food records). 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

A controlled feeding, cross-over design was employed for this study (Figure 1). Following successful completion of screening procedures, participants began a 2-week lead-in feeding period where they consumed a control diet isocaloric to their habitual diet. Following this lead-in period, the participants came to the lab after an overnight fast and a muscle biopsies were obtained from their vastus lateralis using a modified Bergstrom-type needle with suction[16,17]. Following the control diet, participants were placed on a 5-day high-fat diet (HFD), which was isocaloric to the control diet. Following the 5 days of high-fat feeding, the participants again came to the lab after an overnight fast and another muscle biopsy was taken from their vastus lateralis.

Controlled feeding producers

4-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 3 weekdays and 1 weekend day. The research dietitian used the 3-pass method to review habitual diet records with the participants[18]. 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[19]. Both the control diet and HFD operated on a 7-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. Each diet was isocaloric to the participants' daily energy requirements. During the two-week lead-in controlled feeding period participants were required to consume planned meals consisting of approximately 55% carbohydrate, 30% fat (< 10% saturated fat), and 15% protein. The 5-day HFD was composed of planned meals containing approximately 50% fat (45% saturated fat equal to 25% of total calories) 35% carbohydrate, and 15% protein. Diets aimed to provide 3 g of fiber per 500 kcal (± 5 g). All meals were prepared in the Department of Human, Nutrition, Foods and Exercise Metabolic Kitchen. Participants ate breakfast in the Dining Laboratory for Eating Behavior and Weight Management every day and took the remaining food for the day with them. Participants were weighed 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 be instructed to report consumption of all non-study foods.

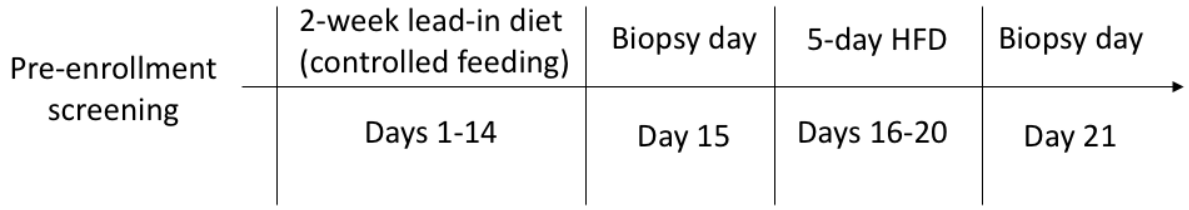

Figure 1. Schematic of research design

Following screening procedures, participants began a 2-week lead-in diet that was isocaloric to their habitual diet. Following the lead-in diet, the participants came to the lab after an overnight fast and a muscle biopsy was take from their vastus lateralis. The participants were then put on a 5-day high-fat diet (HFD). Following the HFD, the participants again came to the lab after an overnight fast and a muscle biopsy was take from their vastus lateralis.

Measurements

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).

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.

Muscle Biopsies. Muscle samples were immediately placed in ice cold phosphate-buffered saline (PBS) to remove blood and connective tissue. Muscle tissue used to assess substrate oxidation was immediately placed in 200μL 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 for mass spectrometry 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 (Thermo Scientific, Pittsburgh, PA), then snap-frozen in liquid nitrogen.

Substrate Metabolism. 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). The samples were then homogenized in a Potter-Elvehjem glass homogenizer (Thomas Scientific, Swedesboro, NJ) at 10 passes across 30 seconds at 150 RPM with a motor-driven Teflon pestle. Substrate oxidation 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. Samples were incubated in 0.5 μCi/mL of [1-^{14}C]-palmitic acid for 1 hour after which the media was acidified with 200 μL 45% perchloric acid for 1 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 of scintillation fluid. The vial's ^{14}C concentrations were measured on a 4500 Beckman Coulter scintillation counter (Indianapolis, IN). Acid soluble metabolites

were determined by collecting the acidified media and measuring ^{14}C levels. Pyruvate oxidation was measured with methods similar to that of fatty acid oxidation with the exception of a substitution of [1-^{14}C]-pyruvate for [1-^{14}C]-palmitic acid. A substrate preference assay, denoted by the percentage decrease in pyruvate oxidation in the presence of free fatty acid, was assessed by measuring [1-^{14}C]-pyruvate oxidation in the presence or absence of non-labeled BSA (0.5%) bound-palmitic acid. Substrate preference is expressed as FFA-suppressed pyruvate oxidation (%), e.g., the decrease of pyruvate oxidation in the presence of FFA.

Enzyme Kinetics. 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 170 µl of a solution containing Tris buffer (0.1 M, pH 8.3), DNTB (1 mM, in 0.1 M in Tris buffer) and oxaloacetate (0.01 M, in 0.1 M Tris buffer). Following a 2-minute background reading, the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) was calibrated and 30 µl of 3 mM acetyl-CoA was added to initiate the reaction. Absorbance was measured at 405 nm at 37°C every 12 seconds for 7 minutes. Maximum CS activity was calculated and reported as µmol/min/mg.

Malate dehydrogenase (MDH) activity was measured spectrophotometrically at 340nm at 37°C. Briefly, ten microliters of sample was pipetted in triplicate in wells. Then, 290 µl 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 5 minutes at 340 nm. The rate of disappearance of NADH was analyzed and expressed relative to protein content. Maximum MDH activity was calculated and reported as µmol/min/mg.

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

Corporation, Sunnyvale California) was calibrated and 15 µl of 2 mM acetoacetyl CoA was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 6 minutes at 37°C. Maximum BHAD activity was calculated and reported as µmol/min/mg.

Mass spectrometry. Frozen muscle tissue samples allocated for mass spectrometry 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 12,000 *g* for 10 min at 4°C to remove insoluble components. Supernatant protein concentrations were determined spectrophotometrically using the bicinchoninic acid assay (BCA) (Thermo Scientific). The samples were transferred to 15 ml Falcon tubes and brought to 1 ml using freshly prepared 100 mM ammonium bicarbonate (AmBic). The samples were then sonicated utilizing a Sonic Dismembrator Model 500 (Fisher Scientific) equipped with a microtip for 5 cycles of 10 sec each at 10% amplitude with 50 sec cooling on ice between each cycle. Protein was precipitated by the addition of 8 ml acetone and 1 ml methanol and incubation over night at -80˚C. Protein was recovered by centrifugation at 3696 x g in a chilled (8˚C) swinging bucket rotor table top centrifuge for 15 minutes. Protein pellets were resuspended at 5 mg/ml in freshly prepared 8 M urea containing 100 mM AmBic. Disulfide bonds were reduced by incubation at 37˚C for 1 hour in the presence of 10 mM dithiothreitol (DTT). Free sulhydryls were then alkylated by incubation in the dark for 30 minutes in the presence of 55 mM iodoacetamide (IAA). Unreacted IAA was quenched by the addition of 55 mM DTT. Urea concentration was brought to 4 M by the addition of 100 mM AmBic, LysC (Wako) was added at a ratio of 1:130, and the digestion was carried out over night at 37˚C. The following day the urea concentration was brought to 1.6 M by the addition of 100 mM AmBic, trypsin (Pierce) was added at a ratio of 1:100, and the digestion was carried out for 4 hours at 37˚C. Digestions were desalted using Oasis HLB 1 cc 10 mg extraction cartridges (Waters). An aliquot (1/20 of each eluate) was set aside for analysis of the total proteome. Samples were dried using a vacuum concentrator then resuspended in water containing 0.1% (v/v) formic acid.

Total proteome (unenriched) samples were analyzed using an Orbitrap Fusion Lumos equipped with an Easy-nLC 1200 UPLC, and an Easy Spray nanospray source (Thermo Scientific). The

column utilized for peptide separation was a PepMap RSLC C18 2 μm 100 A 50 μm x 15 cm (Thermo Scientific). The system also utilized an Acclaim PepMap 100 100 μm x 2 cm as a trapping column. Analysis utilized a 110-minute gradient from 4 to 50% solvent B where A is 0.1% (v/v) formic acid in water and B is 20:80 water:acetonitrile containing 0.1% (v/v) formic acid with the column temperature maintained at 55˚C and the ion transfer tube at 275˚C. Conditions for analyzing total proteome (unenriched) peptides were as follows: 2 μl injection, 400 nl/min flow rate, 2400 V ion spray voltage, MS scans utilizing the orbitrap at 60000 resolution for m/z 350-1550, an agc target of 1e6 with 100 ms max inject time, profile, positive, MSMS targets chosen using MIPS looking for peptide-like isotopic distribution with a z=2-5 and a minimum intensity of 2e3, dynamic exclusion for 120 sec if n=3 within 90 sec, priority given to the most intense with a max cycle time of 3 sec (Top Speed mode), MSMS isolation window of 2 m/z, HCD at 35±5, ion trap rapid scan detection using a normal m/z range with first mass at 110, agc target of 2e4 with a max inject time of 1000 ms, centroid.

Progenesis QI for proteomics software (version 2.4, Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) was used to perform ion-intensity based label-free quantification. Raw files were imported and converted into two-dimensional maps (y-axis = time, x-axis =m/z) followed by selection of a reference run for alignment purposes. An aggregate data set containing all peak information from all samples was created from the aligned runs, which was then further narrowed down by selecting only +2, +3, and +4 charged ions for further analysis. The samples were then grouped in control versus lipidema. A peak list of fragment ion spectra from only the top eight most intense precursors of a feature was exported in Mascot generic file (.mgf) format and searched against the human SwissProt_2017_10 database using Mascot (Matrix Science, London, UK; version 2.4). The search variables that were used were: 10 ppm mass tolerance for precursor ion masses and 0.5 Da for product ion masses; digestion with trypsin; a maximum of two missed tryptic cleavages; variable modifications of oxidation of methionine and phosphorylation of serine, threonine, and tyrosine; ^{13}C=1. The resulting Mascot .xml file was then imported into Progenesis, allowing for peptide/protein assignment, while peptides with a Mascot Ion Score of <25 were not considered for further analysis. Protein quantification and logarithmic transformation was performed using only non-conflicting peptides and precursor ion-abundance

values were normalized in a run to those in a reference run (not necessarily the same as the alignment reference run).

Statistical analysis

Data was screened for outliers, violations of normality and missing data. If suspected, a Q test was performed to identify outliers. If identified, outliers were replaced by the most extreme value in that tail of the distribution. Two-way repeated measures analysis of variance was used to determine group differences in the fasting response pre- and post-HFD. Multiple comparisons were performed using an ANOVA with a Tukey post-hoc analysis. Independent t-tests were used to compare protein or metabolite levels between or within groups, pre and post HFD. All data was expressed as means ± standard error of the mean (SEM). The significance level was set *a priori* at $\alpha = .05$.

Results

Study participant characteristics

Following the high-fat diet (HFD), we identified all 13 participants' percent change in fasting total skeletal muscle fatty acid oxidation (FAO). Study participant percent change in fasting total FAO are shown in Figure 1. We used the median value to divide the participants into two groups. We selected the top subjects that increased total FAO in response to the HFD and classified them as responders (n=4). We selected the subjects that exhibited the greatest decrease in total FAO in response to the HFD and classified them as non-responders (n=3). Only data from these subjects are presented herein. Responders and non-responders' percent change in fasting total, complete, and incomplete skeletal muscle FAO in response the HFD, are shown in Table 1.

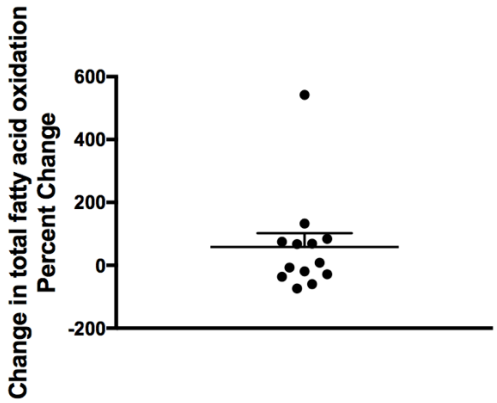

Figure 1. Change in fasting total fatty acid oxidation
Participants percent change in fasting total skeletal muscle FAO in response to the HFD. n=13.

Table 1. Responders and non-responder's change in fatty acid oxidation

Group	Subject	Total Fatty Acid Oxidation (%)	Complete Fatty Acid Oxidation (%)	Incomplete Fatty Acid Oxidation (%)
Responder	15	542.2	1470.0	501.3
	6	133.1	366.7	117.6
	5	84.6	297.5	72.1
	16	74.9	522.2	59.5
Non-responder	12	-28.5	32.4	-31.4
	18	-36.6	2.8	-38.9
	10	-73.9	-66.2	-74.4

The subjects that increased total FAO in response to the HFD are referred to as responders, while the subjects that decreased total FAO in response to the HFD are referred to as non-responders Values expressed as percent change in response to HFD. Responders n=4. Non-responders n=3.

Subject anthropometrics are shown in Table 2. Prior to starting the HFD, compared to non-responders, responders had higher fasting serum triglycerides (TAG) ($P < 0.05$; Table 2). In response to the HFD, responders decreased the amount of circulating serum TAGs ($P < 0.05$; Table 2), while the HFD had no effect on non-responders fasting serum TAGs. Responders and non-responders did not differ in age, height, body mass index (BMI), body mass, lean mass, fat mass, percent body fat or fasting serum free fatty acids (FFA), prior to or following the HFD. The HFD had no effect on BMI, body mass, lean mass, fat mass, percent body fat or serum fasting FFA in either group.

Table 2. Participant characteristics before and after the high-fat diet

Variables	Responders		Non-responders	
	Pre	Post	Pre	Post
Age (years)	22.0 ±0.4	-	22.7 ± 1.8	-
Height (inches)	68.7 ± 1.8	-	70.1 ± 2.6	-
Body mass index (kg m^{-2})	23.1 ± 1.1	23.2 ± 1.2	25.9 ± 2.3	25.8 ± 2.2
Body mass (kg)	71.0 ± 7.3	71.3 ± 7.3	81.4 ± 5.5	81.1 ± 5.4
Lean body mass (kg)	51.8 ± 3.0	52.9 ± 3.2	57.6 ± 2.1	57.6 ± 2.4
Fat mass (kg)	16.2 ± 4.2	15.5 ± 4.0	20.4 ± 3.8	20.1 ± 3.6
Percent body fat (%)	22.9 ± 3.2	21.8 ± 3.1	25.8 ± 3.1	25.4 ± 2.9
Serum free fatty acids (μM)	522.5 ± 150.8	455.2 ± 13.0	354.7 ± 171.4	368.0 ± 94.3
Serum triglycerides (mg/dL)	100.2 ± 11.1	62.7 ± 12.9*	48.1 ± 5.5†	30.6 ± 2.2

Values expressed as mean ± Standard error (SE). *Pre vs. Post HFD ($p<0.05$). †Independent of diet, responder vs non-responder ($p<0.05$). Responders n=4. Non-responders n=3.

Fatty Acid Oxidation

The absolute values of total, complete, and incomplete skeletal muscle FAO of responders and non-responders, pre and post the HFD, are shown in Figure 2. Prior to HFD, responders had less total, complete, and incomplete skeletal muscle FAO than non-responders ($P < 0.05$; Fig. 2A-C). Following the HFD, responders significantly increased complete FAO ($P < 0.05$; Fig. 2A), while no change was observed in the non-responders group. Responders and non-responders' oxidative efficiency, defined as the ratio of complete to incomplete FAO, are shown in Figure 2D. Both the responders and non-responders increased oxidative efficiency ($P < 0.05$; Fig. 2D), in response to the HFD.

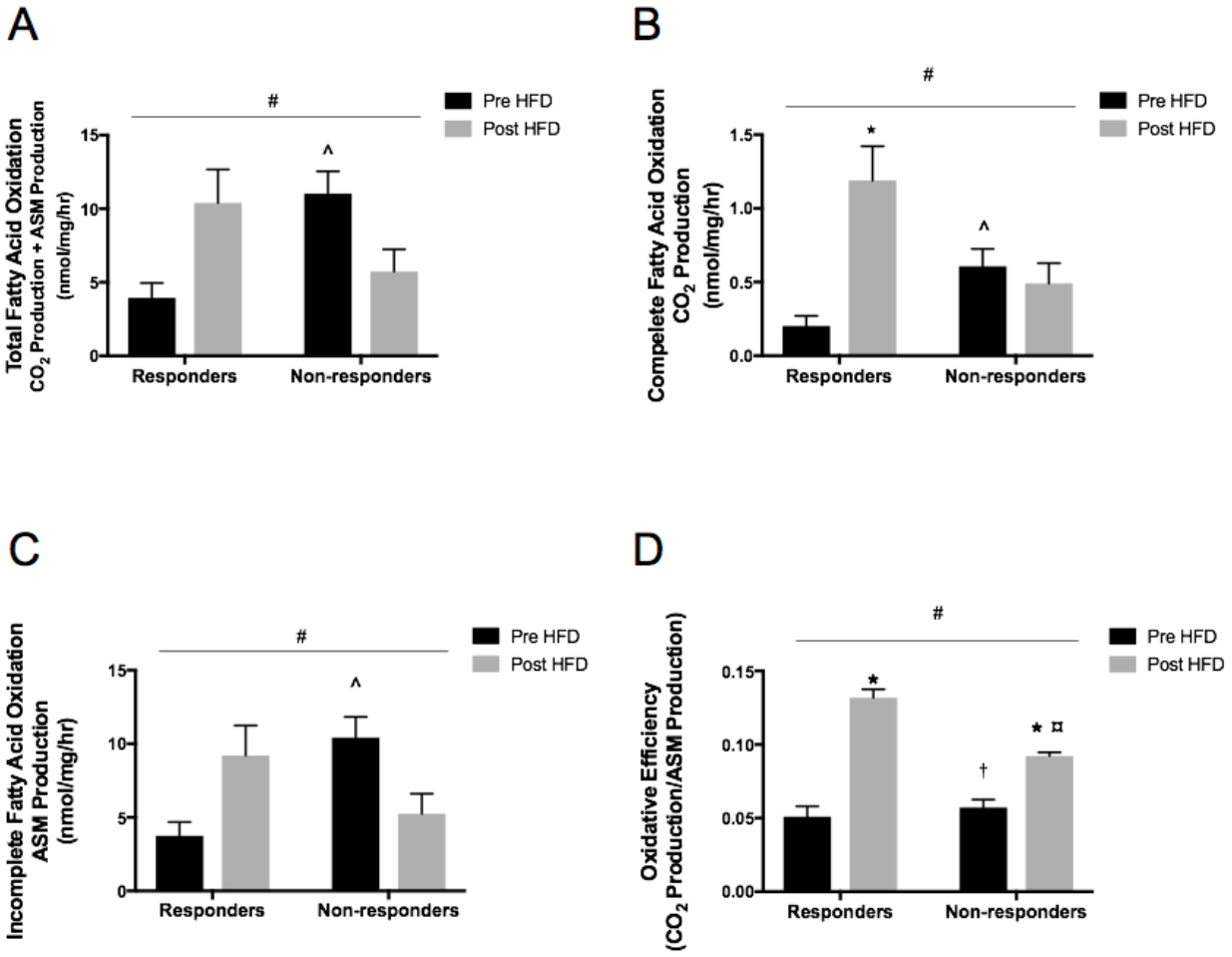

Figure 2. Fatty acid oxidation

Total (a), complete (b), and incomplete (c) fatty acid oxidation of responders and non-responders before and after the high-fat diet. Oxidative efficiency (d), expressed as the ratio of complete to incomplete fatty acid oxidation, of responders and non-responders before and after the high-fat diet. Values are expressed as mean ± SE. *pre vs. post HFD ($p < 0.05$); ^responder pre HFD vs non-responder pre HFD ($p < 0.05$); ¤responder post HFD vs non-responder post HFD ($p < 0.05$); †Independent of diet, responder vs non-responder ($p<0.05$); $^{\#}$Diet x Group (responder vs non-responder) interaction ($p < 0.05$). Responders n=4. Non-responders n=3.

Carbohydrate Oxidation

Responder and non-responder glucose and pyruvate oxidation are shown in Figure 3A and B, respectively. Glucose oxidation was not different between groups, prior to or in response to the

HFD. Pyruvate oxidation exhibited a significant group-diet interaction, which was likely driven by the decrease in pyruvate oxidation in non-responders in response to HFD ($P < 0.05$; Fig. 3B). Responders and non-responders' substrate preference, determined by the capacity for FFA to suppress pyruvate oxidation, are shown in Figure 3C. Substrate preference was not different between groups, prior to or in response to the HFD.

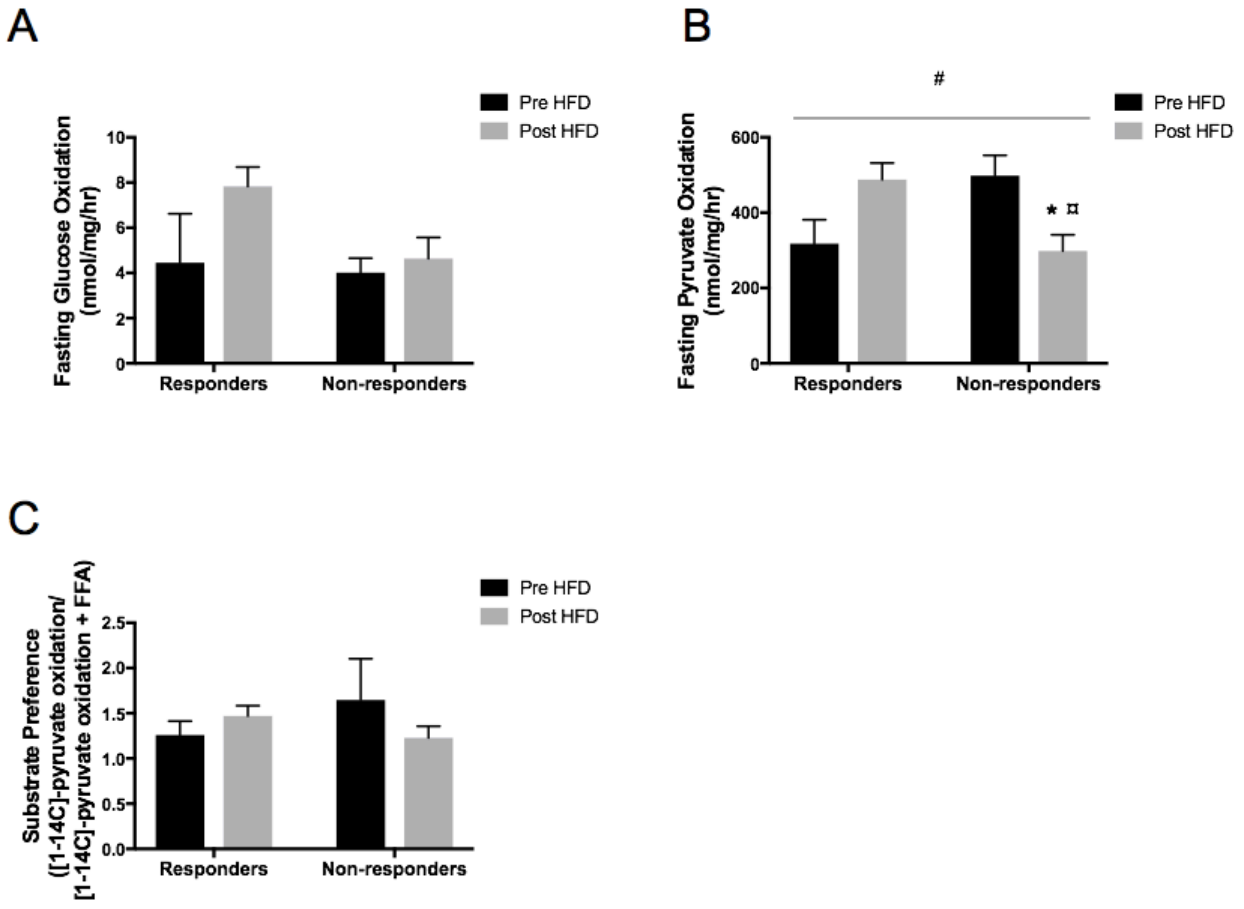

Figure 3. Carbohydrate Oxidation

Glucose (a) and pyruvate (b) oxidation of responders and non-responders before and after the high-fat diet. Substrate preference (c), determined by the capacity for FFA to suppress pyruvate oxidation, in responders and non-responders before and after the HFD. Values are expressed as mean ± SE. *pre vs. post HFD ($p < 0.05$); ¤responder post HFD vs non-responder post HFD ($p < 0.05$); $^{\#}$Diet x Group (responder vs non-responder) interaction ($p < 0.05$). Responders n=4. Non-responders n=3.

Enzymatic Activity

Responders and non-responders enzymatic activities of malate dehydrogenase (MDH), citrate synthase (CS), and β-hydroxyacyl-coenzyme A dehydrogenase (BHAD), pre and post HFD, are shown in Table 3. Responders and non-responders MDH and CS activity did not significantly differ, prior to or in response to the HFD. Independent of diet, there was an overall higher BHAD activity in the responders group compared to non-responders ($P < 0.05$; Table 3). The ratio of BHAD activity to CS activity of responders and non-responders are shown in Figure 4. Independent of diet, the ratio of BHAD activity to CS activity was overall lower in responders compared to non-responders ($P < 0.05$; Fig 4). Following the HFD, responders had a significantly lower ratio of BHAD to CS activity compared to non-responders ($P < 0.05$; Fig 4).

Table 3. Fasting enzymatic activity pre and post the high-fat diet

Enzyme (nmol /mg prot/min)	**Responders**		**Non-responders**	
	Pre	Post	Pre	Post
Malate dehydrogenase	1686.5 ± 164.8	1955.3 ± 205.8	1956.7 ± 205.6	1500.0 ± 310.7
Citrate synthase	124.0 ± 19.8	131.6 ± 16.6	102.1 ± 18.3	73.4 ± 0.4
β-hydroxyacyl-coenzyme A dehydrogenase	37.8 ± 11.9	28.2 ± 2.1	52.6 ± 2.7†	59.4 ± 8.1

Values are expressed as mean ± SE. †Independent of diet, responder vs non-responder ($p<0.05$). Malate dehydrogenase and citrate synthase responders n=4, non-responders n=3. β-hydroxyacyl-coenzyme A dehydrogenase responders n=2, non-responders n=3.

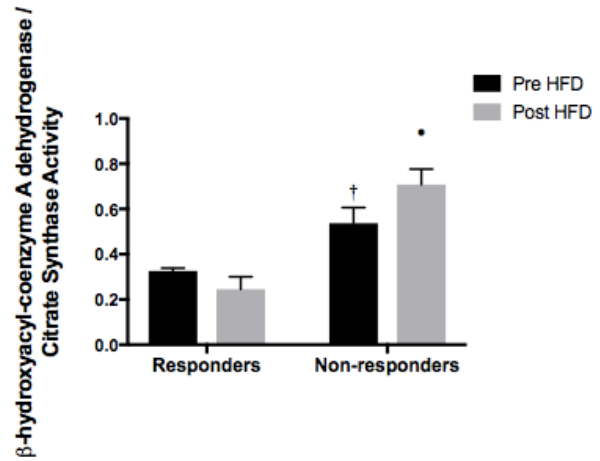

Figure 4. The ratio of BHAD activity to CS activity

Values are expressed as mean ± SE. •responder post HFD vs non-responder post HFD ($p<0.05$); †Independent of diet, responder vs non-responder ($p<0.05$). Responders n=2. Non-responders n=3.

Proteome

Glycolysis. The levels of skeletal muscle proteins involved in glycolysis, pre and post the HFD, are shown in Table 4. Of all the glycolytic enzymes measured, β-enolase was found to significantly different between groups. Independent of diet, responders had lower levels of β-enolase, compared to non-responders ($P < 0.05$; Table 4).

Beta-oxidation. The levels of skeletal muscle proteins involved in beta-oxidation, pre and post the HFD, are shown in Table 4. Of the beta-oxidation enzymes measured, short-chain acyl-CoA dehydrogenase (SCAD) and medium-chain acyl-CoA (MCAD) were found to significantly differ between groups. Independent of diet and compared to non-responders, responders had significantly lower levels of SCAD ($P < 0.05$; Table 4). Prior to the HFD and compared to non-responders, responders had significantly higher levels of MCAD ($P < 0.05$; Table 4).

Tricarboxylic acid cycle. The levels of skeletal muscle proteins and protein subunits involved in the tricarboxylic acid (TCA) cycle, pre and post the HFD, are shown in Table 4. Of all the TCA enzymes measured, the α subunit of mitochondrial isocitrate dehydrogenase (IDH) was found to significantly differ between groups. Independent of diet, responders had overall higher levels of

the α subunit IDH, compared to non-responders ($P < 0.05$; Table 4). Of all the TCA enzymes measured, the HFD only affected the levels of the α subunit of succinnyl-CoA synthetase (SCS). In non-responders, the HFD significantly decreased the of the α subunit of SCS ($P < 0.05$; Table 4), while the levels of responders α subunit of SCS exhibited no change.

Electron transport chain. The levels of skeletal muscle proteins and protein subunits involved in the electron transport chain (ETC), pre and post the HFD, are shown in Table 4. Of the complexes involved in the electron transport chain, the subunits of Complex I (CI) exhibited the most significant changes between groups and in response to the HFD. Independent of diet, responders had overall higher levels of subunit 8 from the α subcomplex of CI ($P < 0.05$; Table 4), compared to non-responders. Prior to the HFD and compared to non-responders, responders had significantly higher levels of subunit 6 from the α subcomplex of CI, the chain 5 subunit of CI, along with subunits 2 and 3 from the flavoprotein subcomplex of CI ($P < 0.05$; Table 4). Non-responders significantly increased the levels of subunit 8 of the iron-sulfur subcomplex of CI ($P < 0.05$; Table 4), while responders exhibited no change. No subunit of Complex II (CII) was found to significantly differ between groups or in response to the HFD.

Prior to the HFD and compared to non-responders, responders had significantly higher levels of of subunits 2 and 6 of Complex III (CIII) ($P < 0.05$; Table 4). Non-responders increased the levels of Complex IV (CIV) subunit 5B in response to the HFD ($P < 0.05$; Table 4), while responders exhibited no changes. Non-responders decreased the of CIV subunit NDUFA4 in response to the HFD ($P < 0.05$; Table 4), while responders exhibited no changes. Responders and non-responders exhibited a group and diet interaction in which responders significantly decreased the levels of CIV subunit 4 isoform 1 in response to the HFD ($P < 0.05$; Table 4).

Independent of diet and compared to non-responders, responders exhibited higher levels of ATP synthase (ATPase) subunit α ($P < 0.05$; Table 4). Prior to the HFD and compared to non-responders, responders exhibited a higher levels of ATPase subunit O ($P < 0.05$; Table 4). Responders decreased the levels of ATPase subunit f6 in response to the HFD ($P < 0.05$; Table 4), while no changes were observed in non-responders.

Table 4. Fasting skeletal muscle proteome pre and post the HFD

Pathway	Uniprot ID	Protein/Protein subunit	Responders		Non-responder	
			Pre HFD (Relative abundance)	**Post HFD** (Relative abundance)	**Pre HFD** (Relative abundance)	**Post HFD** (Relative abundance)
Glycolysis	P19367	Hexokinase-1	0.8 ± 0.06	0.52 ± 0.2	0.3 ± 0.2	0.4 ± 0.04
	P08237; Q01813	ATP-dependent 6-phosphofructokinase	413.6 ± 42.7	513.9 ± 99.3	394.1 ± 91.1	408.7 ± 58.6
	P04075; P05062	Fructose-bisphosphate aldolase A	5736.6± 1355.4	5989.1 ± 1459.8	6227.6 ± 1123.9	5820.3 ± 830.0
	P60174	Triosephosphate isomerase	1750.1 ± 46.1	2060.9 ± 289.9	1861.9 ± 258.6	1912.1 ± 362.4
	P04406; O14556	Glyceraldehyde-3-phosphate dehydrogenase	6348.5 ± 1059.7	9244.0 ± 1169.4	10595.1 ± 2875.3	8423.2 ± 1422.8
	P00558	Phosphoglycerate kinase 1	631.0 ± 61.6	743.1 ± 72.5	603.5 ± 108.3	74210.0 ± 223.7
	P18669	Phosphoglycerate mutase 1	2.2 ± 0.5	2.5 ± 0.4	2.2 ± 0.4	3.0 ± 0.5
	P13929	β-enolase	1340.2 ± 240.5	1678.7 ± 79.4	2696.6 ± 805.7†	2059.8 ± 256.5
	P14618; P30613	Pyruvate kinase	1335.7 ± 142.6	1675.3 ± 147.4	1389.3 ± 128.6	1521.4 ± 355.7
	P08559; P29803	Pyruvate dehydrogenase E1 component subunit α	28.9 ± 3.8	22.8 ± 6.1	19.4 ± 4.6	25.1 ± 4.9
	P11177	Pyruvate dehydrogenase E1 component subunit β	30.8 ± 5.7	33.5 ± 7.4	20.4 ± 6.6	26.8 ± 5.5
Beta Oxidation	P16219	Small chain acyl-CoA dehydrogenase	0.4 ± 0.2	0.7 ± 0.2	1.7 ± 0.4†	1.9 ± 0.3
	P11310	Medium chain acyl-CoA dehydrogenase	18.5 ± 0.3	24.8 ± 7.3	5.6 ± 0.1$^{\wedge}$	12.3 ± 3.9
	P49748	Very long chain acyl-CoA dehydrogenase	66.3 ± 14.7	83.4 ± 21.2	103.5 ± 24.7	70.7 ± 16.3
	P30084	Enoyl-CoA hydratase	11.9 ± 3.4	8.9 ± 2.2	5.4 ± 0.8	7.5 ± 1.7
	Q16836	β-hydroxyacyl-coenzyme A dehydrogenase	34.6 ± 1.2	38.2 ± 6.8	38.6 ± 10.5	26.7 ± 7.2
	P42765	3-ketoacyl-CoA thiolase	4.5 ± 0.5	7.4 ± 2.4	4.5 ± 1.4	4.7 ± 1.9
Tricarboxylic acid cycle	O75390	Citrate synthase	114.5 ± 16.3	109.3 ± 24.7	87.9 ± 3.0	104.2 ± 22.6
	Q99798	Aconitase	196.9 ± 18.6	194.4 ± 34.2	151.5 ± 4.9	149.4 ± 47.9
	P50213	Isocitrate dehydrogenase subunit α	0.4 ± 0.1	0.3 ± 0.06	0.07 ± 0.02†	0.1 ± 0.07
	O43837	Isocitrate dehydrogenase subunit β	2.3 ± 1.1	6.7 ± 2.5	7.2 ± 3.0	2.8 ± 0.7
	P51553	Isocitrate dehydrogenase subunit γ	0.09 ± 0.06	0.03 ± 0.02	0.003 ± 0.0006	0.02 ± 0.02
	Q02218; Q9ULD0	α-ketoglutarate dehydrogenase	25.9 ± 4.3	26.1 ± 5.6	22.0 ± 5.7	21.3 ± 2.8

	P53597	Succinnyl-CoA synthetase subunit α	1.3 ± 0.4	0.5 ± 0.2	0.6 ± 0.1	0.3 ± 0.1*
	Q9P2R7	Succinnyl-CoA synthetase subunit β	18.4 ± 4.9	26.7 ± 6.0	18.5 ± 2.2	14.3 ± 5.5
	P31040	Succinate dehydrogenase flavoprotein subunit	18.7 ± 2.6	17.6 ± 3.7	13.0 ± 0.9	12.0 ± 3.8
	P21912	Succinate dehydrogenase iron-sulfur subunit	26.5 ± 3.8	27.9 ± 4.6	17.8 ± 0.3	19.9 ± 10.0
	P07954	Fumarase	31.2 ± 9.5	30.9 ± 9.0	29.1 ± 4.4	27.8 ± 10.7
	P40926	Malate dehydrogenase	522.5 ± 74.5	455.7 ± 93.7	436.9 ± 40.5	379.7 ± 87.7
Electron Transport Chain	O95299	Complex I alpha subcomplex subunit 10	3.0 ± 1.0	3.0 ± 0.8	3.6 ± 0.5	2.9 ± 0.2
	Q9UI09	Complex I alpha subcomplex subunit 12	2.1 ± 0.8	1.3 ± 0.3	0.8 ± 0.4	1.3 ± 0.3
	Q9P0J0	Complex I alpha subcomplex subunit 13	2.9 ± 0.8	2.0 ± 0.5	1.2 ± 0.1	1.8 ± 0.6
	O43678	Complex I alpha subcomplex subunit 2	2.5 ±0. 9	2.3 ± 0.7	1.0 ± 0.4	2.2± 0.7
	Q16718	Complex I alpha subcomplex subunit 5	4.9 ± 1.6	3.8 ± 1.1	3.3 ± 0.4	4.0 ± 1.1
	P56556	Complex I alpha subcomplex subunit 6	0.9 ± 0.09	0.7 ± 0.2	0.3 ± 0.1^	0.5 ± 0.2
	O95182	Complex I alpha subcomplex subunit 7	5.5 ± 1.7	3.8 ± 1.4	3.3 ± 1.7	4.1 ± 1.8
	P51970	Complex I alpha subcomplex subunit 8	0.7 ± 0.2	0.3 ± 0.1	0.1 ± 0.03†	0.2 ± 0.07
	Q16795	Complex I alpha subcomplex subunit 9	6.2± 1.8	7.5 ± 3.0	3.1 ± 0.4	6.3 ± 2.5
	O75438	Complex I beta subcomplex subunit 1	2.2± 0.9	3.1 ± 1.3	1.1 ± 0.03	2.7 ± 1.3
	O96000	Complex I beta subcomplex subunit 10	13.8 ± 4.8	13.1 ± 3.6	5.6 ± 1.7	9.8 ± 1.7
	Q9NX14	Complex I beta subcomplex subunit 11	4.7 ± 1.8	2.8 ± 0.7	1.6± 0.01	3.2 ± 1.5
	O95139	Complex I beta subcomplex subunit 6	1.9 ± 0.8	1.5 ± 0.5	0.4 ± 0.03	1.4 ± 0.3
	O95169	Complex I beta subcomplex subunit 8	1.1 ± 0.5	0.7 ± 0.2	0.8 ± 0.6	0.9 ± 0.3
	Q9Y6M9	Complex I beta subcomplex subunit 9	0.8 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.2
	P49821	Complex I flavoprotein 1	18.9 ± 3.0	12.3 ± 4.3	9.5 ± 1.9	18.6 ± 6.6
	P19404	Complex I flavoprotein 2	5.8 ± 0.9	5.5 ± 1.7	2.5 ± 0.6^	4.1 ± 1.2
	P56181	Complex I flavoprotein 3	2.1 ± 0.3	1.2 ± 0.6	0.4 ± 0.1^	1.6 ± 0.5
	O75489	Complex I iron-sulfur protein 3	10.9 ± 1.4	11.4 ± 1.0	8.3 ± 0.4	7.8 ± 2.7
	O43181	Complex I iron-sulfur protein 4	4.4 ± 1.6	4.8 ± 1.4	1.9 ± 0.08	3.4 ± 1.9
	O43920	Complex 1 iron-sulfur protein 5	0.4 ± 0.1	1.8 ± 0.8	0.7 ± 0.07	0.7 ± 0.09

	O75380	Complex I iron-sulfur protein 6	2.0 ± 0.7	1.4 ± 0.5	1.1 ± 0.3	1.6 ± 0.6
	O75251	Complex I iron-sulfur protein 7	0.5 ± 0.1	0.4 ± 0.2	0.4 ± 0.3	0.3 ± 0.2
	O00217	Complex I iron-sulfur protein 8	1.7 ± 0.7	0.9 ± 0.5	0.4 ± 0.1	1.6 ± 0.02*
	P28331	Complex I 75 kDa subunit	33.0 ± 4.2	29.9 ± 6.4	27.4 ± 12.0	34.9 ± 5.3
	P03915	Complex I chain 5	0.3 ± 0.05	0.2 ± 0.1	0.1 ± 0.02^	0.1 ± 0.05
	Q99643	Complex II b560 subunit	0.02 ± 0.01	0.05 ± 0.02	0.08 ± 0.06	0.05 ± 0.02
	P31930;O75439	Complex III subunit 1	169.2 ± 41.0	169.7 ± 37.7	112.9 ± 4.6	131.9 ± 29.6
	P22695	Complex III subunit 2	173.0 ± 17.3	152.1 ± 33.2	84.8 ± 8.0^	107.5 ± 34.8
	P07919; A0A096LP55	Complex III subunit 6	9.3 ± 1.6	6.4 ± 1.4	4.3 ± 0.4^	9.1 ± 3.2
	P14927	Complex III subunit 7	26.2 ± 8.9	19.6 ± 5.1	27.4 ± 11.2	31.0 ± 3.6
	O14949	Complex III subunit 8	8.1 ± 2.2	6.5 ± 1.8	4.4 ± 0.4	6.4 ± 0.6
	Q9UDW1	Complex III subunit 9	0.003 ± 0.003	0.03 ± 0.02	0.007 ± 0.006	0.004 ± 5x10^-3
	P47985; P0C7P4	Complex III subunit Rieske	30.6 ± 8.5	34.9 ± 3.3	25.8 ± 2.3	27.7 ± 5.8
	P00395	Complex IV subunit 1	5.7 ± 2.4	7.8 ± 2.3	3.6 ± 3.0	4.0 ± 2.3
	P00403	Complex IV subunit 2	73.1 ± 12.2	91.4 ± 14.8	6550.0 ± 9.6	89.7 ± 29.1
	P13073	Complex IV subunit 4 isoform 1	87.6 ± 17.5	44.7 ± 13.8*	29.8 ± 7.7#	54.3 ± 8.8
	P20674	Complex IV subunit 5A	72.3 ± 11.3	64.2 ± 15.2	78.8 ± 25.1	59.8 ± 2.9
	P10606	Complex IV subunit 5B	67.2 ± 13.5	67.4 ± 12.2	31.9 ± 4.8#	50.1 ± 4.4*
	P14854	Complex IV subunit 6B1	46.7 ± 7.3	41.4 ± 9.4	45.3 ± 16.9	57.7 ± 19.0
	P09669	Complex IV subunit 6C	6.9 ± 0.6	8.0 ± 2.9	2510.0 ± 20.8	5.0 ± 1.2
	P24310	Complex IV subunit 7A1	4.9 ± 0.9	4.9 ± 1.1	3.1 ± 1.8	8.0 ± 2.1
	O00483	Complex IV subunit NDUFA4	23.3 ± 6.2	32.2 ± 5.8	25.5 ± 3.1	10.7 ± 0.3*
	P24539	ATP synthase F(0) complex subunit B1	18.6 ± 5.1	26.1 ± 5.0	16.9 ± 2.8	17.4 ± 6.8
	P25705	ATP synthase subunit α	613.8 ± 74.4	766.4 ± 83.5	524.4 ± 73.1†	483.0 ± 88.0
	P06576	ATP synthase subunit β	909.5 ± 69.8	1076.5 ± 148.0	816.6 ± 83.7	752.2 ± 165.9
	O75947	ATP synthase subunit d	37.8 ± 13.9	56.3 ± 8.1	38.8 ± 4.2	34.8 ± 6.7
	P30049	ATP synthase subunit δ	28.8 ± 11.5	28.0 ± 6.4	17.8± 5.5	20.2 ± 6.0
	P56385	ATP synthase subunit e	31.6 ± 6.4	28.3 ± 9.4	18.1 ± 0.7	21.6 ± 2.1

	P56134	ATP synthase subunit f	7.2 ± 2.0	5.8 ± 1.7	4.1 ± 0.5	4.1 ± 1.7
	O75964	ATP synthase subunit g	25.0 ± 1.4	31.8 ± 4.3	21.0 ± 2.7	21.5 ± 3.9
	P36542	ATP synthase subunit γ	12.3 ± 3.8	12.7 ± 2.7	8.6 ± 1.8	9.6 ± 1.8
	P48047	ATP synthase subunit O	101.3 ± 8.4	108.5 ± 6.2	55.6 ± 13.1^	76.0 ± 15.9
	P18859	ATP synthase-coupling factor 6	5.4 ± 0.7	1.9 ± 0.4*	3.3 ± 1.0	2.6 ± 0.6

Values expressed as mean ± SE. *pre vs. post HFD ($p < 0.05$); ^responder pre HFD vs non-responder pre HFD ($p < 0.05$); †Independent of diet, responder vs non-responder ($p<0.05$); $^{\#}$Diet x Group (responder vs non-responder) interaction ($p < 0.05$). Responders n=4. Non-responders n=3.

Discussion

The current study utilized changes in skeletal muscle fatty acid oxidation (FAO) as the response variable to characterize metabolic adaptation to a high-fat diet (HFD) in humans. Responders were defined as participants that increased FAO and non-responders were defined by the participants that decreased FAO. The HFD-induced increase in FAO observed in the responder group is consistent with other previous research[20-27] and was accompanied by decreased amounts of circulating serum triglycerides (TAG). These data suggest the HFD is promoting the uptake and utilization of fat in the responder group[3,20,28-30]. Our results show that responders and non-responders differing FAO patterns were present without group differences in fasting glucose oxidation prior to and following the HFD. Our primary findings were overall responders contained a higher content of protein subunits involved in Complex I (CI) and ATP synthase (ATPase). However, we did not observe HFD induced proteome remodeling, as of the 81 subunits and proteins identified, only the content of 6 subunits, 2 from the responders and 4 from the non-responders, responded to the HFD.

Our proteomic analysis of medium-chain acyl-CoA (MCAD) [EC 1.3.8.7], the enzyme that catalyzes the first step of beta-oxidation of medium-chain FAs, supported this group disparity in FAO patterns in response to HFDs. Prior to the HFD, responders had a higher content of MCAD, which is indicative of a greater capacity to oxidize fatty acids[31-34]. Our results are consistent with studies demonstrating lower levels of acyl-CoA dehydrogenases were associated with a

decreased rate of fat oxidation and a build-up of fatty acid oxidation intermediates[31,32,34-36]. However, responders had an overall lower content of short-chain acyl-CoA dehydrogenase (SCAD) [EC 1.3.8.1], the enzyme that catalyzes the first step of beta-oxidation of short-chain FAs, eluding to other mechanisms, not only protein content, regulating FAO. SCAD has the ability to be acetylated[37], a post-translational modification which results in an attachment of an acetyl group[38]. Evidence shows acetylation has the ability to directly regulate protein function[39,40], localization[41,42], and stability[43,44]. There is a possibility in our present study SCAD is acetylated, affecting it's functionality and protein content.

Responders exhibited lower levels of incomplete oxidation, which was supported by their observed higher oxidative efficiency and our enzymatic activity assay data. The ratio of β-hydroxyacyl-coenzyme A dehydrogenase (BHAD) to citrate synthase (CS) activity, was overall higher in non-responders. BHAD [EC 1. 1. 1. 35] participates in beta-oxidation and it's activity is indicative of the rate of beta-oxidation. CS [EC 2.3.3.1] catalyzes the first reaction of the tricarboxylic acid (TCA) cycle and it's activity is indicative of the rate of the TCA cycle. A higher ratio of BHAD to CS activity suggests the TCA cycle is oxidizing the acetyl-CoA from beta-oxidation at a lower rate than beta-oxidation is supplying it, resulting in incomplete fatty acid oxidation products[7,8]. Kim et al.[15] observed rats who gained weight in response to a HFD, compared to their counterparts that did not gain weight on the same diet, increased the ratio of BHAD to CS activity and incomplete oxidation. This discrepancy suggests HFD induced incomplete oxidation is selective and regulated by a mechanism not yet known. However, our n size of the responder BHAD activity was 2 due to a lack of muscle sample from some of those participants.

Consistent with previous studies, the HFD did not affect CS protein content or activity [23,45,46], in either group. An overall higher content of the alpha subunit of the TCA enzyme isocitrate dehydrogenase (IDH) [EC 1. 1. 1. 42] was observed in responders. The alpha subunit of IDH is thought to be the catalytic domain of IDH[47]. An overall higher content of this subunit could

suggest that the responder group possess a higher capacity for IDH enzymatic activity. In non-responders, the alpha subunit of the TCA enzyme succinnyl-CoA synthetase (SCS) [EC 1. 2. 1. 76] decreased in response to the HFD. The catalytic domain of SCS is located in the interface of it's alpha and beta subunits[48]. The alpha subunit particularly binds the phosphate group, which is needed for the completion of SCS's reaction[48]. There is a possibility that a decrease in this subunit could decrease enzymatic activity. However, every enzyme involved in the TCA has the possibility to be post-translationally regulated[49-57]; therefore, the protein content observed in our study does not necessarily reflect the activity of the individual proteins or the TCA cycle itself.

Our proteomic analysis showed responders contained an overall lower content of the glycolytic enzyme beta-enolase [EC 4. 2. 1. 11], which conflicts our data showing no differences in carbohydrate oxidation between groups. However, previous research shows decreased content of beta-enolase is associated with increased levels of FAO[25]. Despite our lack of observed carbohydrate oxidation differences, the observed lower content of beta-enolase in responders is consistent with others research[25]. Further, the HFD selectively decreased the oxidation of the product of glycolysis, pyruvate. Non-responders decrease in pyruvate oxidation suggests the HFD is affecting something downstream of glycolysis to reduce the activity of pyruvate dehydrogenase (PDH) [EC 1. 2. 4. 1], the enzyme that converts the resulting pyruvate from glycolysis to acetyl-CoA. Our results show no changes in PDH protein content, suggesting another mechanism for reduced pyruvate oxidation. Evidence shows acetylation plays an inhibitory role on PDH's activity and high-fat feeding is known to promote the hyperacetylation of PDH[58]. Further, PDH can be phosphorylated, the attachment of a phosphate group[59], to regulate it's activity[60-62].There is a possibility that the decreased pyruvate oxidation observed in the non-responder group is due high-fat feeding induced acetylation or phosphorylation. However, it is unclear why responders' pyruvate oxidation did not change in response to the HFD as well.

A little over a third of the subunits identified by our analysis were components of CI [EC 1. 6. 5. 3]. CI is composed of 3 domains, each functioning to either bind and oxidize NADH, transfer electrons, or pump protons[63]. We observed an overall higher content of CI in responders compared to non-responders. Non-responders overall lower content of CI subunits is consistent with previous data showing an association between decreased amounts of CI and decreased content of acyl-CoA dehydrogenases[34]. In response to the HFD, non-responders increased a subunit of CI that participates in transferring electrons, iron-sulfur subunit 8. This increase is consistent with evidence showing an increase in CI subunits following a HFD[15,26], particularly subunits participating in electron transport[15]. However, the studies showing increased content of CI following a HFD used rodent models on a HFD for a minimum of 4 weeks[15,25,26,45,46] and typically were associated with insulin resistance[25,26,46] and/or obesity[15,25,46]. Insulin resistance, the inability of insulin to inhibit hepatic glucose production via gluconeogenesis or glycogenolysis[64-67], and obesity are both associated with diminished oxidative capacity and inability to utilize free fatty acids as a fuel source[28-30,65,68]. There is a possibility that the results from those previous studies are influenced by those metabolic defects and not due to the HFD alone.

Due to Complex II's (CII) [EC 1. 3. 5. 4] mitochondrial role in accepting electrons from $FADH_2$ originating from the TCA cycle and beta-oxidation, it was surprising the HFD had no affect it's content. However, evidence shows that absence of mitochondrial deacetylase sirtuin 3 reduced the activity of CI, Complex III (CIII), and ATPase, while it had no affect on CII activity[69]. This suggests CII is regulated by different mechanisms than the other ETC complexes.

CIII [EC 1. 10. 2. 2] contains 11 subunits that primarily function to transfer electrons through the electron transport chain for ATP production. Responder's had an overall higher content of the 2 of CIII's subunits, subunit 2 and 6. Subunit 2 is a component of CIII's binding site[70] and subunit 6 facilitates interaction of the ETC electron carriers cytochrome c1 and cytochrome c[71-

[73]. A decreased content of CIII may decrease electron transport as well as proton pumping, thereby diminishing the electrochemical gradient and thus the ability to produce ATP[74].

Complex IV (CIV) [EC 1. 9. 3. 1] accepts electrons from CI and CII to reduce oxygen resulting in the production of water. All the subunits found to significantly change in response to the HFD participate CIV activity[75-80]. Following the HFD, responders and non-responder both decreased a subunit of CIV, subunit 4 isoform 1 and subunit NDUFA4, respectively. Evidence shows that decreased content of CIV can lead to reactive oxygen species (ROS) [81], suggesting the HFD may promote ROS production. Further, decreased activity of CIV is observed in cases of insulin resistance[77], which has been associated with HFDs [22,77,82]. Many studies show HFDs stimulate ROS and lead to insulin resistance[83-87]. There is a possibility that diminished oxidative capacity induced by a long-term HFD could participate in the development of insulin resistance through ROS production. However, following the HFD, non-responders also decreased a subunit of CIV, subunit 5B. It seems counterintuitive that non-responders would simultaneously decrease and increase subunits that stimulate CIV activity but the mechanism behind this is not yet known.

ATPase [EC 3. 6. 3.14] is the complex that concludes the ETC and produces ATP. ATPase is composed of three components including a catalytic domain, a membrane channel and a stalk acting as a bridge between the preceding components[88]. Responders contained an overall higher content of a subunit located in the catalytic domain of ATPase, subunit alpha[88]. Further, prior to the HFD, responders had a higher content of a subunit located in the central stalk of ATPase, subunit O[88]. Our results suggest responders have an increased capacity to produce ATP. Following the HFD, responders decreased the f6 subunit, which is located in the central stalk[88], suggesting the HFD is selectively promoting the destabilization of ATPase, and possibly a decreased in ATP production, in the non-responder group.

A limitation of our study is the small n size. A larger n size would provide more robust data. Our present study attempted to characterize differential metabolic adaptation to short-term high-fat feeding in humans. Our results are consistent with human studies showing an absence of protein changes in response to short-tern high-fat feeding[13,27]. Evidence showing HFD induced proteome changes were accompanied by insulin resistance [22,25,46] and not high-fat feeding alone. Further, a majority of the studies showing protein changes used rodent models. Mice develop insulin resistant at a faster rate than humans[46], making insulin resistance a confounding variable. To our knowledge, this study is one of the few that provides insight on the effect of isocaloric short-term high-fat feeding on the human skeletal muscle proteome. Our results show that changes in proteome alone cannot explain differential responses to macronutrient composition and that metabolic responses may be individual specific. This data suggests other mechanisms, such as post-translation modification, are involved in the regulation metabolic adaption. A future direction is to analyze changes in post-translational modifications of proteins important to substrate metabolism in response to a HFD. Post-translational modifications respond to environmental cues at a faster rate than changes in protein levels. There is the possibility that the duration of short-term HFDs may not be long enough to see changes at the protein level. Analyzing changes in post-translational modifications of metabolic proteins may provide useful information on skeletal muscle's initial response to high-fat feeding.

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CHAPTER V

Conclusions and Future Directions

We utilized changes in skeletal muscle fatty acid oxidation (FAO) as the response variable to characterize metabolic adaptation to a high-fat diet (HFD) in humans. Participants that increased FAO in response to the HFD were term responders, while the participants that decreased FAO were termed non-responders. Primary findings include: 1) the HFD-induced differential FAO patterns were present without group differences in fasting glucose oxidation prior to and following the HFD, 2) skeletal muscle of responders exhibited an overall higher content of subunits from Complex I (CI) and ATP synthase (ATPase), and 3) the HFD did not induce proteome remodeling due to the content of very few proteins or protein subunits changing in response to the diet. There is a possibility that a high-fat diet of a longer duration would give more insight into proteome remodeling in response to the HFD. However, our findings show that changes in proteome alone cannot explain differential responses to macronutrient composition and that metabolic responses may be individual specific.

Future studies should analyze changes in post-translational modifications of proteins important to substrate metabolism in response to a HFD. Post-translational modifications respond to environmental cues at a faster rate than changes in protein levels. Analyzing changes in post-translational modifications, such as acetylation, of metabolic proteins may provide useful information on skeletal muscle's initial response to high-fat feeding. Evidence has shown the acetyl-CoA utilized for acetylation in fasting conditions is derived from FAO. Analyzing this post-translational modification that is tightly intertwined with fat oxidation may provide useful information on metabolic adaption to high-fat feeding. A major limitation of our study was the small n size. Future studies with larger n sizes would provide more robust data.

APPENDICES

Appendix A: Informed Consent for Participants of Investigative Projects

Department of Human Nutrition, Foods and Exercise

Virginia Tech

TITLE: **Effect of a High Fat Diet on Muscle Metabolism**

INVESTIGATORS: **Kevin P. Davy, Ph.D.**
Mathew W. Hulver, Ph.D.
Madlyn I. Frisard, Ph.D.
Brenda M. Davy, Ph.D., R.D.
Elaina Marinik, Ph.D.
Mary Elizabeth Baugh, M.S., R.D.
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Nabil Boutagy, Ph.D.
Loren Weldon, EMT-I

MEDICAL DIRECTOR: **Jose Rivero, M.D.**

PURPOSE:

The amount of fat in the diet may influence the risk for diabetes and cardiovascular disease by altering metabolism (how the body gets energy from food in our diet). However, it is not clear how muscle tissue and the cardiovascular system responds to different amounts of fat in the diet. Therefore, the purpose of this study is to determine how a high fat diet influences metabolism in muscle and blood vessels. Sixty males and females will be included in this study.

METHODS:

You are being asked to be involved in a study that involves eating a high fat meal (for example, two sausage and egg biscuits) on three (3) occasions; before and after 14 days of eating a diet similar to your usual diet and again after 5 days of eating a high-fat diet.

During the first 14-day period you will be given all your food. This food will have the same number of calories you usually eat. The food given to you will have 55% of the calories from carbohydrates, 30% from fat, and 15% from protein. After this 14-day period, you will participate in a high fat diet for 5 days. The high fat diet will contain the same number of calories you normally eat so you should not gain or lose weight. You will also be provided food during the high fat portion of the study so that 40-50% of all the calories you eat come from fat.

Blood samples and muscle biopsies will be taken at six time points during the 30-day study, before and after the high fat meal which will occur on three (3) occasions: before and after 14 days of eating a diet similar to your normal, habitual diet period, and again after the 5-day high fat diet. The additional tests are described below under Session 1

You will be required to complete session 1, session 2, the take home tests and any other optional sessions identified with a checked box. There will be approximately 25-30 visits if you choose to participate in the study. The actual number and order of visits may depend on your schedule, the availability of the study staff, and the number of sessions you are required to complete. In addition, the order may differ from the order of appearance in this document. You will undergo Session 1 one time and all others selected (including session 2 and take home tests) three times (before and after 14 days of eating your typical diet and again following the 5-day high fat diet).

If you agree to be involved in this study you will first have to fill out a health history questionnaire. The additional tests are described below under Session 1. Your results may be discussed with the study medical director to determine if you can be a subject. You may be able to be a subject if you are between 18 and 40 years of age. If you are female, you will be required to be taking an oral contraceptive so that when your menstrual cycle begins and how long it lasts will be known as accurately as possible. You will not be able to participate if you are pregnant. Your body mass index (a measure of obesity) must be less than or equal to 30 kg/m^2. If you smoke or have high blood pressure, heart disease or diabetes then you cannot be in this study. You will not be able to participate if your cholesterol is too high or have other health problems that would make it unsafe for you to be in the study. You will not be able to participate if you have lost or gained more than 5 pounds in the last 6 months. You will not be able to participate if exercise more than twice per week at a moderate to hard level (e.g., exercise that causes you to breathe hard and sweat) or less than 5 days/week of running or cycling for an hour. If you use any medication or nutritional supplements that might influence the study variables or have taken antibiotics in the last month

then you will not be able to be in this study. You will not be able to be a subject if you have an allergy to lidocaine or bupivacaine, or have food allergies (for example, gluten allergy).

Session 1 (You will complete this session only one time at the beginning of the study)

- **Overnight Fast:** You will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process.
- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. Your height and weight will also be measured at this time. Your body weight will be measured on a standard digital scale. Your height will be measured with a standard stadiometer (ruler on the wall). Your waist, hip, and neck circumference will be measured using a measuring tape.
- **Blood Pressure:** You will be asked to sit quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Maximal Oxygen Consumption:** Maximal oxygen consumption will be measured while exercising on a treadmill. Before the test begins you will be asked to warm-up for 5-10 minutes at a comfortable speed on a treadmill. You will then be asked to run or walk at a fast speed and then the angle of the treadmill will be increased every one or two minutes until you cannot exercise any longer. You will be fitted with a mouthpiece and nose clip so that we can collect and measure the amount of oxygen and carbon dioxide your breath in and exhale out. The test will take approximately 8-12 minutes. Following the test, we will have you cool-down for 5 minutes at a comfortable walking speed.
- **Physical Activity Questionnaire:** You will be asked a series of questions to estimate your usual physical activity level, which will require about 15 minutes to complete.
- **Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure glucose, cholesterol, blood cells, and other factors to determine your eligibility.
- **Pregnancy Test**: If you are female you will be required to have a pregnancy test. This will require you to collect 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study.

Approximate time required: 1.5 hour

Session 2 (you are being asked to complete this session three times; before and after 14 days of eating your typical diet and again after 5 days of eating a high fat diet)

- **Overnight Fast:** You will need to avoid eating or drinking for 12 hours and having caffeine-containing foods or drinks for 24 hours before to this visit. This is to make sure that your eating does not influence the test results.
- **Body Composition:** This test is to measure your body fat. You will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 5 minutes and there is no pain associated with the procedure. This procedure will be performed once at the beginning of the study, once after the 2-week habitual dietary period, and once at the end of the study. Your weight and height will also be measured at this time.
- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.
- **Muscle Biopsy:** You should not take aspirin, ibuprofen or other non-steroidal, anti-inflammatory medications (such as Advil, Motrin, Celebrex or Vioxx), or other medications or substances that may affect bleeding or bruising, for 72 hours prior and after this procedure. This procedure is used to sample a small amount of muscle (about 350-450 mg or about the size of 2-3 erasers on a pencil) from underneath the skin from the thigh. The actual biopsy site will be on the top of either the right or left leg half way between the knee and the hip.

  You will be asked to undergo this procedure 6 times, before and after the high fat meal during the following testing periods: before and after the diet similar to your usual diet and again after the high fat diet. Neither a physician nor nurse may be present during the procedure. This procedure will be performed by a study investigator (Kevin P. Davy, Ph.D.) or co-investigator (Mathew Hulver, Ph.D.) who has been specifically trained to perform the biopsy. You will be lying down and your skin will be cleansed with iodine-type solution (Providine or Betadine). If you are allergic to iodine, we will use chlorhexadine which does not contain iodine. A sterile drape will be placed over the area and your skin and muscle tissue will be numbed by injecting numbing medication (lidocaine/bupivacaine) into the area with a small needle. If you allergic to lidocaine or bupivacaine, you cannot participate in this study. Then, a small incision (about 1/4 of an inch) will be made in the skin and a needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle. Some suction may be applied to the other end of the needle to help remove the muscle.

After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleaned with saline and will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE bandage. You will be asked to keep the ACE bandage on for at least 10-15 minutes. You may take Tylenol for any discomfort you may experience following the biopsy. We will use the biopsy samples to measure factors which contribute to inflammation.

You will be provided with instructions on how to care for the biopsy sites as well as what to look for if a problem were to occur.

- **Catheter and Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure various hormones that influence your metabolism (how your body burns calories and produces body heat) and cardiovascular system (the heart, blood vessel and lungs). Blood will be collected before the meal challenge and at 1, 2, 3, and 4 hours after the meal. The catheters will remain in your arm throughout the entire test.
- **Meal Challenge:** You will be asked to eat a test meal consisting of two breakfast sandwiches (e.g., egg and sausage). Blood will be collected before and 1, 2, 3, and 4 hours after the meal and a muscle biopsy will be performed before and approximately 4 hours after the meal.
- **Fuel Use**: You will be asked to lie under a clear, plastic hood or canopy immediately following the meal challenge The hood will be placed over your head and upper body and will be used to collect all the air you expire so that we can measure how much fat and carbohydrate you use as fuel during the 4-hour period. You will be allowed to use the bathroom and stretch your legs at specific times during the test if needed.

Approximate time required: 5 hours

If this box is checked you will be required to complete Session 3 ☐

Session 3 (you are being asked to complete this session three times; before and after 14 days of eating your typical diet and again after 5 days of eating a high fat diet)

- **Overnight Fast:** You will have to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat.

- **Blood Pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Arterial Stiffness**: To measure arterial stiffness, the blood flow and diameter in the arteries in your chest, neck and leg will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low-frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck and leg.
- **Endothelial Function:** To measure endothelial function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation of a blood pressure cuff on your forearm for 5 minutes. We will also measure blood flow and diameter of your brachial artery after placing nitroglycerine (0.4 mg) under your tongue. This procedure takes about 30 minutes to complete.
- **Intestinal Permeability Testing:** You will be asked to empty your bladder before consuming about a cup of a sugar drink. You will then drink an additional 2 cups of water to make collection of urine easier. You will be asked to collect and save your urine. Urine will be collected in two containers. The first will be used for collecting your urine between the point at which you consume the sugar drink and 5 hours after and another between 6 and 24 hours after consuming the sugar drink. You will be provided with urine collection containers and asked to return them to the lab or we will arrange to pick them up at the end of each collection period.

Approximate time required: 1.5 hour

If this box is checked you will be required to complete Session 4 ☐

Session 4 (you are being asked to complete this session three times; before and after 14 days of eating your typical diet and again after 5 days of eating a high fat diet)

- **Overnight Fast:** You will need to avoid eating or drinking for 12 hours and having caffeine-containing foods or drinks for 24 hours before to this visit. This is to make sure that your eating does not influence the test results.
- **Intravenous Glucose Tolerance Test (IVGTT).** Two small plastic tubes (intravenous catheters) will be placed in each of two arm veins (different arms) and about 3 tablespoons of blood will be taken to measure hormones or proteins that influence your metabolism and cardiovascular system. We will

then inject a small amount of glucose (0.3 mg/kg body weight) and insulin (0.03 unit/kg body weight) into your veins (insulin is a hormone which helps your body's cells metabolize glucose). We will draw a small amount of blood (less than one half teaspoon) about 28 times over a 3-hour period. A registered nurse will be present to perform this test with the assistance of investigators.

Approximate time required: 4 hours

Take-Home Tests

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day) as part of baseline screening.
- **Stool Collection:** You will be asked to collect a stool sample and bring it to the laboratory in the container provided on 3 occasions.
- **Urine Collection:** You will be asked to collect a 24-hour urine sample to bring to the laboratory on three occasions (each time you come in for session two). This test will only be required if you are required to complete Session 3.

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all of the scheduled experiments.
- Follow all participant instructions for each session.
- Record any food you eat that has not been provided by the investigators.
- Return any uneaten food that has been provided by the investigators.
- Follow physical activity instructions provided by the investigators.
- Carefully read the instructions on consuming any food provided to you.

RISKS OF PARTICIPATION

- **Catheter and Blood Draw:** Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000.

There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in catheter placement and blood draws will minimize these risks.

- **HIV/AIDS:** Your blood will be tested for the presence of HIV if one of the study investigators is exposed to your blood. There will not be any cost to you for this test. The results will be sent to your primary care physician or the study medical director, Dr. Jose Rivero, if you do not have a primary care physician. He/she will discuss them with you and provide you with the necessary referral for further evaluation and/or counseling if your results are positive. The results of your test will remain confidential.
- **Muscle Biopsies:** If you are allergic to lidocaine, you will not be allowed to participate in this study. There may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but you are not expected to experience discomfort during the biopsy procedure. Bruising in the area of the muscle biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately after the procedure to limit this potential effect and its accompanying tenderness. There is a slight risk of infection at the biopsy site. There is a small risk that you will become lightheaded, dizzy, or anxious before, during or after the procedure. There is also a small risk of fainting. If you feel dizzy, lightheaded or feel like you might faint before, you should sit down or lie down immediately to avoid falling. These reactions are usually temporary and resolve within a short time after sitting or lying down. If these feeling do not go away soon after sitting or lying down, you should call 911 or have someone take you to the nearest emergency room. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. We did have one individual faint and hit their head after leaving the laboratory. This required a trip to the emergency room and stitches. However, this occurred only once in the over 350 biopsies performed in our research studies. These risks are minimized by having a trained individual perform the procedure. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.
- You will likely receive a scar from each of the biopsies performed but these are expected to be very small. These scars usually turn a purple color in the weeks to months following the biopsy and then fade considerably over time. The study staff will show you several pictures of examples of the scarring (greater than 1 year old) that can occur following similar biopsy procedures. It is important that you understand that these are just examples of the scarring that can occur. The actual scar you receive may be smaller or larger or differ in coloring. Individuals with darker skin (e.g., African

Americans, Hispanics and Asians) tend to scar more than those with lighter skin. You should consider this before you agree to participate.

- **DEXA Scan:** The amount of radiation that you will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however, the exact increase in such risk is not known.
- **Maximal Oxygen Consumption:** There is a small risk of injury (e.g., sprained ankle), complications requiring you to go to the hospital, heart attack, or even death. In studies involving people with heart disease, the risk of hospitalization was 1 in 500 tests <0.20%). The risk of heart attack was 1 in 2,500 tests (0.04%) and death, 1 in 10,000 tests (0.01%). The risks are likely to be lower in young, healthy subjects. Only experienced staff members will conduct these tests and you will be monitored throughout the test for signs of problems. You will be tired after this test and may have sore muscles for a few days.
- **Fuel Use:** There is a small risk of feeling anxiety associated with having your face covered with the plastic hood.
- **Arterial Stiffness:** There are no known risks associated with these procedures.
- **Endothelial Function:** Some pain or discomfort may be experienced when the blood pressure cuff is inflated. However, this pain/discomfort is temporary and resolves within a short time after completing or stopping the procedure.
- **Sugar Drink:** Potential risks related to ingestion of the different types of sugar found in the sugar/water drink may be associated with gastrointestinal symptoms such as gas, bloating, and diarrhea.
- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.
- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION

Your participation will provide you with:

- Information on your fitness and body composition.

- Information on your blood pressure, cholesterol and glucose tolerance

COMPENSATION

You will be compensated $25 for completing each muscle biopsy and $50 for each IVGTT (if completed). Muscle biopsies will be performed before and after each test meal and the test meals (3 total) will occur at the following time points before and after your typical diet and again after the high fat diet ($150 total). The IVGTT may will be performed before and after your typical diet and again after the high fat diet ($150 total). You can receive an additional $100 for completing the high fat feeding period. You can receive up to $400. If you are not asked to complete the IVGTT, you can receive up to $250.

CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by subject numbers, without anything to identify you by name. In the event that any of your tests indicate that you may have a heart problem, Dr. Rivero or investigators may want to share this information with your doctor but he will request your approval first.

FREEDOM TO WITHDRAW

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain muscle, body fat or other measurements that are necessary for the study.

INJURY DURING PARTICIPATION IN THIS STUDY

Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if injured as a result of your participation in this study. Any expenses that you incur including emergencies and long term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH

This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

SUBJECT PERMISSION

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:

- Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; After hours: 540-230-0486
- Chairman, Institutional Review Board for Research Involving Human Subjects:
- David Moore, Associate Vice President for Research (540) 231-4991

Name of Subject (please print)_____

Signature of Subject_____ Date_____