Skeletal muscle acetylation in response to an acute and chronic high fat diet

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ABSTRACT

The past thirty years have seen a dramatic rise in obesity worldwide owing to a change in dietary composition, quantity of food consumed- positive energy balance, and a more sedentary life style[1, 2]. Accompanied with obesity is a chronic low grade inflammatory state defined by increased circulating cytokines and an increase in gene expression promoting inflammation[3-5]. Multiple health risks are associated with obesity such as cardiovascular disease, insulin resistance, and type II diabetes[6, 7].

Advances in mass spectrometry have made wide scale proteomic studies possible and are redefining cell and molecular biology[8-10]. One such area of that has become of considerable interest is protein acetylation which is observed in most cellular processes such as cell cycle regulation, gene expression, subcellular localization, metabolism, muscle contraction, protein stability, apoptosis, and more[10-13]. Metabolic proteins are highly susceptible to acetylation with almost all showing the capacity to be acetylated[9, 13].

Our research, using an obese mouse model fed a chronic high fat diet and a lean control mouse model fed a standard chow diet, showed numerous differences in the acetylome between obese and lean animals in a fasted state. As well as, differences in the acetylome's of both animal models upon receiving a high fat meal. We showed that almost every mitochondrially located metabolic protein in obese animals is hyper-acetylated in a fasted state compared to lean animals and that upon feeding lean animals have a greater response in the change to their metabolic acetylome. We show that in the fed state lean and obese mice have almost completely different

acetylomic profiles of mitochondrial and glycolytic metabolic proteins. Furthermore, we have observed possible new regulatory mechanisms utilizing acetylation to 1) determine the fate of the co-factor NADH in glycolysis and 2) control an ATP producing reaction in glycolysis.

GENERAL ABSTRACT

In recent decades obesity rates have been on the rise because of a change in diet that is high in fat and sugar. Associated with obesity are increased risks of cardiovascular disease, insulin resistance, and type II diabetes. Obesity is also known to promote the expression of genes responsible for a low grade inflammatory state.

Utilizing mass spectrometry, it is now possible to measure the addition or removal of modifying groups, termed posttranslational modifications (PTMs), to proteins tissue wide. Acetylation is one such PTM whose addition or removal can both promote or inhibit protein activity. Posttranslational modifications are one way of regulating metabolism by altering the ability to oxidize fats, carbohydrates, or proteins. The modifying group for acetylation, acetyl CoA, is also the product of carbohydrate, fat, and amino acid metabolism making acetylation an integral regulator of metabolism.

Our research measured the changes in acetylation to metabolic proteins between lean and obese mouse models as well as the effect of a single high fat meal on acetylation changes in lean and obese models. We showed hyper-acetylation due to obesity inhibits changes in acetylation of metabolic proteins unlike lean animals which show a greater number of acetylation changes in response to fasting and feeding.

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TABLE OF CONTENTS

CHAPTER I: INTRODUCTION

Essential to biological systems homeostasis is the regulation of proteins modulating those pathways. One of the most prominent forms of regulation is that of protein posttranslational modifications (PTM) affecting the activity and integrity of those proteins. Within the last decade acetylation as a PTM governing protein activity has come to the forefront of research with the advancement of high throughput mass spectrometry[1]. Acetylation of a protein is the addition of an acetyl group to a lysine residue which changes the amino acid's charge and can alter the interaction of that protein in its environment as well as the conformational shape[2]. The addition of an acetyl group can interfere and inhibit further protein modifications such as phosphorylation[3], and has also been shown to promote ubiquitination leading to degradation[4]. Studies in skeletal muscle and liver, highly metabolic tissues, have shown that acetylation can modulate enzymatic activity [5-7].

Metabolic pathways are uniquely positioned to regulation by acetylation as the required substrate necessary, acetyl CoA, is produced at the intersection of carbohydrate, fat, and amino acid metabolic pathway[8]. The abundance of acetyl CoA in the mitochondria specifically results in higher acetylation rates of resident proteins when compared to any other cellular region[8]. Tissues with higher metabolic function, such as liver and skeletal muscle, exhibit higher acetylation rates although the expression is observed in a tissue specific manner where often the effects of acetylation, rates of, and times of occurrence vary[5]. Protein acetylation patterns observed in tissues from metabolically flexible, healthy, individuals will be referred to as normal acetylation patterns when in comparison to observed protein acetylation patterns from individuals afflicted with some form of metabolic dysfunction- resulting from obesity or type II diabetes.

As reported by the Center for Disease Control and Prevention (CDC) and the World Health Organization (WHO) rates of obesity have more than doubled in not only the United States but also worldwide[9, 10]. The causes for this pandemic of obesity will not be the focus of this analysis, more the effects of obesity on acetylation of the proteins that govern metabolic activity. As is already known, obesity and type II diabetes have robust effects on metabolic pathways perturbing normal healthy substrate selection based on a fasted or fed status and substrate availability[11]. Little data exists as to the effects of obesity on skeletal muscle acetylation of metabolic proteins with no data available on obesity's effects on acetylation of metabolic proteins during the fasted to fed transition. While many metabolic proteins have shown to be acetylated, for the majority, the exact nature of how this affects their activity is not clear. Resolving how acetylation is affected in a state of overfeeding and obesity may pose a better understanding of the role of acetylation in metabolic dysregulation. Our hypothesis was that high fat diet-induced obesity creates an unchanging state of hyper-acetylation and that hyper-acetylation may in part contribute to metabolic inflexibility by inhibiting substrate switching. We further hypothesize that in healthy mice a single high fat meal can result in acetylome changes within the proteins of metabolic pathways promoting fat oxidation. Proof of these hypothesizes would demonstrate how dynamic and integral a process acetylation is, and how necessary acetylation is to maintain healthy metabolic function.

CHAPTER II: REVIEW OF THE LITERATURE

Introduction

Over the past decade acetylation has come to the forefront as a metabolic regulatory mechanism as integral and important as that of phosphorylation or ubiquitination[1, 6]. Advances in mass spectrometry have made possible whole tissue acetylome research identifying thousands of acetylated proteins and potential acetylation targets. Observed with obesity and type 2-diabetes is a correlation of disturbed acetylation and deacetylation patterns of metabolic proteins which may further exacerbate the complications to metabolic function associated with those disease states[5, 7, 12]. This review will focus on the role of acetylation in regulating metabolism, how acetylation levels change between the fed and fasted states, the effect of caloric restriction on acetylation, and the consequences of obesity and type 2-diabetes on acetylation status. From the current research, conclusions will be drawn as to the implications of acetylation's role in regulating metabolism and how perturbations to the acetylome of metabolic proteins affect metabolic flexibility.

Acetylation

Acetylation of proteins is a covalent modification where by an acetyl group is added to the ε-amino group of a lysine within a protein [13, 14]. More specifically, acetylation results in the generation of an amide between acetyl CoA (a thioester) and a lysine residue (an amine)[15]. The sulfur atom of the thioester (acetyl CoA) is extremely prone to nucleophilic acyl substitutions from carbon and nitrogen atoms[15]. The addition of an acetyl group to the lysine residue results in the neutralization of the positively charged amino acid and its ability to interact with other amino acids and proteins altering multiple facets of its function from transcriptional activation to

enzymatic activity [13, 16, 17]. Protein acetylation is regulated by acetylases that add acetyl groups, and deacetylases that remove acetyl groups[13]. To date twenty-two acetylases have been discovered, comprised of three major families: GCN5, CBP/p300, and MYST[18]. Two families of deacetylases encompass the eighteen known deacetylases: Zn^{2+}- dependent histone deacetylases and sirtuins[19, 20]. The acetyl group is a component of acetyl Coenzyme A (acetyl CoA), a crucial molecule at the intersection of glycolysis, fatty acid oxidation, ketogenesis, amino acid metabolism, and the TCA cycle [6]. Because acetyl CoA is integral to most metabolic activity it is perfectly situated to function as a sensing molecule for the nutritional state of the cell, and tissues which will be discussed in future sections [6].

Acetylation has become a prominent focus of study in recent years as high throughput examination by mass spectrometry has made volume proteomic studies more viable. In 2006, Kim et al. (2006) became the first to publish a proteomic analysis of acetylation in HeLa cells and mouse liver mitochondria using mass spectrometry identifying 195 acetylated proteins from HeLa cells and 133 acetylated mitochondrial proteins from mouse liver[21]. The liver mitochondria used was gathered from two groups of mice, one fasted and the other fed, and produced small subgroups uniquely acetylated in either the fasted or fed state.[21] While the majority of acetylated proteins, 62% were common to both fasted and fed animals, 14% were only acetylated in the mitochondria of fed animals, and 24% in the liver mitochondria of fasted animals[21]. Not only was this the first analysis using mass spectrometry of the acetylome but the first description of differences in the acetylome between fasted and fed states. They produced peptides through a tryptic digestion followed by immunoprecipitation using and anti-acetyl lysine antibody followed by Nano-HPLC-MS/MS[21]. Protein digestion followed by immunoprecipitation for mass spectrometry is the method used for acetylome analysis. Shortly after Kim et al. (2006),

Choudhary et al. (2009), and Zhao et al. (2010) produced even larger data sets from human cell culture and liver tissue[6, 16, 21, 22], with a consistent observation that a large majority of acetylation occurred in the mitochondria[6, 16, 21, 22]. To date the list of target proteins for acetylation is in the thousands.

Acetylation levels vary among tissues in the body depending on energy demand and storage capacity. Among those tissues that exhibit higher levels of metabolic activity, such as skeletal muscle and liver, acetylation appears to play a prominent role in regulating metabolic pathways[22].

All proteins require the actions of an acetylase and deacetylase to complete the acetylation cycle. To date, no acetylase has been observed to affect mitochondrial metabolic proteins. Unlike other cellular regions, mitochondria are unique in that acetylation can occur without the action of an acetylase [23]. The high levels of acetyl groups from acetyl CoA and the pH of the mitochondria provide and environment where enzymes can be acetylated without an acetylase[23]. This can account for the lack of acetylases discovered residing in the mitochondria that are necessary for acetylation to occur in the cytosol and nucleus. A recent theory suggests that the low pH of the mitochondria depresses the pKa of lysine residues generating an environment where acetylation can occur upon an increase in acetyl CoA[24]. While acetylases are not necessary, deacetylation is still crucial and various groups of deacetylases are present and required. Most metabolic deacetylases belong to one family known as - the Sirtuins

Sirtuins

Sirtuins are a nicotinamide adenine dinucleotide (NAD^+)-dependent family (SIRT1-7) of deacetylases involved in metabolic regulation, most notably in the mitochondria[25-28]. Sirtuin 1 and 2 are located in the cytoplasm, 3, 4 and 5 reside in the mitochondria, and 6 and 7 are located in the nucleus[28]. Nicotinamide adenine dinucleotide is reduced by a sirtuin and later oxidized in the TCA cycle resulting in an acetylated protein with O-acetyl-ADP-ribose and nicotinamide (NAM) as byproducts [29, 30]. Sirtuins are dependent on NAD^+, and until very recently the supposition was that they are highly sensitive to the NAD^+/NADH ratio, but new evidence has called in to question this assumption and further research is needed[31].

Nicotinamide adenine dinucleotide is a coenzyme that functions in metabolism as an electron carrier and therefore comes in two forms; the oxidized form NAD^+, and the reduced form NADH [29]. Electron transfer is NAD's main function, but as will be detailed later it has another important role as a substrate in the addition and removal of acetyl groups mediated by members of the sirtuin family. Nicotinamide adenine dinucleotide is used to shuttle electrons produced through the breakdown of substrates in the cytoplasm and outer mitochondrial matrix into the inner mitochondria matrix to drive oxidative phosphorylation. Using shuttle systems and intermediate proteins, NADH is oxidized and reduced transferring its electrons to other proteins oxidizing and regenerating itself in a series of reactions to eventually deliver electrons for the electron transport chain (ETC) in the inner mitochondrial matrix. The glycerol-3-phosphate shuttle transfers electrons from NADH to FAD forming $FADH_2$. The pentose phosphate pathway uses NAD to generate NADPH necessary for oxidizing free radicals and as a precursor in the production of nucleotides.

Gluconeogenesis largely occurs in the reverse order of glycolysis using the same enzymes using NADH and ATP, and is required for fatty acid synthesis. Nicotinamide adenine dinucleotide is a sensitive sensor of cellular energetic status as small accumulations of the reduced form, NADH, indicates energy demands are being met and functions as a negative feedback regulator of PDH.

Over 2,500 acetyl sites have been identified on mitochondrial proteins; much of this has been elucidated through proteomic studies of SIRT3 function in the liver[16, 32]. Not all tissues will show the same level of acetylation and are largely governed by higher levels of metabolic function, such as the liver, which displays higher levels of acetylation[5].

Sirtuin 1 (SIRT1) regulates metabolic tissues including skeletal muscle, liver, and adipose tissue, by controlling transcription factors and metabolic proteins [33-35]. Sirtuin 1 is the master regulator of mammalian metabolic homeostasis through its control over glucose and lipid metabolism, oxidative phosphorylation, mitochondrial biogenesis, and the many genes involved in these processes[33, 34]. Of the many transcriptional regulators under SIRT1's influence: p53, PGC-1α, FOXOs, LXR, PPAR-α, and SREBP-1c are directly deacetylated by SIRT1 [34, 35]. Targets of SIRT1 activate multiple metabolic pathways with SREBP-1c and LXR promoting fatty acid synthesis, FOXOs increasing gluconeogenesis, and PPAR-α stimulating lipid metabolism and mitochondrial biogenesis[35]. In mice, SIRT1 exhibits protective effects against metabolic disorder from a high fat diet[36]. Overexpression of SIRT1 is shown to improve insulin sensitivity[37, 38], which is associated with increases in PGC-1α[39]. Sirtuin 1's role in metabolic disorders will be described later in the obesity and type II diabetes section.

Sirtuin 1's function is modulated by several post-translational modifications, though the extent and number of PTMs is not known [40]. For example, in addition to multiple sites for

acetylation, sites for SUMOylation have also been mapped on SIRT1 [40, 41], which leads to an increase in its activity[41]. Exactly how many PTMs and how their interaction with one another affect SIRT1 expression and activity is unclear, but the data available points to a complex landscape with implications of novel therapeutic discoveries.

Sirtuin 2 (SIRT2) operates in the cytoplasm to regulate gluconeogenesis by increasing PEPCK's function[4]. In adipocytes research has shown SIRT2 is vital for normal glucose transporter 4 (GLUT4) translocation in response to insulin by deacetylating and thereby inhibiting a protein controlling GLUT4 storage vesicles[42]. Similar effects were seen in skeletal muscle of SIRT2 KO mice[42]. Sirtuin 2's role in mediating the effects of insulin and regulating gluconeogenesis will be discussed in later sections. Sirtuin 2 also has multiple non-metabolic roles such as adipocyte differentiation[43].

Like the other Sirtuin members, SIRT3 is vital to metabolic regulation and loss of SIRT3 causes metabolic dysfunction; this will be detailed more thoroughly in later sections [5, 44]. Sirtuin 3 is localized in the mitochondria and affects metabolism in skeletal muscle, liver, kidneys, heart and the brain [5]. While SIRT3's affects are mediated throughout numerous tissues the result of its function are opposite between substrate producing tissues and those that are limited to metabolite use only[5, 44]. In the liver, SIRT3 plays a role in regulating almost every metabolic function as well as in the production of reactive oxygen species [5, 32, 45, 46]. Later sections will detail how intertwined SIRT3 is in metabolic regulation. Opposite effects are seen from SIRT3 function in liver compared to skeletal muscle where fasting promotes SIRT3 function in the liver fasting decreases SIRT3 expression in skeletal muscle [44].

As with other sirtuins, SIRT3 activity is more robust in tissues with higher metabolic activity, such as the liver and skeletal muscle. Sirtuin 3 regulates the TCA cycle and oxidative

phosphorylation as well as functions to coordinate substrate utilization between tissues through its deacetylase activity[5]. Results from Dittenhafer-Reed et al. (2015) suggests an interesting tissue specific dynamic between tissues that are obligatory users of metabolites and those that also regulate and produce substrate for extra-tissue utilization[5]. Sirtuin 3 KO mice showed a pattern of dynamic fluctuations in acetylation status in proteins of tissues that were end users of substrate, brain, heart, and skeletal muscle[5]. Compared to tissues that are producers of metabolic substrates, such as the liver and kidneys, which contain more acetylation sites but show less change overall in acetylation levels compared to tissues like skeletal muscle and the brain [5]. They have suggested the need of skeletal muscle, brain, and heart to regulate acetyl CoA usage, as metabolite supplies are limited, resulting in acetylation status modification occurring more frequently[5]. The liver and kidney have larger volumes of acetyl CoA present and regulation occurs slower but on a larger scale compared to skeletal muscle, brain, and heart tissue[5]. As stated previously, conditions inside the mitochondria promote acetylation, but in skeletal muscle and heart, acetyl CoA is used rapidly. In the liver and kidneys, acetyl CoA pools may remain longer, unused in a regulatory role, leading in part to higher levels of acetylation, and in turn greater expression of SIRT3[5].

Sirtuin 4 (SIRT4) functions during the fed state to promote lipid anabolism while suppressing fatty acid oxidation through deacetylation and inhibition of malonyl CoA decarboxylase (MCD) leading to the accumulation of mitochondrial malonyl CoA and CPT1 inhibition[47]. In addition to MCD's inhibition of mitochondrial fatty acid import through CPT, conversion of acetyl CoA to malonyl CoA by MCD increases malonyl CoA levels leading to an inhibition of ACC[48]. Little is known about SIRT 4 function and so far, MCD appears to be SIRT4's only target[47]. SIRT4 KO mice show protection against diet induced-obesity and

increased exercise tolerance[47]. Sirtuin 4 is a weak deacetylase and may serve other functions and has been suggested to regulate other PTMs[49].

As with SIRT4, Sirtuin 5 (SIRT5), also possesses weak deacetylase capabilities[50]. Sirtuin 5 deficiencies do not have any observable effects on acetylation[51]. However, carbamoyl phosphate synthetase 1 (CPS1) has been identified as a target of SIRT5[52]. It is likely that CPS1 deacetylation is performed by multiple sirtuins, as the absence of SIRT5 has no effect on its deacetylation. Sirtuin 5's main function has been hypothesized to be as a desuccinylase and demalonylase[53]. Cell lines with a SIRT5 deficiency show significant increases in malonylated and succinylated proteins [53]. This suggests SIRT5's function is more as a desuccinylase and demalonylase rather that a deacetylase.

Sirtuin 6 (SIRT6) is localized to the nucleus but has been shown to exhibit some control over glycolysis and to the metabolic response to exercise[54]. More recently SIRT6 was shown to directly affect oxidative phosphorylation and mitochondrial biogenesis by deacetylating general control non-repressed protein 5 (GCN5) which can acetylate, and inhibit, PGC-1α[55]. These effects have only been observed in the liver and it was shown that SIRT6 is a regulator of gluconeogenesis through its effects on GCN5[55].

SIRT7 has little function in skeletal muscle and like SIRT6 is localized in the nucleus[56]. Sirtuin 7 deletion in mice is associated with reduced life expectancy and a reduced capacity for DNA repair[57]. The exact mechanism behind how SIRT7 affects life expectancy and DNA repair is unknown.

Metabolic Pathways

Insulin Signaling

Acetylation's role in the fed state begins with insulin's release modulating the insulin signaling pathway, and the switch to glucose uptake and utilization as well as inhibition of fatty acid oxidation. The insulin-signaling pathway described below details multiple sites of regulation through phosphorylation followed by acetylation. Acetylation of many proteins in the insulin-signaling pathway works to change the conformational state of those proteins to either inhibit phosphorylation or aid in phosphorylation [3].

For skeletal muscle, insulin signaling originates upon insulin binding to transmembrane insulin receptor proteins initiating a signaling cascade that culminates in GLUT4 translocation to the cell membrane and glucose uptake[42]. Insulin receptor binding causes a conformational change, activating its kinase activity, and acting as the starting point for the activation or suppression of metabolic pathways [58]. Activation of the IR has multiple outcomes progressing down two pathways. The first is mediated by insulin receptor substrates (IRS) and facilitates metabolic function; the second, mediated by IRS and Src homology 2-domain containing (SHC) provides cues for continued cell growth and proliferation or apoptosis [58]. Processing of dietary carbohydrate depends on the former process where IR causes IRS phosphorylation, and in skeletal muscle the specific IRS isoform involved in the carbohydrate metabolic pathway is IRS-1. Insulin receptor substrate 2 (IRS-2) is active in skeletal muscle metabolism and is involved in lipid synthesis [58]. Phosphorylated IRS-1 binds and activates PI3-kinase (PI3K) which then phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce phosphatidylinositol (3,4,5)-triphosphate (PIP_3) [58]. Phosphatidylinositol 4,5-bisphospahte/PIP_3 is membrane bound and upon binding of free cytoplasmic 3-phosphoinositide-dependent protein kinase 1 (PDPK-1,

aka PDK-1) activation of PDPK-1 occurs [58]. Protein kinase B/Akt and atypical PKC are targets for PDPK-1 phosphorylation and activation; Akt initiates Glut4 translocation to the cell membrane for glucose transport [58]. Protein kinase B/Akt is only partially activated by PDPK-1, for full activation Akt must be again phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) [58].

Activation of IR causes binding and phosphorylation of IRS proteins leading to IRS activation, but IRS proteins are subject to more forms of posttranslational modifications. Acetylation of IRS proteins has opposing effects between the two IRS isoforms, where acetylation of IRS1 promotes phosphorylation the opposite is true for IRS2 [3]. This may be due to the location of acetylation sites on IRS1 that lie within its pleckstrin homology domain and how they affect IRS1 recognition and binding of phosphorylated PIP_3 [3]. Activation of IRS proteins by phosphorylation induces binding of IRS and PI3K proteins, which are comprised of several subunits. Multiple subunits of the PI3K enzyme, p85α, p85β, p50α/p55α, p110α, and p110β, have been shown to contain acetylation sites in non-muscle tissue, but the nature of how acetylation affects the function of PI3K, and its interaction with its downstream effectors is unclear [3, 59]. Skeletal muscle appears to have far fewer acetylation sites for PI3K with only the p55α subunit having been recognized as containing any sites but this could change with future studies[60]. As with many proteins and enzymes in metabolic pathways there are numerous acetylation sites that have been discovered through mass spectrometry, but the actual level and functionality of their acetylation remains to be revealed[6, 13]. Following IRS/PI3K binding and activation, PI3K phosphorylates membrane bound PIP_2 forming PIP_3 which recruits PDK1 for activation that in turn phosphorylates and activates Akt (PKB). Akt and PDK1 both contain acetylation sites in their kinase domains, pleckstrin homology domains; and in cardiac tissue

deacetylation of Akt2 was shown to increase its affinity for binding with PDK1[3, 61]. In skeletal muscle of fasted mice, deacetylation of Akt followed insulin stimulation but not in fed mice[61]. Ensuing Akt activation, Akt stimulates mTORC2 promoting a positive feedback mechanism where mTORC2 increases Akt function. Mammalian target of RICTOR complex 2 depends on acetylation of multiple lysine residues within the rictor complex to enhance stability to the mTORC2 protein; without acetylation at these residues decreased phosphorylation of Akt was observed [3, 62].

The negative regulators of the insulin-signaling pathway are also modulated by acetylation. Phosphatase & tensin homolog (PTEN) and protein tyrosine phosphatase 1B (PTP1B) have lysine residues that are targets of acetylation[3]. Dephosphorylation of PIP_3 by PTEN inhibits further transduction of the insulin-signaling pathway[63]. The available data points to acetylation in the phosphatase domain of PTEN inhibiting its ability to dephosphorylate PIP_3 and halting insulin signaling[64]. In HEK293 cells, acetylation of Lys125 and Lys128 inhibits PTEN's ability to dephosphorylate PIP_3[64]. Sirtuin 1's action is necessary for PTEN function, at least in embryonic stem cells, where a SIRT1 KO was observed with increased PTEN acetylation[65]. Dephosphorylation of IR by PTP1B inhibits insulin-signaling[66]. Much less is known as to how acetylation affects PTP1B, only that acetylation sites are present leaving it as conjecture to whether acetylation affects it in much the same way as PTEN.

Adenosine monophosphate- activated protein kinase (AMPK) acts to regulate metabolism, through various actions, one of which is AMPK's function to monitor AMP/ATP ratios in the cell. As stated previously small increases in AMP levels are indicative of increased energetic demand [67]. Adenosine monophosphate- activated protein kinase activates oxidative phosphorylation and mitochondrial biogenesis while turning off ATP consuming processes

through posttranslational modifications and transcriptional regulation [67]. As important to AMPK's role as a metabolic sensor monitoring AMP/ATP levels, AMPK covalently modifies numerous proteins and enzymes involved in metabolism. Posttranslational modifications mediated by AMPK regulate multiple metabolic pathways promoting catabolism of substrates while simultaneously inhibiting anabolic pathways. The actions of AMPK are facilitated either directly through phosphorylation as is the case with glycogen phosphorylase, glycogen synthase, and ACC or indirectly by increasing the activity of other proteins like sirtuins. In response to insulin signaling AMPK serves a vital role in metabolic flexibility through promoting glucose uptake [68]. In contracting muscle AMPK promotes GLUT4 translocation and glucose uptake and in non-muscle cells AMPK functions similarly on GLUT1 [69]. Fatty acid oxidation is also dependent on AMPK phosphorylating ACC2 thereby inhibiting production of malonyl CoA [69]. Mitochondrial biogenesis is partially dependent on AMPK's actions in a SIRT1 dependent manner by increasing NAD supplies [69]. In a non-insulin stimulated state GLUT4 is sequestered in intracellular compartments known as GLUT4 storage vesicles (GSVs)[70]. Glucose transporter 4 is trapped in its GSVs by tether containing a UBX domain for GLUT4 (TUG) protein which when acetylated is in its functional state[71]. Insulin stimulation relies on SIRT2 deacetylation of TUG, followed by cleavage and release of GLUT4 and translocation to the cell surface membrane[42].

Glycolysis

Simplistically, glycolysis is the conversion of one glucose molecule to two molecules of pyruvate for the introduction into the tricarboxylic acid (TCA) cycle in the presence of oxygen. In aerobic conditions pyruvate is shuttled into the mitochondria where it is converted to acetyl

CoA in the first step of the TCA cycle. Almost every step of glycolysis has the potential to be controlled through posttranslational modifications. The initial phase of glycolysis is the phosphorylation of glucose to glucose 6-phosphate (G6P) by hexokinase in skeletal muscle or glucokinase in the liver, both are ATP consuming reactions [48]. From this point forwards, all the steps of glycolysis are the same regardless of the tissue. Glycogen and galactose also enter at this stage through conversion to G6P. Glucose-6-phosphate proceeds through a series of oxidation reactions splitting the six carbon G6P into two, three carbon pyruvate molecules producing the energy necessary to convert ADP into ATP and NAD^+ into NADH. Pyruvate can either be converted into lactate (anaerobic) via lactate dehydrogenase, or enter the mitochondria for oxidation [48].

Important regulatory steps in glycolysis are the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate via phosphofructokinase (PFK), and the final step where phosphoenolpyruvate is converted to pyruvate via pyruvate kinase. These two steps represent key regulatory checkpoints with almost total control over the continuance or inhibition of glycolysis. As they are such vital steps in the glycolytic process, they are subject to numerous allosteric and covalent controls. Posttranslational regulation of proteins via phosphorylation in glycolysis is not the predominant means for regulation for the enzymes acting in the main sequence of glycolysis. Allosteric feedback is far more common as they respond more rapidly to the fluid changes in nutrient availability and energy demand. While covalent modifications are not as prevalent in glycolysis as regulation by allosteric feedback, recently it has become apparent that acetylation not phosphorylation may be the covalent modification controlling glycolysis.

All glycolytic enzymes contain acetylation sites and in theory can be acetylated. However, the protein that is most often recorded as being acetylated is pyruvate kinase[22]. Pyruvate kinase represents a heavily regulated checkpoint in glycolysis, as proceeding forward would result in the generation of pyruvate and its conversion to acetyl CoA, both of which represent an irreversible reaction, and in the latter's case full commitment to oxidation in the TCA cycle.

In addition to pyruvate kinase being regulated by phosphorylation, acetylation of pyruvate kinase marks it for ubiquitination and proteosomal degradation [4]. There are four isoforms of pyruvate kinase; M1, M2, L, and R, for muscle 1, muscle 2, liver, and blood. Isoform M1 is found in most cells; M2 is turned off after fetal development except in cases of cancer [4]. Acetylation at Lys-305 leads to decreased pyruvate kinase M1 and ultimately degradation [4]. While PK-M has multiple acetylation sites, Lys-305 is conserved among all four isoforms [4].

Gluconeogenesis/ Glycolysis

During the fed state when glucose supplies were plentiful the majority was stored in the liver and skeletal muscle in the form of glycogen for later use[72]. Glycogen from the liver and glucose from gluconeogenesis will be the primary source to keep the blood glucose supply at an adequate level for all tissues of the body, and while skeletal muscle has glycogen reserves they remain stored to meet energetic demands[72]. Approximately the same percentage of enzymes in the gluconeogenic pathway are also capable of being acetylated but as with glycolysis the actual frequency of acetylation is unknown[22].

As with pyruvate kinase in glycolysis, phosphoenolpyruvate carboxykinase (PEPCK) is an irreversible step with a separate catalytic enzyme [4]. Phosphoenolpyruvate carboxykinase is an

enzyme that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and is one of the regulatory steps in gluconeogenesis, as well as glyceroneogenesis, and amino acid metabolism. Zhao et al. (2010), showed four acetylation sites on PEPCK through mass spectrometry analysis, and that substrate availability determines the acetylation status of PEPCK[4]. Glucose availability leads to a decrease in PEPCK acetylation where a lack of glucose and increased reliance on amino acids causes an increase in acetylation [4]. Furthermore, they demonstrated that acetylation has no direct effect on PEPCK but rather tags it for proteosomal degradation [4]. Phosphoenolpyruvate carboxykinase is acetylated by acetyltransferase complex consisting of P300 and BAT3 leading to an interaction with UBR5 and E3 ligase targeting PEPCK for degradation [4]. A member of the Sirtuin family, SIRT2, deacetylates PEPCK and is thus responsible for a portion of fasted state metabolism [4].

In skeletal muscle the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate via PFK1 is one of the few irreversible steps of glycolysis, and is another committal step to the utilization of the glucose molecule. Once passing the PFK checkpoint, glucose is committed to glycolysis and this cannot be undone in skeletal muscle- pyruvate must be transported to the liver for gluconeogenesis to regenerate the glucose molecule. In the liver fructose-1,6-bisphosphate (ALDOA1) catalyzes the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate. Acetylation of ALDOA1 has been observed through multiple mass spectrometry proteomic surveys, and site-specific acetylation of ALDOA1 inhibits its function resulting in a slow-down of gluconeogenesis[60].

Cytokine production of IL-6 can inhibit gluconeogenesis thorough binding and activation of signal transducer & activator of transcription 3 (STAT3)[73]. Binding of PEPCK and glucose-6-

phosphatase promoter regions by STAT3 prevents their transcription[74]. Sirtuin 1 is required to deacetylate STAT3, which causes a decrease in STAT3 activity promoting gluconeogenesis[72].

Acetylation's effects and actual occurrence in both glycolysis and gluconeogenesis remains ambiguous at best, but both pathways illustrate an alternative function of acetylation. Pyruvate kinases and PEPCK are marked for degradation through acetylation, whether/or how many metabolic enzymes are degraded because of acetylation is unclear but demonstrates the dual modality of acetylation as a posttranslational modification.

Glycogenolysis/ Glycerol Synthesis

During the fed state when glucose supplies are plentiful, roughly two-thirds of available glucose is stored in the liver and skeletal muscle in the form of glycogen for later use[75]. Glycogenolysis proceeds with the de-branching of the glucose molecule through glycogen phosphorylase's activity that cleaves the glucose molecule while adding a phosphate group producing glucose 1-phosphate. The phosphate group of glucose-1-phosphate is transferred to carbon six generating glucose-6-phosphate via phosphoglucomutase, glucose-6-phosphate can enter glycolysis but only in the liver can it be converted back to glucose, as only the liver possesses glucose-6-phosphatase.

Glycogen anabolism and catabolism are regulated by glycogen synthase and glycogen phosphorylase, both of which whose function depends on covalent modification. As they represent opposing reactions, covalent modifications have differing effects. Where glycogen phosphorylase is dependent on phosphorylation by glycogen phosphorylase kinase to function, acetylation has the opposing effect. Acetylation of glycogen phosphorylase has been shown to reduce glycogen catabolism by promoting protein phosphatase 1 interaction [3, 76]. Glycogen

phosphorylase kinase and protein phosphatase 1 have also been shown to have acetylation sites, but the level of acetylation that occurs and the mechanism through which this operates is unclear [3, 76].

There are relatively few steps in glycerol synthesis compared to other metabolic pathways; still acetylation is an integral link to glycerol synthesis function. Glycerol synthesis links carbohydrate and lipid metabolism pathways, the formation of TAG requires glycerol and fatty acids. Glycerol-3-phosphate dehydrogenase converts dihydroxyacetone from glycolysis into glycerol-3-phosphate in a reduction reaction utilizing an electron from NADH. Glycerol-3-phosphate dehydrogenase is inhibited by acetylation [3, 60]. This is currently the extent of our understanding of acetylation's role in the glycerol synthesis pathway.

Tricarboxylic Acid Cycle

The tricarboxylic acid (TCA) cycle is a series of eight oxidation reactions that produces ATP & NADH. Through the TCA cycle and the electron transport chain (ETC), the energy required to convert ADP to ATP (GDP→ GTP), NAD^+ to NADH, and FAD^+ to $FADH_2$ is generated creating an electrochemical gradient driving ATP synthase. The initial formation of acetyl CoA links the metabolism of carbohydrates, fats, and amino acids as all three forms of energy are first converted to acetyl CoA. While most catalyzing enzymes of the TCA cycle can be acetylated, as with other metabolic pathways the level of acetylation for most of the TCA cycle enzymes and under what circumstances acetylation occurs is currently not known.

Isocitrate dehydrogenase (IDH) is an enzyme that catalyzes a decarboxylation reaction of isocitrate producing α-ketoglutarate, and in the process generates NADH and CO_2[77]. This is an irreversible step and is allosterically regulated by the precursor and product, coenzymes, and

substrates to conserve isocitrate. Isocitrate dehydrogenase 2 (IDH2) like IDH1 can catalyze a decarboxylation of isocitrate, but reduces $NADP^+$ as an electron acceptor and occurs in the mitochondrial matrix (TCA cycle) or in the cytosol[78]. Kim et al (2006) demonstrated that IDH2 is deacetylated in response to feeding[21]. Isocitrate dehydrogenase enzyme activity is inhibited by acetylation, and for function to resume must be deacetylated by SIRT3 [77-79].

Succinate dehydrogenase (Sdh) links the TCA cycle to the electron transport chain of the inner mitochondria and Sdh is subject to regulation by phosphorylation and acetylation[80]. Succinate dehydrogenase activity is inhibited by acetylation and for the TCA cycle to continue must be deacetylated by SIRT3 [77, 80]. As with IDH, Sdh is acetylated in response to feeding as first described by Kim et al (2006)[21]. Cimen et al. (2010) demonstrated the interplay between acetylation of a subunit of Sdh and SIRT3 through studies in mice liver. Deacetylation of Sdh is not completely necessary in the presence of high levels of NADH and $FADH_2$ in which there is no need to generate acetyl CoA [80]. It is only when levels of NAD^+ and FAD increase that the TCA cycle must continue and SIRT3's deacetylase activity is necessary [80].

β-Oxidation

Fat represents by far the most energy dense source of substrate available for metabolism. Through β-oxidation, where two carbons are sequentially removed with each round, generates acetyl CoA to enter the TCA cycle. Activation of fatty acids through the addition of acyl CoA before entry into the mitochondria is required. Fatty acyl CoAs are transported into the inner mitochondrial matrix via the transmembrane protein CPT1, and is inhibited in the presence of malonyl CoA. Acetyl CoA is necessary for the generation of fatty acyl CoA, and promotes CPT1 transport of fatty acyl CoAs into the mitochondrial matrix.

Long-chain acyl-CoA dehydrogenase (LCAD) catalyzes the first reaction of the β-oxidation pathway, and in the process reduces FAD to $FADH_2$[48]. Bharathi et al. (2013) demonstrated *in vitro* through chemical acetylation that LCAD has multiple lysine sites where acetylation is possible, but only four where acetylation was demonstrated to occur[81]. Through their previous studies, they have shown SIRT3 knockout lead to a state of hyper-acetylation of LCAD, and a significant decrease in fatty acid oxidation[81]. Two lysine residues in close proximity when acetylated, Lys-318 and Lys-322, result in a significant decrease in LCAD activity, and protein modeling suggested that acetylation at these lysines caused a conformational change shifting neighboring residues and impeding substrate binding[81]. Bharathi et al. (2013) have hypothesized this mechanism may govern the other acyl-CoA dehydrogenases which share a similar amino acid residue pattern[81]. To what extent the *in vitro* model matches *in vivo* function has not been determined.

Enoyl CoA hydrotase/3-hyrdoxyacyl CoA dehydrogenase (EHHADH) catalyzes the second and third steps in β oxidation, and has four different lysine residues that can be acetylated[16, 22]. Acetylation of EHHADH increases its function and acetylation is concurrent with increased fatty acid supply[77]. SIRT3 inhibits EHHADH's activity through its deacetylase activity[16, 22]. Long-chain acyl CoA dehydrogenase (LCAD) is hyper-acetylated on one lysine residue, Lys-42, inhibiting its function [77]. SIRT3 deacetylation of LCAD increases its activity [77].

Malonyl CoA decarboxylase (MCD) partial regulates substrate selection by promoting fatty acid oxidation through the inhibition of acetyl CoA carboxylase's conversion of acetyl CoA to malonyl CoA- inhibiting CPT1 and therefore fatty acid entry into the mitochondria. Malonyl CoA decarboxylase function is promoted through acetylation; to date this is the only known PTM that governs its function [47].

Little is known about acetylation's effects on the remaining enzymes involved in β-oxidation, but from what data is available acetylation has mixed effects on β-oxidation and while MCD is not part of the β-oxidation pathway it is necessary for import of fatty acids into the mitochondria. Whether acetylation is a negative feedback mechanism to slow β-oxidation down in response to increasing acetyl CoA levels, and how the appropriate enzymes are targeted is unknown and should be considered a promising area of future research.

Amino Acid Metabolism/ Urea Cycle and Nitrogen Metabolism

Amino acid metabolism occurs to some extent but amino acids are more important for maintaining body protein supplies due to degradation and synthesis. Catabolism of amino acids occurs during the fed state when energy demand is high; amino acid catabolism will also occur in a starvation state to maintain blood glucose levels.

Glutamate dehydrogenase (GDH) converts glutamate and NAD^+ to α-ketoglutarate and NADH. Deacetylation increases GDH activity and in the liver, and there are up to 11 different acetylation sites on GDH [16, 21, 22, 77, 79]. Regulation by SIRT3 & 4 is one of only a few examples of multiple sirtuins regulating the same enzyme [77]. The liver is the main site for the metabolism of non-branch chain amino acids as they can be converted to glucose in time of starvation or intense physical activity; branch chain amino acids are metabolized predominately in skeletal muscle, but under normal fed and fasted conditions is a negligible contributor to the overall substrate pool[82].

The urea cycle begins with a transamination or deamination reaction to remove the nitrogen group from the amino acid creating an α-keto acid that will be converted into a TCA

cycle intermediate for oxidation. All the enzymes in the urea cycle and involved in nitrogen metabolism have been shown to be acetylated[16, 21, 22, 83].

Carbamoyl phosphate synthetase 1 (CPS1) CPS1 has up to 12 different acetylation sites and deacetylation by SIRT5 increases its function [21, 22, 52, 77]. Activation of CPS1 by SIRT5 is critical for the conversion of ammonia to carbamoyl phosphate to handle excess ammonia [84]. So many acetyl targets suggest a gradient of function modulated by acetylation level. In the liver of mice it has been shown that starvation or a high protein diet increases CPS1 activity in a SIRT5 dependent manner[52]. Both situations would require a metabolic shift towards protein degradation and amino acid metabolism, and ultimately and increase in urea cycle function[52]. Ornithine carbamoyl transferase (OTC) is acetylated at up to three different sites, and OTC function is inhibited by acetylation [21, 22, 77]. Yu, W. et al (2009) showed that acetylation of Lys-88 inhibited OTC function, and that OTC is acetylated in the presence of high glucose concentration[85]. SIRT3 deacetylates OTC leading to its activation [72]. Argininosuccinate lyase (ASL) is acetylated to inhibit function [22, 77]. ASL is acetylated in the presence of high glucose concentrations [77]. Little is known about glutamate dehydrogenase (GLDH) acetylation other than it is a SIRT3 target for deacetylation [84]. Whether deacetylation by SIRT3 activates or inhibits GLDH function is speculative at this moment.

Transcriptional Regulators

Forkhead box O1 (FOXO1) protein is activated in the fasted state in response to glucagon release, and promotes the expression of gluconeogenic and lipogenic genes. Multiple covalent modifications regulate FOXO activity; Akt phosphorylation inactivates FOXO marking it for ubiquitination and degradation while MAPK phosphatase 3 activates FOXO1 [72, 84]. There are

multiple FOXO homologues found in skeletal muscle that are differentially regulated by acetylation[86]. FOXO1 is acetylated by p300/CBP decreasing its ability to bind to target gene promoter regions in a fasted state [72, 86]. Unlike FOXO1, acetylation by p300/CBP has the opposite effect on FOXO3a under similar conditions[86]. This differential regulation of FOXO homologues may point to the necessity of regulation by Sirtuins and Akt to ensure proper transcription and protein activity among FOXO homologues[86].

NF-κB contains two subunits and one of the most abundant, p65/ Rel A, is acetylated at multiple lysine residues inhibiting its function. HDAC3 deacetylates NF-κB at each residue with different effects to its function depending on which lysine is deacetylated. Deacetylation at Lys-221 promotes binding with IκK, and export to the nucleus whereas deacetylation at Lys-310 impairs transcriptional ability without changing export to the nucleus; HDAC3 deacetylation at Lys-122/123 keeps NF-κB active in the nucleus [84]. HDAC3 activity is inhibited in the cytoplasm by IκK and requires ubiquitination and degradation of IκK mediated by TNF-α for activation of NF-κB [84].

Sirtuin 1 regulates SREBP-1c through deacetylation leading to increased activity and expression of SREBP-1c and its target genes in the skeletal muscle of healthy mice[33]. Acetylation sites on SREBP-1c at Lys-289 and Lys-309 leads to decreased stability by interfering with its association capabilities and ultimately ubiquitination and degradation [33]. In skeletal muscle, it was shown using gene electro-transfer that over expression of SIRT1 leads to increased SREBP-1c promoter transactivation confirming that in skeletal muscle SIRT1 regulates SREBP-1c gene expression [33]. This pathway is not unidirectional, but functions as a positive feedback loop in which increased SREBP-1c expression then further activates SIRT1 thereby further increasing SREBP-1c gene expression [33]. This was indicated when viral

transfection of SREBP-1c mRNA into skeletal muscle induced increased SIRT1 expression [33]. These findings are contradictory to previous studies in the liver of fasted mice where loss of SIRT1 results in an increase in SREBP-1c expression while in liver of obese mice fed a high fat diet when SIRT1 is active SREBP-1c expression is decreased[33, 87, 88].

As previously mentioned, LXR induces SREBP-1c expression, although it is unknown the exact mechanism in which this occurs [33]. In SIRT1 KO mice LXR acetylation is significantly increased in gastrocnemius muscle and Li et al showed that re-introduction of SIRT1 via viral transfection in human myotubes results in decreased LXR-β acetylation [33]. Li et al. also showed that acetylation at Lys-432 and Lys-433 in HEK293T cells and deacetylation at these lysine residues result in ubiquitination and degradation [33].

A family of inner mitochondrial membrane proteins known as uncoupling proteins function to reduce the electrical gradient by allowing passage of protons from the inner to outer mitochondrial compartments[89]. This effectively reduces the ability of the electron transport chain to complete oxidative phosphorylation, and as a consequence can prevent the build of ROS products[89]. Uncoupling proteins promote fat oxidation, which in turn inhibits glucose oxidation[90]. In pancreatic β cells SIRT1 acts as a positive regulator of insulin by binding the uncoupling protein 2 (UCP2) promoter region and inhibiting UCP2 gene expression[91]. Sirtuin 1 is partially responsible for controlling UCP3 gene expression preventing increased gene expression through its deacetylase activity, and while not completely responsible for UCP3 expression SIRT1 is a potent inhibitor[92].

Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha regulates genes controlling metabolism as well as mitochondrial biogenesis, acts as a transcription factor, and is regulated through several posttranslational modifications [35, 93]. In skeletal muscle, PGC-1α has

been described as the "master" regulator of mitochondrial biogenesis whose expression is elicited through exercise and AMPK[94]. Phosphorylation of PGC-1α by AKT inhibits gluconeogenesis and fatty acid oxidation; PGC-1α increases AKT function through positive feedback [58]. Many of AMPK's effects on metabolic function are mediated through PGC-1α where AMPK phosphorylation of PGC-1α increases its function and in turn its own expression[94].

Inhibition of PGC-1α is also accomplished through acetylation and PGC-1α is reliant on SIRT1 to regain functionality when acetylated[35]. The ω-3 fatty acid oleic acid was demonstrated to increase fatty acid oxidation through the PKA/SIRT1/PGC-1α transcription pathway in skeletal muscle[35]. Oleic acid and not other fatty acids such as palmitate which can induce an inflammatory response was shown to increase fatty acid oxidation in a PGC-1α dependent manner[35]. Gerhart-Hines, et. al. (2007) showed that fasting induced PGC-1α deacetylation via SIRT1 in skeletal muscle was necessary for the transcription of mitochondrial OXPHOS genes[95]. Inhibition of SIRT1 by nicotinamide or acetylation of PGC-1α through GCN5 leads to the opposite outcome with downstream targets of PGC-1α showing decreased levels of gene expression in myotubes[95]. Though there are multiple lysine acetyltransferase families each with members who bind PGC-1α to date only GCN5 has been directly observed to acetylate and inhibit PGC-1α[96].

Effects of Exercise, Caloric Restriction, Obesity, & Type II Diabetes

Acetylation status fluctuates in most metabolite producing tissues as well as tissues that are consumers of metabolic fuels depending on nutritional status. Almost all enzymes in the metabolic network have acetylation sites with few exceptions, and while many metabolic enzymes can be acetylated, acetylation is most prominent in the mitochondria [6]. In response to

transitioning between fed and fasted states, it is of the utmost importance that the tissues of the body, skeletal muscle and liver, in particular, respond to changing substrate availability appropriately. Acetylation has proven to be a mediator in transitionary periods that is crucial for healthy fuel switching through regulation of metabolic pathways. Yang, Li et al. (2011) measured acetylation levels of multiple tissues, liver, skeletal muscle, heart muscle, brown and white adipose tissue, brain, and kidneys, in the fasted and re-fed state and measured 733 acetylated peptides from 337 proteins [6]. Of these 58 proteins showed a 3-fold or greater change in acetylation levels where 38 of these proteins were metabolic proteins or chaperones [6]. In the transition from fasted to fed states, acetylation patterns change significantly in the enzymes of glycolysis, TCA cycle, β-oxidation, and oxidative phosphorylation [6]. Of equally great importance, Yang, Li et al. (2011) showed tissue specific acetylation patterns between fasted and fed states illustrating the dynamic intricacies of metabolic control by acetylation. Acetylation of metabolic enzymes function to regulate metabolism, specifically the fasted to fed transition and vice versa, but acetylation is also linked to metabolic dysfunction [7]. Recently Davies et al. (2016), showed the effects of a high fat diet on control mice in skeletal muscle where when subject to chronic high fat feeding there was an increase of 222% in acetylation compared to their control mice fed a low-fat diet [7]. This is evidence to the influence of a western style diet on metabolic regulation and the impact a habitual high fat diet can have in modifying the acetylome.

Both obesity and caloric restriction increase acetylation in mitochondrial proteins pointing to the varied nature of acetylation, and how it affects proteins differently. However, sirtuins responses to caloric restriction and obesity are considerably different, and may highlight a potential mechanism for the observed difference in affect between the two states. Acetylation both increases and decreases protein function highlighting the need for specificity among

sirtuins, and how they contribute to metabolic flexibility or inflexibility depending on the nutritional context.

For decades, it has been known that the insulin-signaling pathway is highly regulated by phosphorylation, but only recently has acetylation been shown to be so critical for proper transduction of this pathway. Based on the evidence it appears acetylation modifies tertiary structure allowing or inhibiting phosphorylation in healthy individuals. Consuming a diet habitually high in fat can lead to incomplete oxidation of fatty acids resulting in fatty acid intermediaries that directly interfere with the insulin-signaling pathway[97]. It remains to be shown what effect these fat intermediaries have on acetylation status, and whether glucose uptake and insulin-signaling transduction disruption is a result of acetylome perturbation. Members of the sirtuin family show altered function in response to a high fat diet, and it maybe a plausible hypothesis that a high fat diet and the resulting fat intermediaries can interfere with the acetylases and deacetylases responsible for normal insulin signaling. Posttranslational modifications are vital to the translation of insulin's effects through the insulin-signaling pathway and not only obesity but an excess of long chain fatty acids or incompletely oxidized fatty acids can disturb metabolic homeostasis[97]. Diacylglycerols and ceramides can inhibit protein kinase C and Akt inhibiting insulin signaling transduction and Glut4 translocation[97].

Pyruvate dehydrogenase complex (PDC) links glycolysis to the TCA cycle, and the PDC is highly regulated by posttranslational modification to ensure oxidation of pyruvate, a gluconeogenic precursor, when necessary. Given PDC's position at the crossroads of multiple metabolic pathways regulating carbohydrate catabolism and gluconeogenesis, it is subject to several forms of posttranslational modification. The pyruvate dehydrogenase complex is regulated by phosphorylation at the E1α subunit by pyruvate dehydrogenase kinase (PDK),

which does so in response to cellular cues such as the accumulation of pyruvate and pyruvate dehydrogenase phosphatase's (PDP) removal of the phosphor group [44]. Phosphorylation of PDC is removed in response to insulin as well as the accumulation of phosphoenolpyruvate and AMP. SIRT3 has been shown to regulate PDC in skeletal muscle and liver with differing effects [44]. Deletion of SIRT3 has dramatic effects on glucose and fatty acid oxidation preceding metabolic inflexibility culminating in a reduction in PDC function with a concurrent increase in lactate and pyruvate [44]. Inhibition of PDC through decreased SIRT3 activity in skeletal muscle mirrors the effects of metabolic inflexibility with a preference for the oxidation of fatty acids over carbohydrates, even in a fed state [44]. Acetylation of PDC occurs on the same subunit, E1α, as that of the reversible phosphorylation also regulating its function [44]. A cellular increase in NAD^+ levels functions in two significant regulatory roles in glycolysis that are critical for metabolic flexibility, 1) it activates PDC and 2) triggers SIRT3 deacetylation of PDC leading to increased function [44]. In metabolically flexible skeletal muscle, the onset of the fasting period leads to a decrease in SIRT3 activity and hyper-acetylation of PDC switching skeletal muscle substrate preference for fat oxidation [44].

Associated with obesity and type 2-diabetes is a state of chronic low-grade inflammation, in part mediated by NF-κB activation[34]. Nuclear factor kappa-light-chain-enhancer of activated B cells promotes an inflammatory state through activation of cytokine and chemokine genes furthering monocyte/macrophage recruitment[98, 99]. In healthy metabolically flexible individuals down regulation of NF-κB's regulatory subunit p65 by SIRT1 deacetylation leads to its continued inactivation [34]. There is a strong correlation between decreased SIRT1 expression and metabolic syndrome[100]. Caloric restriction has been shown to mitigate the effects of obesity and prolong life span and induce SIRT1 activity [34]. SIRT1 also acts as a deacetylase on CREB

regulated transcription coactivator 2 (CRTC2) to suppress gluconeogenesis in times of prolonged fasting [72]. In a fasted state CRTC2 activates cAMP response element binding protein (CREB) which in turn activates gluconeogenic genes in the liver, however, CRTC2 is not solely responsible for CREB activation[72]. All evidence to SIRT1's function indicates it is necessary to maintaining metabolic flexibility and exerts protective anti-inflammatory effects. Sirtuin 1's function can be increased by decreasing use of NAD supplies by other enzymes, and is currently an area of research as a potential therapeutic target for metabolic disorders[101].

Deficiencies in SIRT3 whether occurring naturally or through genetic modification can lead to obesity and type 2-diabetes through hyper-acetylation of oxidative pathways in the mitochondria[102]. Associated with type 2-diabetes is a reduction of SIRT3 function resulting in decreases in Akt function and glucose uptake[46]. Chronic high fat feeding can partially mimic SIRT3 ablation in that mice fed a chronic high fat diet exhibit decreased SIRT3 expression in the liver which in turn results in a hyper-acetylated state of mitochondrial proteins with limited deacetylase capacity[102, 103]. Among the hyper-acetylated proteins is long-chain acyl-CoA dehydrogenase, a component of the β-oxidation pathway, and vital for oxidation of fatty acids[102]. Hirschey et al. (2013) have proposed that a partial mechanism in the development of metabolic syndrome is brought about by decreased SIRT3 function[102]. Short term fasting elicits the opposite effect on SIRT3 function with an increase in SIRT3 expression[45]. Two members of the sirtuin family, SIRT1 and SIRT3, appear to be engaged in almost every facet of metabolic control and are core components of substrate selection and utilization required for organismal metabolic flexibility as demonstrated by ensuing metabolic dysfunction in sirtuin knockout models. Metabolic dysfunction from obesity is linked to mitochondrial dysfunction from

overabundance of substrate, and limited electron acceptors resulting in potentially deleterious superoxide formation[104].

The neutralization of reactive oxygen species (ROS) is partially dependent on SIRT3 function as it deacetylates superoxide dismutase 2 (SOD2), which increases SOD2 activity[44]. Superoxide molecules are transformed into hydrogen peroxide and O_2 by SOD2[105]. Diminished SIRT3 function affects ROS production and oxidative stress response, as well as fatty acid metabolism[44]. Restoration of healthy mitochondrial function and reduction of ROS production can be achieved through caloric restriction leading to a shift metabolic plasticity and reduced substrate flux[32]. The mitochondrial response to caloric restriction is mediated through SIRT3 regulation of oxidative mitochondrial proteins[32]. Herbert et al. (2013) measured total and mitochondrial acetylation levels in a mouse model in response to caloric restriction and noticed two significant trends with an increase in total and mitochondrial acetylation accompanied by a small decrease in acetylation of select mitochondrial proteins[32]. Using a SIRT3 KO model Hebert et al. (2013) confirmed that the observed mitochondrial deacetylation they observed was due to SIRT3 action [32]. A similar response was noticed by Hirschey et al (2011) in that fasting promoted increased levels of SIRT3 expression[102].

The effect of acetylation on most glycolytic enzymes has not been elucidated but mounting evidence suggests that acetylation inhibits the utilization of carbohydrates and acetylation could potentially be prominent as a regulatory mechanism in the fed to fasted transition. Evidence of the effects of obesity on metabolic control of glycolysis through disruptions to acetylation patterns can be extrapolated from a recent study in which $CrAT^{skm-/-}$ mice were fed a habitual diet high in fat and the acetylome from muscle samples analyzed through mass spectrometry [7]. The knockout of CrAT in skeletal muscle led to an acetyl CoA

build up in the mitochondria accompanied by an increase in mitochondrial protein acetylation and a decrease in acetylation of non-mitochondrial metabolic proteins [7]. Buildup of mitochondrial acetyl CoA not accompanied by a metabolic shift away from glycolytic metabolite usage towards mitochondrial oxidation can be likened to the effects of obesity and type 2-diabetes in which there is increased substrate flux in the mitochondria accompanied with reduced energy demands and an overall state of metabolic inflexibility[104]. Skeletal muscle CrAT KO mice fed a high fat diet show impaired whole body glucose usage beyond that of the $CrAT^{fl/fl}$ controls[7]. Decreases in non-mitochondrial protein acetylation accompanied with increased mitochondrial protein acetylation may be indicative of a preference for energy production through non-oxidative pathways in a type 2-diabetic state in which an increase in blood glucose levels from both recently digested food and an aberrant glycogenolysis pathway must be dealt with.

The liver has been the subject of a large portion of acetylomic profiling, and is the only tissue to examine the effects of acetylation on gluconeogenesis. Associated with type 2-diabetes is a failure to inhibit gluconeogenesis, but whether this is mediated by a failure of acetylase activity on ALDOA1 is speculation. There is still much to clarify about acetylation's status in a fasted state and skeletal muscle, as an obligatory user of gluconeogenic products, would be a solid target for research in understanding acetylation's role in glucose uptake and utilization in a fasted state. As the rates for obesity and type 2-diabetes continues to skyrocket worldwide and associated with these conditions is a failure to suppress gluconeogenesis, a potential mechanism maybe with decreased acetylation rates of not only glycolytic enzymes but also those of gluconeogenic.

How obesity and type 2-diabetes effects acetylation's role in glycogen break down and glycerol synthesis, and ultimately TAG synthesis, needs research. Associated with obesity and type 2-diabestes is an increase in postprandial glucose levels in part mediated by a failure to suppress glycogenolysis and gluconeogenesis[106]. As stated previously, associated with type 2-diabes is metabolic inflexibility in which glucose is the predominant substrate with less fatty acid oxidation and increased storage, and while most increases in acetylation from obesity are mitochondrial there is a decrease in non-mitochondrial metabolic proteins. This may represent a mechanism through which the over reliance of a metabolically inflexible state for glucose is mediated as acetylation has an inhibitory effect on many non-mitochondrial proteins as exhibited by pyruvate kinase and glycogen phosphorylase.

Multiple proteomic studies measuring the acetylome have noted acetylation of ATP synthase although the role acetylation plays is unclear. Acetylation of ATP synthase slows down ATP production as confirmed in mouse liver of SIRT3 knockouts[107]. Sirtuin 3 and ATP synthase activity were noticeably altered in response to exercise, caloric restriction, fasting, and a high fat diet[107]. Obesity and type 2-diabetes leading to mitochondrial flux and ROS production has already been documented, and there is evidence to their effect on ATP production. A high fat diet was shown to increase the acetylation of an ATP synthase subunit[107].

Caloric restriction has a significant impact on the TCA cycle where under these conditions citrate synthase, aconitase, malate dehydrogenase, and succinate dehydrogenase show a multiple fold increase in acetylation in response to caloric restriction in murine embryonic fibroblasts [32]. Caloric restrictions effects on acetylation was measured in liver extract from mice fed a diet that was in a stepwise fashion decreased from 10% down to 40% by 16 by mass spectrometry[83]. A chronic high fat diet also increases the level of protein acetylation in skeletal

muscle with most of the target proteins residing in the mitochondria [7]. The same affect was observed in response to build up of acetyl CoA through CrAT knockout leading to increased mitochondrial protein acetylation and decreased levels of acetylation of proteins in the glycolytic pathway [7]. Increased acetylation of mitochondrial metabolic proteins suppresses fatty acid oxidation in conjunction with decreased glycolytic acetylation promoting glucose oxidation pointing to a condition of increased reliance on glycolytic products for energy production and a decrease in oxidation. This is in conjunction with what is known about type 2-diabetes where there is an increase in FA import but a decrease in FA oxidation [108]. Both situations combined suggest a mechanism for increased mitochondrial substrate flux without a corresponding demand for ATP leading to mitochondrial dysfunction and stress. Deacetylation of certain β-oxidation enzymes ameliorates mitochondrial flux and will be discussed in detail later[81]. Paradoxically, both caloric restriction and high fat feeding cause increased mitochondrial acetylation while the overall effect to metabolic flexibility are polar opposites. A chronic high fat diet appears to promote disruption in a multifaceted process causing dysfunction starting with the insulin signaling pathway, followed by perturbations to cytosolic and mitochondrial oxidative pathways, and finally damaging the hormone producing cells of the pancreas over a sustained period.

The underlying mechanistic effects for the difference in outcome between caloric restriction and a habitual high fat diet maybe due to the carbon source attached to the acetyl CoA. Acetylation of TCA cycle enzymes has mixed effects and may be due not only to the carbon source of substrates and where they enter the TCA cycle as carbohydrates, fatty acids, and amino acids are all oxidized in the mitochondria and can enter at multiple sites but also the tissue and its function as a producer, user, or storage site of metabolic fuel.

In an obese and diabetic state where there is an excess of fatty acids transported into the mitochondria coupled with decreased FA oxidation rates possibly overloading oxidative capacity [108, 109]. Exceeding oxidative capacity results in partially oxidized lipid intermediates that interfere with glucose uptake and insulin signaling [108, 109]. Fatty acid overload demonstrates why effective metabolic signaling is paramount to metabolic flexibility. As stated previously Hirschey et al. (2011) demonstrated the effects of fasting and high fat feeding on SIRT3 function which in turn promoted decreased LCAD acetylation in the latter, and increased acetylation in the former[102].

Caloric restriction alters mitochondrial metabolic protein acetylation levels, but caloric restriction's affects extend to the β-oxidation pathway as well[83]. Liver extract from mice fed a diet that was stepwise decreased to 40% by 16 weeks were measured for acetylation via mass spectrometry[83]. Under caloric restriction only hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase enoyl-CoA hydratase (HADHA) was shown to be hyper-acetylated, and at this time it is unknown if other β-oxidation enzymes are affected by caloric restriction[83]. Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase enoyl-CoA hydratase catalyzes the last three steps of β-oxidation.

Skeletal muscle from high running capacity rats have decreased mitochondrial acetylation levels in fat oxidation pathways, and increased fatty acid oxidation capacity during exercise compared to lower running capacity rats[110]. In addition to higher fatty acid oxidation during exercise in skeletal muscle, high running capacity rats show an increase in fat oxidation over the entire body, and decreased glucose utilization compared to their lower running capacity counterparts[110]. As stated previously decreased fat oxidation accompanied with increased glucose dependency is a hallmark of metabolic inflexibility and type 2-diabetes[111]. Overall

decreased mitochondrial protein acetylation appears to be necessary for maintenance of metabolic flexibility in skeletal muscle and is responsible for increased fat oxidation.

Mitochondrial fusion is dependent on inner mitochondrial membrane proteins and outer mitochondrial membrane proteins; mitofusin 1 (MFN1) and mitofusin 2 (MFN2) are outer membrane fusion proteins [112]. Fasting promotes a switch to beta-oxidation, which also correlates with greater risk of oxidative damage [113]. Mitofusin 1 is subject to multiple modes of covalent modification including ubiquitination by Parkin, which marks it for degradation [114]. As measured in skeletal muscle upon entering a fasting state HDAC6 deacetylates MFN1 promoting mitochondrial fusion [113]. Failure of increased fusion to occur does not hinder energy production, but leads to an increase in ROS generated [113]. Generation of ROS products is a hallmark of obesity, type 2-diabetes, and of a metabolically inflexible state. While not necessary for metabolic regulation MFN 1 & 2 are critical components for mitochondrial function, and indicators of the development of a disease state.

Amino acid metabolism is tightly regulated to insure adequate demands to supply protein synthesis, but diets high in protein and low in carbohydrates can increase amino acid oxidation in the TCA cycle. Acetylation regulates amino acid metabolism and the urea cycle that handles the disposal of nitrogenous amine group through inhibition of both cycles. Deacetylation, mediated by sirtuins, is necessary for both processes to occur. This may in part be accomplished through acetylation and deacetylation of TCA cycle enzymes allowing intermediates into the TCA cycle while simultaneously inhibiting entry and oxidation of acetyl CoA derived from carbohydrates or fatty acids as suggested by acetylome patterns observed during the fed to fasted transition [40]. Associated with type 2-diabetes is an increase in amino acid metabolism and a sign of sever metabolic dysfunction[82]. A starvation state, 3+ days, or if suffering from type 2-diabetes is

normally when amino acid metabolism becomes a major contributor to energy production, but it is possible to purposely induce a state of greater amino acid metabolism through caloric restriction. Acetylation of urea cycle enzymes functions to down regulated their enzymatic activity, and in response to glucose and in a fed state it would expected to observe high acetylation levels as was observed with ASL. Does an obese and diabetic state lead to decreased acetylation levels of urea cycle enzymes in response to increased amino acid metabolism? This is currently unknown and a possible area of research.

Under caloric restriction the need to generate glucose via gluconeogenesis becomes greater and amino acids become a vital source of gluconeogenic precursors as they can be converted to pyruvate, acetyl CoA, and succinyl CoA. Sirtuin 3 activity increases under caloric restriction deacetylating and promoting an increase in function of enzymes involved in amino acid metabolism [32]. Accompanied with increased amino acid metabolism is the need to dispose of nitrogen whose enzymes are positively regulated by SIRT3 [32].

Increased exercise is interconnected with increased branch chain amino acid metabolism[115]. Recently in skeletal muscle from high capacity rats increased deacetylation of branch chain amino acid metabolism pathway enzymes was observed with increased branch chain amino acid metabolism during exercise[110]. This deacetylation was not associated with an increase in SIRT3 expression but rather an increase in SIRT3 enzymatic function[110]. Increased exercise capacity is similar to the effects of caloric restriction[116].

As with SIRT1, AMPK increases skeletal muscle metabolism in mice through an increase in SIRT4 activity [67]. Also in mice, there is a correlation between increases in phosphorylation and SIRT1 activity in concert with exercise [93].Though AMPK function is hardwired into the aforementioned metabolic processes, its actions can be negated when the situations of metabolic

inflexibility and mitochondrial dysfunction arise as associated with obesity and type 2 diabetes. Overloading the mitochondria with substrate is known to produce fatty acid intermediates that decrease GLUT4 translocation as well as insulin production which would feedback negatively on AMPK activity. Aberrant substrate selection in metabolic inflexibility occurs either despite AMPK activity or in concurrence with decreased AMPK signaling abrogating AMPK's effects. In skeletal muscle, caloric restriction of AMPK knockout mice was observed to exhibit a degree of metabolic dysfunction characterized by decreased glucose tolerance parallel with decreased levels of SIRT1 and PGC-1α[117]

Cellular concentrations of the oxidized form of NAD, NAD^+, are key to the activation of sirtuins as they are obligatory users in their deacetylase activity and AMPK controls catabolic, and anabolic processes and thus the NAD^+/NADH ratio. Lower NAD^+/NADH ratios would indicate a reliance, or preference, for glycolysis over fat oxidation for energy generation in skeletal muscle[25]. Caloric restriction leads to an increase in NAD^+ levels where the opposite is true for over consumption[118, 119]. In liver cells a decrease in AMPK accompanied with decreased SIRT1 levels concomitant with increased glucose and lactate levels were observed[120]. By increasing NAD^+ levels, AMPK increases SIRT1 deacetylase activity, which promotes PGC-1α and several FOXO transcription factors [67, 69, 117].

It is well known that PGC-1α is a crucial component in the pathway for mitochondrial biogenesis[121, 122]. Whether from nutritional deprivation, or exercise induced mitochondrial biogenesis, PGC-1α's increased transcriptional activity is paramount[123, 124]. The evidence for the role of SIRT1 to PGC-1α's response to both nutritional deprivation and exercise in skeletal muscle can be paradoxical when the collective body of research is assessed. It has been shown that metabolic stress leads to an increase in PGC-1α deacetylation by SIRT1, followed by

increased transcriptional activation of PGC-1α targets, culminating in increased mitochondrial gene transcription and biogenesis [40, 125, 126]. The time frame of SIRT1 expression and activation varies sometimes and can be contradictory to one another[40]. It has been proposed that for SIRT1 it is not the level of protein expression but SIRT1 activity level that is the key to the functionality of PGC-1α [40].

Acetylation of PGC-1α by GCN5 decreases PGC-1α's ability to induce gluconeogenic genes in the liver [72]. SIRT1 is not the only member of the Sirtuin family to act on PGC-1α as SIRT3 also increases PGC-1α activity through activation of AMPK [44]. SIRT3 regulates lipid metabolism through its downstream effects on PGC-1α, and in addition to a decrease in PDC function in the development of metabolic syndrome is a decrease in PGC-1α function in accordance with decreased SIRT3 activity [44]. Chronic exercise is shown to induce mitochondrial biogenesis correlating with increased SIRT1 deacetylation of PGC-1α in wild type mice [93]. The interaction between PGC-1α, its acetylases and deacetylases, and PGC-1α's targets are a prime example of the intricate and complicated nature of metabolic regulation through acetylation. Acetylation represents but one of multiple mechanisms for metabolic regulation, and disturbances, as from obesity, to any point of the process has ramifications for multiple other pathways.

In a fasted or starvation state UCP2 gene expression is increased shifting substrate preference away from carbohydrates. Uncoupling protein 3 (UCP3) is a mitochondrial membrane transporter predominately expressed in skeletal muscle whose function is to lower membrane potential thereby protecting against fatty acid overload and ROS production[92]. In addition to protection from ROS damage UCP3 expression has been linked with increased fatty oxidation in muscle[127]. Fasting has been shown to induce UCP3 gene expression in rat muscle through

increases in UCP3 mRNA levels in a fasted state[128]. This data underscores UCP3's importance to not only regulation of fatty acid metabolism but also its role in the switching of substrate choice for oxidation.

Whether directly or indirectly, all the previously mentioned transcription factors are influenced through SIRT1. This reliance on SIRT1 function leaves transcriptional regulation open to disturbance in an obese and type 2-diabetic state as SIRT1 activity is suppressed in an obese and type 2-diabetic state. Sirtuin 1 exhibits decreased function in response to obesity and insulin resistance, although this was reported in adipose tissue and how this translates to skeletal muscle and liver is unclear. What can be assumed is that transcription for genes regulating TAG storage and release can be disrupted in response to SIRT1 suppression. A chronic low-grade inflammatory state is observed in the obese and type 2-diabetics associated with NF-κB expression feeding back into and further promoting the inflammatory state. In response to NF-κB expression T-cells and B-cells express receptors for activation/ recruitment/ maturation, and increase inflammatory interleukin cytokine production. Alterations to transcriptional pathways have consequences extending into all metabolic pathways disrupting substrate metabolism and storage, and promoting an inflammatory state exacerbating the disease state extending beyond metabolism into other body systems.

CONCLUSIONS

Metabolism is a highly-ordered process with mechanisms in place to determine substrate preference and utilization. The human body has adapted to fasting and re-feeding with transient overfeeding having no long-term consequences over the course of one hundred thousand plus years. This historic model of feeding has radically changed over the last one hundred years with

most the developed world having access to abundant food supplies, and the food itself changing in composition to an extremely processed composite of food stuffs that are as energy dense as anything available to our ancestors if not much more so. Over the last thirty years the rates of obesity worldwide have more than doubled, and we are witnessing the emergence of a chronically ill class of the population suffering the effects of dietary habits we have adopted[9, 10]. Obesity and type 2-diabetes have been shown to alter the metabolic landscape causing widespread dysregulation resulting in an observable metabolic phenotype termed metabolic inflexibility[104]. With metabolic inflexibility the body is no longer able to respond to hormonal cues, does not exhibit robust substrate switching capacity, and floods the mitochondria with substrate well beyond its oxidative capacity feeding back and exacerbating the disease state[104]. At the core of metabolism are signaling mechanisms in place taking cues from substrate availability and energetic demands, and when those mechanisms breaks down the results are severe.

Acetylation as a posttranslational modification has been shown to be as vital for the regulation of normal metabolic flux as better-known modifications such as phosphorylation and ubiquitination[32]. As with other regulatory mechanisms, a high fat diet and obesity have an adverse effect on acetylation of the enzymes in all the pathways governing what is healthy metabolic substrate selection and utilization[102]. While almost all enzymes in glycolysis, TCA, fatty acid oxidation, and amino acid metabolism have been shown to contain regions capable of acetylation, not all are utilized for regulation, at least as far as it has been shown, or are disturbed through the adverse effects of metabolic syndrome and inflexibility[22, 102]. However, those that are modified show the integral nature of acetylation as mode of metabolic regulation. **Table 1** illustrates how embedded acetylation is in metabolic regulation.

TABLE 1

Protein	Pathway	Acetylated	Effect
Hexokinase	Glycolysis	Yes	Unknown
Phosphoglucose Isomerase	Glycolysis	Unknown	Unknown
Phosphofructokinase 1	Glycolysis	Yes	Unknown
Aldolase	Glycolysis	Yes	Unknown
Triose Phosphate Isomerase	Glycolysis	Yes	Unknown
Glyceraldehyde 3-Phospahte Dehydrogenase	Glycolysis	Yes	Unknown
Phosphoglycerate Kinase	Glycolysis	Yes	Unknown
Phosphoglyceromutase	Glycolysis	Yes	Unknown
Enolase	Glycolysis	Yes	Unknown
Pyruvate Kinase	Glycolysis	Yes	↓ Activity
Pyruvate Carboxylase	Gluconeogenesis	Yes	Unknown
Phosphoenol Pyruvate Carboxykinase	Gluconeogenesis	Yes	↓ Activity
Fructose 1,6-bisphosphatase	Gluconeogenesis	Yes	↓ Activity
Glucose 6-Phosphatase	Gluconeogenesis	Yes	Unknown
Pyruvate Dehydrogenase	TCA Cycle	Yes	↓ Activity
Citrate Synthase	TCA Cycle	Yes	Unknown
Aconitase	TCA Cycle	Yes	Unknown
Isocitrate Dehydrogenase	TCA Cycle	Yes	↓ Activity
α-ketoglutarate Dehydrogenase	TCA Cycle	Yes	Unknown
Succinyl CoA Synthetase	TCA Cycle	Yes	Unknown
Succinate Dehydrogenase	TCA Cycle	Yes	↓ Activity
Fumarase	TCA Cycle	Yes	Unknown
Malate Dehydrogenase	TCA Cycle	Yes	↑ Activity
Pyruvate Carboxylase	TCA Cycle	Yes	Unknown
Uridyl Transferase	Glycogen Synthesis	Yes	Unknown
Glycogen Synthase	Glycogen Synthesis	Yes	Unknown
UDP-GP	Glycogen Synthesis	Yes	Unknown
Glycogen Phosphorylase	Glycogen Metabolism	Yes	↓ Activity
PGM3	Glycogen Metabolism	No	N/A
(ACC)	FA Metabolism	Yes	Unknown
(FAS)	FA Metabolism	Yes	Unknown
(ACS1)	FA Metabolism	Yes	↓ Activity
(CAT)	FA Metabolism	Yes	Unknown

CPT	FA Import	Yes	↓ Activity
(LCAD)	β-Oxidation	Yes	↓ Activity
(EHHADH)	β-Oxidation	Yes	↑ Activity
(HADH)	β-Oxidation	Yes	↑ w/ CR
(ACAT)	β-Oxidation	Yes	Unknown
(PCC)	β-Oxidation	Yes	Unknown
(MUT)	β-Oxidation	Yes	Unknown
Glutamate Dehydrogenase	AA Metabolism	Yes	↓ Activity
Carbamoyl Phosphate Synthetase 1	Urea Cycle	Yes	↓ Activity
Ornithine Carbamoyl Synthetase	Urea Cycle	Yes	↓ Activity
Argininosuccinate Lyase	Urea Cycle	Yes	↓ Activity
Glutamate Dehydrogenase	Urea Cycle	Yes	Unknown

[22, 77, 83]

Acetylation is proving to be a fundamental part of metabolic substrate determination as well as utilization, and while more is becoming known there is still much to learn. As the table above displays, the area of acetylome research in metabolism is wide open with the effects of mostly unknown. Furthermore, how high fat feeding (whether acute or chronic), obesity, type 2-diabetes, and an inflammatory state affect acetylation of metabolic enzymes needs much research. What is known is that obesity and high fat feeding are associated with increased global and mitochondrial acetylation as is caloric restriction. In addition to the metabolic acetylome other PTMs are proving to be as integral to healthy metabolism and require further elucidation as to their role in governing metabolism.

Caloric restriction induces cellular stress that in turn modulates acetylase and deacetylase activity and ultimately mitochondrial biogenesis through SIRT1, AMPK, and PGC-1α[129]. Increased mitochondrial acetylation levels are accompanied with caloric restriction[32, 83]. As does short term fasting while long-term fasting sees a decrease in mitochondrial acetylation levels[21, 45]. The effects on mitochondrial acetylation patterns between caloric restriction and high fat feeding are similar while the overall effects to health are polar opposites. To elucidate the

mechanistic differences that must be occurring between caloric restriction and high fat feeding the exact proteins that are being acetylated, and what lysines are being acetylated in these two metabolic states needs to be examined. The key to the disparity between the overall outcome and the acetylation levels must be the small group of mitochondrial proteins deacetylated while overall acetylation was increased with caloric restriction[32]. The expression and functionality level of SIRT3 should not be overlooked as high fat feeding leads to a decrease in SIRT3 expression where caloric restriction an increase in SIRT3 expression[45, 102]. Although, both previously referenced studies were focused on SIRT3 function in the liver and skeletal muscle, skeletal muscle often is operating opposite to that of the liver[44]. Though much research has been done on SIRT3, as it is a mitochondrial deacetylase, SIRT1 must not be over looked due to its effects on most metabolic transcription factors. Together SIRT1 & 3 influence almost all metabolic processes. Acetylation of metabolic proteins has opened the possibility of new therapeutic agents through modulating sirtuin activity. Altering the NAD^+/NADH ratio has been one hypothesis put forth as an idea in regulating SIRT3 activity thereby increasing mitochondrial fat oxidation. With an enhanced understanding of how the acetylome affects global metabolism will certainly come new possibilities in combating the growing pandemic of obesity and type 2-diabetes.

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CHAPTER 3: SPECIFIC AIMS

STUDY GOAL

The overall goals of this project are to investigate in mice 1) the response in skeletal muscle to a chronic high fat diet as well as a single high fat meal, and how this response varies between non-obese and obese conditions, and 2) in response to a chronic high fat diet and a single high fat meal how the acetylome is altered, and the variation in acetylome patterns between obese and non-obese mice skeletal muscle.

SPECIFIC AIMS AND HYPOTHESES

Specific AIM 1: To determine the normal metabolic and acetylation responses, in skeletal muscle, to a high fat meal challenge in healthy, non-obese C57bl/6j mice.

Specific AIM 1 Hypothesis 1: We hypothesize that in response to a single high fat meal challenge, healthy non-obese mice will show a preference for fat oxidation in a fed state.

Specific AIM 1 Hypothesis 2: We hypothesize that in response to a single high fat meal challenge, healthy non-obese mice will exhibit an acetylomic profile of metabolic proteins that supports increased fat oxidation, i.e. increased β-oxidation acetylation and increased glycolytic acetylation.

Specific AIM 2: To determine the adaptive metabolic and acetylation responses in skeletal muscle of obese C57bl/6j mice to a chronic high fat diet, and if normal metabolic and acetylation response to a high fat meal challenge is altered in obese C57bl/6j mice.

Specific AIM 2 Hypothesis 1: We hypothesize that in response to a chronic high fat diet, obese mice will exhibit signs of metabolic inflexibility in substrate selection, i.e. heightened glucose and lowered fat oxidation, and that their metabolic proteins will all be hyper-acetylated.

Specific AIM 2 Hypothesis 2: We hypothesize that in response to a single high fat meal challenge obese mice will exhibit mixed substrate oxidation levels, and that their acetylomic profiles for metabolic proteins will not be that changed from their fasted state acetylation levels.

CHAPTER IV: EXPERIMENTAL DESIGN

EXPERIMENTAL DESIGN

Mice

Diet Induced Obesity (DIO) mice were fed a diet consisting of 60% fat, and control mice (DIO Controls) fed a diet with 10% fat; both of which were purchased from Jackson Laboratories.

Substrate	DIO (Kcal%)	Control (Kcal%)
Protein	20	20
Carbohydrate	20	70
Fat	60	10

Twenty-four mice were purchased at 20 weeks of age with each group, DIO and control, having an N size of 12. The mice were housed in the Integrated Life Science Building (ILSB) vivarium while they acclimated to their new environment until 24 weeks of age. All animal handling and experiments were performed under an approved IACUC protocol (#14-004). Twenty-four hours prior to sacrifice body composition was assessed using a Bruker LF90 NMR. The mice were for fasted 12 hours, and gavaged with a high fat meal challenge 3 hours prior to sacrifice (SAC). Eight mice were sacrificed each day with four mice from each group being sacrificed. In addition, each group was divided into two further groups with half of the mice receiving a high fat gavage and the remainder a water gavage.

Blood Collection

Blood was collected via syringe puncture of the heart, and placed in serum separator tubes. Blood serum was analyzed for hormonal and metabolite differences between DIO and control mice. The collected serum was also used to determine the effect of a high fat gavage on both control and DIO mice hormones as well as a measurement of blood metabolite levels. The data gathered from blood work is supporting evidence for the gavage effect and any acetylation variances.

Tissue Collection

Red muscle was dissected from the gastrocnemius and quadriceps femoris. The muscle collected was divided up into three portions where one leg was collected for mass spectrometry. The other leg was divided in half- one portion for radioactive metabolism studies and metabolic assays, and the other half for any future studies.

The muscle collected for mass spectrometry was washed in cold PBS and frozen in liquid nitrogen dry. Further homogenizing and sonication was performed in 9 M urea buffer, pH 8.0 as specified by Cell Signaling Technologies. The samples were homogenized and sonicated at power setting 4 at 30-second intervals, with a rest for 1 minute on ice in between intervals. Global acetylation patterns were assessed via mass spectrometry. Muscle for metabolic studies

were washed in PBS and homogenized immediately in SET buffer. The remaining muscle was washed in cold PBS, and flash frozen in cell lysis buffer containing protease and phosphatase inhibitors.

Mass Spectrometry

Protein samples were trypsin digested and immunoprecipitated with an acetyl lysine antibody followed by concentration for Synapt G2-S mass spectrometer (Waters Corporation, Milford, MA). Mass spectrometry was used to generate an acetylomic profile in skeletal muscle of both DIO and control mice. Ionization patterns produced by the mass spectrometer were analyzed by ProteinLynx Global Server v. 3.0.2 that generated the list of acetylated proteins from the peptide sequences. This approach generated an overall acetylomic profile in skeletal muscle, but cytosolic and mitochondrial metabolic proteins are the focus for analysis in this study. A quantitative analysis of the gavage data within the control and DIO group was performed as well as a quantitative between the DIO and control groups. Mass spectrometry data provided the bulk of the research data for analysis in the differences between a chronic and acute high fat diet within groups and between groups.

Fatty Acid Oxidation

Fat oxidation capacity was measured through the production of CO_2 and acid soluble metabolites using 1-^{14}C palmitate. Pyruvate metabolism was measured using an approach like that of palmitate oxidation with 1-^{14}C pyruvate. Metabolic flexibility was measured by this formula:

((Pyruvate Activity- Pyruvate with FA Activity)/ Pyruvate Activity) X 100%

In the presence of FA, pyruvate oxidation is lowered, and the degree to which pyruvate oxidation is lowered is metabolic flexibility. Oxidation and metabolic flexibility data was used to support the role of acetylation in metabolic regulation and effect of a chronic and acute high fat diet.

Metabolic Enzymes

Studies measuring the oxidative capacity of metabolic pathways was performed using 1-^{14}C palmitate and 1-^{14}C pyruvate. These studies determined the oxidative capacity and substrate preference between DIO and control mice as well as the effect of a single high fat gavage with in these groups on enzymes in the TCA cycle, β-oxidation, and the electron transport chain. Assays for citrate synthase, malate dehydrogenase, β-hydroxyacyl-CoA dehydrogenase, and cytochrome C were used to measure the effect of a high fat diet and gavage on metabolic function and to further investigate acetylation's role in metabolic regulation.

STATISTICAL ANALYSIS

Mass Spectrometry:

Software automatically determined peptide and fragment mass tolerances. Searches in ProteinLynx Global Server (PLGS) was limited to no more than 5% false discovery rate. The protein database was searched for all Mus musculus entries and downloaded from UniProt excluding those defined as fragments of a larger protein. A random entry for each true protein entry was appended using PLGS prior to peptide identification searches. Results from PLGS was be imported into IsoQuant (http://www.immunologie.uni-mainz.de/isoquant/) limiting the peptide false discovery rate to 1% and the protein false discovery rate to 5%. IsoQuant matched peaks across multiple samples and reported the signal intensity of each peptide across all samples. Signal intensity has been shown previously to be roughly linearly correlated to the

molar amount of that peptide. Probable significance of samples was preliminarily determined using MultiBase and Excel add on followed by graphing averaged sampled values in GraphPad Prism. Any further suspected significant samples were detected manually through a search of the compiled data set. Statistical analysis performed using GraphPad Prism software was calculated by Two Way ANOVA with a Tukey post-hoc test for multiple comparisons and T-tests when measuring the difference within groups and between groups when measuring the effect of the gavage. Statistical significance was set at a 95% confidence interval ($P \leq 0.05$).

EXTENDED METHODS

Mice:

Mice were purchased from Jackson Laboratories; 12 DIOs, catalog number 380050, 12 DIO Controls, catalog number 380056. The DIO mice were raised on a diet make up of 20% (Kcal) protein, 20% (Kcal) carbohydrate, and 60% (Kcal) fat. The make-up of the control diet was 20% (Kcal) protein, 70% (Kcal) carbohydrate, and 10% (Kcal) fat. These diets were continued once the mice are received. A high fat diet of the same composition as was fed to them by Jackson Laboratories was purchased from Research Diets, catalog numberD12492. A control diet of the same composition as was fed to them by Jackson Laboratories, was also purchased from Research Diets, catalog number D12450B. Both control and high fat diet were changed twice a week due to degradation of the high fat diet. Mice were received at 19 weeks of age and sacrificed around 23 to 24 weeks of age. Mice were fasted 12 hours (overnight) prior to sacrifice.

Body Composition:

Twenty-four hours prior to sacrifice mice were weighed and body composition determined using a LF90 NMR Analyzer by Bruker housed in the ILSB vivarium. Fat mass percentage and fat weight in grams as well as lean mass percentage and lean mass in grams was measured and recorded for each animal.

Gavage:

Both control and DIO mice received a gavage, with half of both groups receiving the high fat gavage. The gavage was made with 141.7 g Carnation condensed whole milk, 9 g Kroger brand granulated white sugar, and 14 g Crisco pure vegetable oil. The high fat gavage was 5 Kcal with a caloric make up of 21.4% saturated fat, 40.8% unsaturated fat, 27.1% carbohydrate, and 10.7% protein. Those mice that did not receive the high fat gavage were given a DI water gavage. Each gavage was 300 μL and mice were given the gavage 7 minutes apart. The gavage was administered 3 hours prior to sacrifice. The gavage volume was determined in consultation with Virginia Tech veterinarian staff as well as vivarium staff to be a safe but effective volume the animals could handle without causing undue discomfort as described by IACUC protocol 14-004.

Sacrifice:

Mice were euthanized in a carbon dioxide chamber followed by cervical dislocation as described by IACUC protocol 14-004 with a 7-minute separation between mice in accordance with the gavage times. Blood was collected using a syringe puncture to the heart. Gastrocnemius and quadriceps femoris muscles were taken from the hind legs. After extraction from the leg, muscle was placed in cold 10X PBS. Red muscles were separated out for studies. Muscle was separated, dried, and then placed in cold 10X PBS for distribution. Distribution went as follows:

Mass spectrometry- 1 quadriceps and 1 gastrocnemius; Metabolism studies- 1 quadriceps; 1 gastrocnemius for future studies.

Blood Serum Analysis

Blood was collected via syringe puncture of the heart and placed in serum separator tubes (Fisher Scientific, catalog number 36597). Blood was allowed to sit for 30 minutes for clotting to occur, followed by centrifugation at 12,000 RPM, at 4^{o}C for 10 min. Supernatant was collected and frozen at -80^{o}C. Serum samples were analyzed for glucose and lactate levels using YSI machine located in house. Free fatty acids were measured using a Free Fatty Acids Half-Micro Test, Roche, Cat# 11-383-175-001. Insulin was measured using an ELISA from ALPCO; cat#: 80-INSMSU-E01

Protein Digestion- Mass Spectrometry:

Red muscle was thawed on ice, to which 300 µL of 9 M urea buffer was added. Urea buffer was made to Cell Signaling Technologies specifications: 20 mM HEPES- pH 8.0, 9 M Urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate[1]. Upon addition of the urea buffer, the samples were minced with surgical scissors as finely as possible. To the samples, two scoops of magnetic zirconium oxide protein beads (Next Advance, catalog number ZrO10) were added, and the tubes wrapped in parafilm around the caps. The samples were homogenized in a Bullet Blender at setting 7 for 5 minutes, 3 to 4 times. Afterwards the samples were spun down in a micro-centrifuge at 12,000 RPM for 15 minutes at 4^{o}C. The supernatant was collected and transferred to new tubes; the original sample tubes were saved at -80^{o}C in case the need for re-processing arises. The supernatant was sonicated 3 times at power setting 4 for 15 seconds with a one minutes rest period on ice in between sonicating[1].

To the original sample tube, 100 μL of urea buffer was added and transferred to a new tube free of magnetic beads, then re-minced and transferred back to the tube with beads, re-homogenized, and re-sonicated as previously described before combining samples. Samples were frozen in -80°C freezer until analysis.

Frozen samples were placed on ice to thaw, and then washed with 1 mL 8 mM urea, and 50 mM ammonium bicarbonate was added[2]. Samples were spun at 12,000 g, RT, for 12 minutes to thaw and wash solution simultaneously. Sample and wash buffer was transferred to 15 mL conical tubes, and MgCl was added for a final concentration of 2 mM. Acetylated BSA was added- volume was based on concentration of protein- 90 ng acetylated BSA per 5 mg protein[2]. Tubes were inverted by hand several times to mix gently. The tubes were placed in a shaker incubator at 32°C for 15 min. Dithiothreitol (DTT) was added for a final concentration of 10 mM[1]. Ammonium bicarbonate was added to adjust the urea concentration for a final urea concentration of 1.8 mM. Samples were placed in shaker incubator at 32°C for 25 minutes. Samples were removed from the shaker incubator and allowed to cool to room temperature to which 1/10 volume of iodoacetamide was added and tubes placed in the dark at RT for 20 minutes[2]. Trypsin suspended in acetic acid of a final concentration at 0.25 mM was added- trypsin volume determined by protein concentration[2]. Sample were placed in shaker overnight at 32°C[2]. All tubes were washed with 100% acetonitrile (MeCN) and air dried before use.

Sep-Pak C_{18} Purification of lysate Peptides:

In a hood trifluoroacetic acid (TFA) was added to samples for a final volume of 1% TFA[1]. pH was measured using a pH strip to ensure acidity[1]. Samples were placed on ice to rest for 5 minutes. Purification of peptides was performed at room temperature on 0.7 mL Sep-Pak columns from Waters Corporation catalog number WAT051910. Sep-Pak columns were attached

to a reservoir and held in place by a tube rack. All washes were performed by gravity; no vacuum suction was applied. The filters were washed with 5 mL 100% MeCN[1]. The filters were washed with solvent A in sequential 1, 3, and 6 mL washes to remove MeCN[1]. The samples were added to the filters and allowed to pass through by gravity flow[1]. The pass-through liquid was stored in -80ºC freezer in case later steps do not work. The filters were washed with two sequential 4 mL washes of solvent A followed by 2 elution washes with 3 mL 100% MeCN. Samples were lyophilized in high vacuum liquid nitrogen vacuum overnight. All tubes were washed with 100% MeCN and allowed for remaining solution to evaporate before use.

Acetyl Peptide Immunoprecipitation:

IAP buffer (buffer comes with Cell Signaling Kit) was centrifuged to solubilize as it precipitates out when frozen, and 1X PBS and Cell Signaling beads (CS PTM Scan Acetyl Lysine Motif IAP Beads; Cat #: 13362s) were placed on ice along with ultra-pure water. Samples thawed at RT. 1200 µL IAP buffer was added to cleaned 50 mL conical tube and volume brought up to 12 mL with ultra-pure water; 1.4 mL buffer dilution was added to the sample[1]. The samples were then mixed through pipetting up and down. One milliliter 1X PBS was added to samples, and samples were inverted 5X followed by centrifugation at 2,000 g, RT, for 30 seconds[1]. Most of the supernatant was removed with some left so as not to pull off IP beads. The process of washing, spinning, and supernatant removal was repeated 4X, while in between the samples were kept on ice[1]. Samples were then spun at 1,780 g, 15 minutes, 24ºC. Samples were transferred to a bead tube and placed in a walk-in cooler (4ºC) in a rotator overnight.

Samples were spun at 2,000 g for 30 seconds at RT following which the supernatant was transferred to a new tube and stored, only in case following later test showed a lack of protein[1]. Five milliliters IAP buffer and 0.280 µL (0.25% final concentration) buffer solution was

prepared to which 1 mL buffer detergent was added to samples, and tubes were inverted 5X[1]. Samples were spun at 2,000 g, 30 seconds, RT, afterwards the supernatant was removed and discarded. Samples were washed with 1 mL IAP buffer alone then inverted 5X and spun 2,000 g, 30 seconds, RT and the supernatant discarded[1]. Samples were washed with 1 mL ultra-pure water, inverted 5X, and spun at 2,000 g, 30 seconds, RT following which the supernatant was discarded[1]. This was repeated 2X for a total of 3 washes[1]. Fifty microliters of 15% TFA, alcohol dehydrogenase 1 digested by trypsin (Waters Corp.; Cat # 186002328-1; 1.2 fM/µL- quantitative control) was added and mixed by tapping[1, 2]. Samples sat for 10 minutes at RT and tapped every few minutes. The samples were then centrifuged at 2,000 g, 30 seconds, RT and the supernatant saved in new tubes. The beads were washed again with 0.15% TFA and allowed to sit for 10 minutes at RT with tapping every few minutes, followed by another spin at 2,000 g, 30 seconds, RT and the supernatant was added to the previous elution[1]. Samples were then spun in a centrifugal concentrator at 1,000 g, 37^{o}C for 1 hour under a low vacuum to dry off sample volume for concentration necessary for mass spectrometry runs. Samples were frozen at -80^{o}C. All tubes were washed with 100% MeCN and allowed to air dry before use.

Mass Spectrometry:

Two percent MeCN, 0.1% TFA, 98% ultra-pure water solution was prepared for peptide resuspension. Samples were resuspended in 35 µL suspension solution. Samples were placed in sonicating water bath for 20 minutes to fully resuspend. Samples were spun at 13,000 g, 10 minutes, RT to pellet down any remaining beads.

LC-MS/MS analysis via ESI: Triplicate runs (10 µl injections) of each resolubilized peptide sample were separated through an Acquity I-class UPLC system (Waters Corporation, Milford, MA). The mobile phases were solvent A (0.1% (v/v) formic acid in LC/MS grade water) and

solvent B (0.1% (v/v) formic acid in LC/MS grade acetonitrile). The separation was performed using a CSH130 C18 1.7 µm, 1.0 x 150 mm column (Waters Corporation, Milford, MA) at 50 µL/min using a 110-minute gradient from 3-40% solvent B. The column temperature was maintained at 45˚C.

Column effluent was analyzed using a Synapt G2-S mass spectrometer (Waters Corporation, Milford, MA) using an HDMSe (high-definition (e.g. ion mobility separation) mass spectrometry with alternating scans utilizing low and elevated collision energies) acquisition method in continuum positive ion "resolution" MS mode. Source conditions were as follows: capillary voltage, 3.0 kV; source temperature, 120˚C; sampling cone, 40 V; desolvation temperature, 350˚C; cone gas flow, 50 L/hr; desolvation gas flow, 500 L/hr; nebulizer gas flow, 6 bar. Both low energy (4 V and 2 V in the trap and transfer region, respectively) and elevated energy (4 V in the trap and ramped from 20 to 50 V in the transfer region) scans was 1.2 seconds each for the m/z range of 50 to 1800. For ion mobility separation, the IMS and transfer wave velocities were 600 and 1200 m/sec, respectively. Wave height within the ion mobility cell was ramped from 10 to 40 V.

For lock-mass correction, a 1.2 second low energy scan was acquired every 30 seconds of a 100 fmol/µL [Glu1]-fibrinopeptide B (Waters Corporation, Milford, MA) solution (50:50 acetonitrile: water supplemented with 0.1 % formic acid) infused at 10 µL/min introduced into the mass spectrometer through a different source which was also be maintained at a capillary voltage of 3.0 kV. The data for lock-mass correction was collected but not applied to sample data until data processing.

Peptide/ Protein Identification by Software:

Mass spectrometric data from each chromatographic run were processed and analyzed utilizing ProteinLynx Global Server v. 3.0.2 (PLGS, Waters Corporation, Milford, MA). Average chromatographic and mass spectrometric peak width resolution was determined automatically by the software for peaks eluting from 3.4 to 93.4 minutes. Mass values were lock-mass correction based on the exact m/z value of the +2 charge state of [Glu1]-fibrinopeptide B (785.842). Peaks were defined based on the low energy, elevated energy and bin intensity thresholds of 200, 20 and 200 counts, respectively. Peptide identification was performed utilizing trypsin specificity and the possibility of 3 missed cleavages as search parameters. Other search parameters were a fixed carbamidomethylation of cysteine residues and variable modifications of acetylation of lysine residues, oxidation of methionine residues and formation of pyrrolidone carboxylic acid at the N-terminus of a peptide if the amino-terminal residue is glutamine. The searches required at least 2 fragment ion matches per peptide, five fragment ion matches per protein and 1 peptide per protein. Peptide and fragment mass tolerances were automatically determined by the software. Searches in PLGS was limited to no more than 5% false discovery rate. The protein database search was all Mus musculus entries downloaded from UniProt excluding those defined as fragments of a larger protein. A random entry for each true protein entry was appended using PLGS prior to peptide identification searches.

Metabolic Fatty Acid Oxidation and Enzyme Assays:

Red gastrocnemius and quadriceps femoris was manually excised and washed in cold PBS. Approximately 50 mg of muscle was placed in 200 μL of modified sucrose EDTA medium (SET Buffer) on ice[3]. SET buffer contains 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and

1 mM ATP, pH 7.4[3]. Muscle samples were minced with scissors followed by the addition of SET Buffer to produce a final 20-fold dilution (wt: vol)[3]. The samples were homogenized in a Potter-Elvehjem glass homogenizer at 10 passes across 30 seconds at 150 RPM with a motor-driven Teflon pestle[3].

Radio labeled fatty acid ([1-^{14}C]- palmitic acid) from (Perkin Elmer, Waltham MA) was used to measure $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of red muscle from gastrocnemius and quadriceps femoris[3]. 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 $^{14}CO_2$ [4]. The $^{14}CO_2$ was trapped in a tube containing 1 M NaOH, which was then be placed into a scintillation vial with 5 mL scintillation fluid[4]. The vial's ^{14}C concentrations was measured on a 4500 Beckman Coulter scintillation counter. Acid soluble metabolites were determined by collecting the acidified media and measuring ^{14}C levels. Pyruvate oxidation was measured with methods like that of fatty acid oxidation, except for a substitution of [1-^{14}C]-pyruvate for [1-^{14}C]- palmitic acid. Metabolic flexibility was assessed by measuring [1-^{14}C]-pyruvate oxidation ± non-labeled BSA (0.5%) bound-palmitic acid. Flexibility was denoted by the percentage decrease in pyruvate oxidation in the presence of free fatty acid. It was expressed as the ratio of CO_2 production with labeled pyruvate over CO_2 production with labeled pyruvate in the presence of palmitate. Oxidative efficiency was calculated by dividing CO_2 production (complete palmitate oxidation) by acid soluble metabolites (ASMs, incomplete oxidation) and expressed as a ratio[4].

Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloacetate. CoASH reduces DTNB and CS activity was determined from the reduction of DTMB over time. Ten microliters of a 1:5 diluted muscle homogenate was added, in duplicate,

to 170 μL of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 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 were measured at 405nm at 37°C every 12 seconds for 7 minutes. Maximum CS activity were calculated and reported as μmol/min/mg.

Malate dehydrogenase reversibly catalyzes the oxidation of malate to oxaloacetate, and the rate of the disappearance of NADH was measured spectrophotometrically at 340nm at 37°C. Briefly, 10 uL of sample were pipetted in triplicate in wells. Then, 290ul of reaction media (0.1 M potassium phosphate buffer, pH 7.4 plus 0.006 M oxaloacetic acid, prepared in potassium phosphate buffer plus 0.00375 M NADH, prepared in potassium phosphate buffer) was added to the wells and samples were read for 5 minutes at 340nm. The rate of disappearance of NADH was analyzed and expressed relative to protein content. Data is expressed as means ± SEM.

For the determination of β-hydroxyacyl-CoA dehydrogenase, oxidation of NADH to NAD was measured. In triplicate, 35 μL of whole muscle homogenate was added to 190 μL of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) was calibrated and 15μl of 2mM acetoacetyl CoA was added to initiate the reaction. Absorbance were measured at 340 nm every 12 seconds for 6 minutes at 37C. Maximum BHAD activity was calculated and reported as μmol/min/mg.

Cytochrome C oxidase maximal enzyme activity will be measured based on the oxidation of ferrocytochrome C to ferricytochrome C by cytochrome C oxidase. Absorbance was measured

at 550nm every 10 seconds for 7 minutes. Maximum COX activity was calculated and reported as μmol/min/mg.

Protein Concentration Determination:

Samples were diluted in DI water to a 1:5 ratio and vortexed. Ten microliter samples were taken from each sample plated in triplicate on a 96 well clear flat bottom plate along with 9 standards, (Fisher Scientific- catalog number PI23225), in triplicate.

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CHAPTER 5: Acetylation in Skeletal Muscle in Response to an Acute and Chronic High Fat Diet

Abstract

In recent years, advancements in mass spectrometry have made possible wide scale analysis of posttranslational modifications of proteins. One such modification is acetylation, and multiple previous studies have identified thousands of proteins subject to modification by acetylation[1, 2]. Metabolic proteins are highly susceptible to acetylation with no cellular region more so than the mitochondria due to high concentrations of acetyl CoA- the precursor molecule necessary for acetylation[3, 4].

The purpose of this study was two-fold; 1) to examine the normal metabolic response to high fat meal challenge and the adaptive response to a chronic high fat, and 2) to profile the acetylomic response to an acute and chronic high fat diet between lean and obese mice.

Our study revealed significant differences between the acetylomes of lean control mice fed a diet low in fat and obese mice fed a chronic high fat diet. Almost every protein from the β-oxidation pathway, tricarboxylic acid cycle, and electron transport chain exhibited significantly heightened levels of acetylation between the two animal models in a fasted state. Our study also showed significantly lower levels of acetylation between the two animal models in glycolytic proteins. Obese animals showed a 33% reduction in changes of acetylation to metabolic proteins during the fasted to fed transition compared to lean mice. The findings from our study provided further evidence that acetylation functions as a regulatory mechanism for metabolism, and that obesity is associated with hyper-acetylation of mitochondrial metabolic proteins.

Introduction

Over the last 30 years the world has seen an increase in obesity with worldwide rates above one-quarter of the population, and above forty percent in some developed countries.[5, 6]. Varying degrees of insulin resistance, hyperglycemia, and hyperlipidemia are associated with obesity, and are all hallmarks of type 2-diabetes[7, 8]. This is in large part due to a chronic situation of positive energy balance stemming from a westernized diet high in fat and sugar where caloric input exceeds energetic (ATP) demands. The preceding situation culminates in carbon source flux into the mitochondria from multiple metabolites generating acetyl CoA, and partially oxidized fatty acids, promoting metabolic inflexibility. Partially oxidized fatty acids are known to further inhibit insulin signaling and glucose uptake in a negative feedback loop desensitizing skeletal muscle[8-10].

Acetylation, as a posttranslational modification, alters protein enzymatic activity resulting in inhibition or upregulation of enzymatic activity[11, 12]. Multiple studies have shown that the enzymatic function of metabolic proteins is highly susceptible to modification by acetylation[2, 3, 12-14]. Increased mitochondrial acetyl CoA concentrations from a chronic positive energy balance is directly linked to perturbations in covalent modifications of enzymes governing mitochondrial oxidative pathways[15]. In a state of metabolic inflexibility carbons and electrons/protons are delivered into the TCA cycle and ETC by means of increased acetyl CoA from both carbohydrate and fat[16, 17]. It is unclear how much protein acetylation levels differ between lean and obese animals, and what effects a single high fat meal may have on the acetylome.

The objective of this study was two-fold; 1) to examine the normal metabolic response to a high fat meal challenge and the adaptive response to a chronic high fat diet, and 2) to profile

the acetylomic response to an acute and chronic high fat diet between lean and obese mice. We hypothesized that obese mice in a fasted state would show hyper-acetylation of all metabolic proteins compared to lean, healthy animals which would be used as an acetylomic baseline. We also hypothesized that acetylation would change during the fed to fasted transition, and that healthy animals would show significant changes to their acetylome postprandially. We hypothesized obese animals would exhibit signs of metabolic inflexibility, altered substrate preference, and perturbed acetylation patterns of metabolic proteins.

Methods and Materials

Mice

Diet induced obesity (DIO) mice are from Jackson Laboratories- New Brunswick, New Jersey, Cat# 380050, as are our lean mice fed a standard diet, Cat# 380056. Male mice were purchased at 18 weeks of age, and housed in our vivarium at room temperature (20-22 ^{o}C) under normal light and dark phases until the age of 24 weeks when they were sacrificed. Obese mice were started on a diet high in fat upon weening as our control mice were kept on a diet low in fat upon weening. Mice were fed the same diets as used at Jackson Laboratory; DIO and control diets were purchased from Research Diets. DIO diet, Cat# D12492, make-up: 20% (Kcal) protein, 20% (Kcal) carbohydrate, and 60% (Kcal) fat. Control Diet, Cat# D12450B, make-up: 20% (Kcal) protein, 70% (Kcal) carbohydrate, and 10% (Kcal) fat. Mice were fasted 12 hours (overnight) prior to sacrifice. All animal handling and experiments were performed under an approved IACUC protocol.

High Fat Meal Challenge

The high fat meal challenge was 5 Kcal with a caloric make up of 21.4% saturated fat, 40.8% unsaturated fat, 27.1% carbohydrate, and 10.7% protein. Control mice received a DI water gavage. The high fat challenge was 300 μL and administered 3 hours prior to sacrifice.

Body Composition

Twenty-four hours prior to sacrifice mice were weighed, and body composition was determined using a LF90 NMR Analyzer by Bruker.

Blood Serum Analysis

Blood was collected via syringe puncture of the heart and placed in serum separator tubes, Fisher Scientific, Cat# 36597. Serum samples were analyzed using a YSI analyzer for serum glucose and lactate. Free fatty acids were measured using a Free Fatty Acids Half-Micro Test, Roche, Cat# 11-383-175-001. Blood serum insulin was measured using an ELISA by ALPCO; cat#: 80-INSMSU-E01.

Muscle Preparation- Mass Spectrometry

Muscle samples were flash frozen in liquid nitrogen and upon thawing 300 μL of 9 M urea buffer was added. Urea buffer was made to Cell Signaling Technologies specifications: 20 mM HEPES- pH 8.0, 9 M Urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate[18]. Samples were homogenized on ice first with surgical scissors followed by the addition of magnetic beads, Next Advance, Cat# ZRO10, and further homogenization in a Bullet Blender setting 5 for 5 min (x3). Samples were centrifuged at 12,000 RPM for 10 min to separate out the protein from cellular debris.

Protein Extraction, Digestion, Peptide Immunoprecipitation, & Resuspension

Samples were washed with 1 mL 8 mM urea and 50 mM ammonium bicarbonate[19]. Acetylated BSA was added; volume is based on concentration of protein- 90 ng acetylated BSA per 5 mg protein[19]. Dithiothreitol (DTT) was added, for a final concentration of 10 mM[18], to reduce cysteine bonds. Ammonium bicarbonate was added to adjust the urea concentration for a final urea concentration of 1.8 mM. Samples were alkylated with 1/10 volume of iodoacetamide[19]. Trypsin suspended in acetic acid, of a final concentration at 0.25 mM, was added- trypsin volume was determined by protein concentration[19]. Samples were placed in a shaker overnight at 32^{o}C[19].

In a fume hood, trifluoroacetic acid (TFA) was added to samples for a final volume of 1% TFA[18]. pH was measured using a pH strip to ensure acidity[18]. Purification of peptides was performed at room temperature on 0.7 mL Sep-Pak columns from Waters Corporation, Cat#: WAT051910. Samples were lyophilized under a high vacuum- liquid nitrogen vacuum overnight.

Peptides were immunoprecipitated using Cell Signaling beads (CS PTM Scan Acetyl Lysine Motif IAP Beads; Cat #: 13362s). Peptides were re-suspended with 50 µL of 15% TFA; alcohol dehydrogenase 1 was digested by trypsin (Waters Corp.; Cat # 186002328-1; 1.2 fM/µL- quantitative control) [18, 19]. Samples were spun in a centrifugal concentrator at 1,000 g, 37^{o}C for 1 hour under a low vacuum to dry off sample volumes for concentration necessary for mass spectrometry runs. Samples were frozen at -80^{o}C. Two percent MeCN, 0.1% TFA, 98% ultra-pure water solution was prepared for peptide resuspension. Samples were resuspended in 35 µL suspension solution.

Mass Spectrometry

LC-MS/MS analysis via ESI: Triplicate runs (10 µl injections) of each resolubilized peptide sample were separated through an Acquity I-class UPLC system (Waters Corporation, Milford, MA). The separation was performed using a CSH130 C18 1.7 µm, 1.0 x 150 mm column (Waters Corporation, Milford, MA) at 50 µL/min using a 110-minute gradient from 3-40% solvent B. The column temperature was maintained at 45˚C.

Column effluent was analyzed using a Synapt G2-S mass spectrometer (Waters Corporation, Milford, MA) using an HDMSe (high-definition (e.g. ion mobility separation) mass spectrometry with alternating scans utilizing low and elevated collision energies) acquisition method in continuum positive ion "resolution" MS mode.

Peptide/ Protein Identification:

Mass spectrometric data from each chromatographic run was processed and analyzed utilizing ProteinLynx Global Server v. 3.0.2 (PLGS, Waters Corporation, Milford, MA). Average chromatographic and mass spectrometric peak width resolution was determined automatically by the software for peaks eluting from 3.4 to 93.4 minutes. Peptide identification was performed utilizing trypsin specificity, and the possibility of 3 missed cleavages as search parameters. Other search parameters were a fixed carbamidomethylation of cysteine residues, and variable modifications of acetylation of lysine residues, oxidation of methionine residues, and formation of pyrrolidone carboxylic acid at the N-terminus of a peptide if the amino-terminal residue was glutamine. The searches require at least 2 fragment ion matches per peptide, five fragment ion matches per protein and 1 peptide per protein. Peptide and fragment mass tolerances were automatically determined by the software. Searches in PLGS were limited to no more than 5% false discovery rate. The protein database search was all *Mus musculus* entries downloaded from

UniProt excluding those defined as fragments of a larger protein. A random entry for each true protein entry was appended using PLGS prior to peptide identification searches.

Skeletal muscle substrate metabolism.

Red gastrocnemius and quadriceps femoris were manually excised and washed in cold PBS. Approximately 50 mg of muscle placed in 200 μL of modified sucrose EDTA medium (SET Buffer) on ice[20]. SET buffer contains 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 1 mM ATP, pH 7.4[20]. Muscle samples were minced with scissors followed by the addition of SET Buffer to produce a final 20-fold dilution (wt: vol)[20]. The samples were homogenized in a Potter-Elvehjem glass homogenizer at 10 passes across 30 seconds at 150 RPM with a motor-driven Teflon pestle[20]. Radio labeled fatty acid ([1-^{14}C]- palmitic acid) from (American Radiolabeled Chemicals, St. Louis, MO.) was used to measure $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of red muscle from gastrocnemius and quadriceps femoris[20]. Samples were incubated in 320ul 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 $^{14}CO_2$ [34]. The $^{14}CO_2$ trapped in a tube containing 1 M NaOH, was then placed into a scintillation vial with 5 mL scintillation fluid[34]. The vial's ^{14}C concentrations was measured on a 4500 Beckman Coulter scintillation counter.

Assays for citrate synthase, malate dehydrogenase, β-hydroxyacyl-CoA dehydrogenase, and cytochrome C were used to measure the effect of a high fat diet and gavage on metabolic function and to further investigate acetylation's role in metabolic regulation. Citrate Synthase: Ten microliters of a 1:5 diluted muscle homogenate was added, in duplicate, to 170 μL of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 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 were measured at 405nm at 37°C every 12 seconds for 7 minutes. Malate dehydrogenase: 10 uL of sample were pipetted in triplicate in wells. Then, 290ul of reaction media (0.1 M potassium phosphate buffer, pH 7.4 plus 0.006 M oxaloacetic acid, prepared in potassium phosphate buffer plus 0.00375 M NADH, prepared in potassium phosphate buffer) was added to the wells and samples were read for 5 minutes at 340nm. The rate of disappearance of NADH was analyzed and expressed relative to protein content. NADH Dehydrogenase: In triplicate, 35 µL of whole muscle homogenate was added to 190 µL of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) was calibrated and 15µl of 2mM acetoacetyl CoA was added to initiate the reaction. Absorbance were measured at 340 nm every 12 seconds for 6 minutes at 37C. Cytochrome C oxidase: Cytochrome C oxidase maximal enzyme activity will be measured based on the oxidation of ferrocytochrome C to ferricytochrome C by cytochrome C oxidase. Absorbance was measured at 550nm every 10 seconds for 7 minutes. Enzyme activity is reported in the ***Supplemental Figures*** section.

Statistics

Mass Spectrometry

Software automatically determined peptide and fragment mass tolerances. Searches in ProteinLynx Global Server (PLGS) was limited to no more than 5% false discovery rate. Results from PLGS were imported into IsoQuant (http://www.immunologie.uni-mainz.de/isoquant/)

limiting the peptide false discovery rate to 1% and the protein false discovery rate to 5%. IsoQuant matches peaks across multiple samples and reports the signal intensity of each peptide across all samples. Signal intensity has been shown previously to be roughly linearly correlated to the molar amount of that peptide. Probable significance of samples was preliminarily determined using MultiBase an Excel add on. Acetylation levels in obese mice were expressed relative to data from fasted, fasted control mice (averaged for every protein and set to 1). P-values and standard error mean was calculated using GraphPad Prism software.

Results

Supporting Data/ Mouse Model Characterization

Diet induced obese mice have been used as a model for the characterization of obesity and type 2 diabetes[21-24]. They were chosen because when fed a high fat diet they exhibit symptoms of obesity and type 2 diabetes such as hyperinsulinemia, hyperglycemia, and hypertension when allowed to feed *ad libitum* compared to mice on a control diet[23, 24]. Mice were fed either a control (10% fat, 70% carbohydrate, and 20% protein), or high fat (70% fat, 20% carbohydrate, and 10% protein) diet from weaning until ~ 24 weeks of age, at which time they were euthanized for study. As shown in **Table 1**, the high fat diet resulted in significantly higher body mass, fat mass, and lean mass, as well as hyperglycemia and insulinemia. No differences were observed in fasting serum FFA concentrations. Fasting blood L-lactate concentrations were significantly higher in mice fed a high fat diet.

Table 1

Body Composition & Blood Measurements

Measurement	Lean- Mean	Obese- Mean	P-value
Body Mass (g)	29.69±0.36	44.92±0.87	< 0.0001
Fat Mass (%)	7.25±0.51	21.86±1.08	< 0.0001
Fat Mass (g)	2.17±0.17	10.01±0.61	< 0.0001
Lean Mass (%)	65.49±0.66	51.32±1.11	< 0.0001
Lean Mass (g)	19.43±0.25	22.87±0.33	< 0.0001
Glucose (mmol/L)	7.61±0.82	12.53±0.52	0.0005
Insulin (pmol/L)	10.51±3.26	659.40±159.5	0.002
FFA (mmol/L)	0.054±0.006	0.05±0.007	0.5812
L-lactate (mmol/L)	4.56±0.31	7.16±0.60	0.0031

TABLE 1: Values of lean and obese animals were obtained from measurements taken in a fasted state. Body mass, fat mass, lean mass as a percentage and weight in grams measured by NMR. Glucose and L-lactate were measured on a YSI analyzer. Insulin was measured by ELISA and FFA by a colorimetric assay. All values are expressed as mean±SEM, P-value was derived from independent t-tests, and all statistics were calculated by GraphPad Prism

Measures of fatty acid oxidation (FAO), pyruvate oxidation, and metabolic flexibility measurements were assessed in our obese and lean mouse models, presented in **Figure 1**.

Figure 1 (A-F)

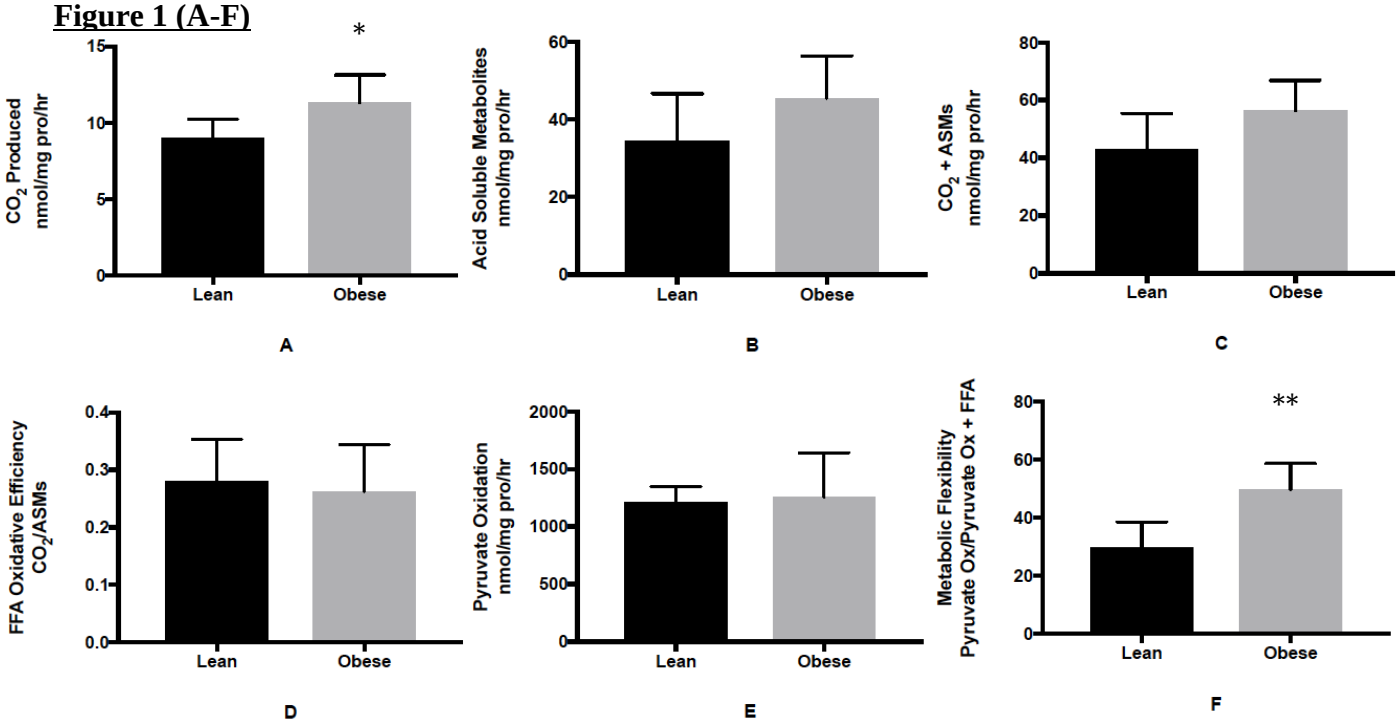

FIGURE 1: Figure 1A-F were performed under a 12 hour fast. CO_2 produced is complete oxidation of FAs. Incomplete oxidation is also presented as ASMs (Acid Soluble Metabolites). CO_2 + ASMs is total oxidation of FAs. Metabolic Flexibility is determined by the levels of Pyruvate Dehydrogenase activity without media/ Pyruvate Dehydrogenase activity+ Fatty Acid Media. Data is presented as mean±SEM. *= $p<0.05$; **= $p<0.005$.

Complete FAO (**Figure 1A**) and metabolic flexibility (**Figure 1F**) were significantly higher in obese animals compared to lean controls. No differences were observed between groups for FAO incomplete oxidation (**Figure 1B**), total fatty acid oxidation (**Figure 1C**), oxidation efficiency (**Figure 1D**), or pyruvate oxidation (**Figure 1E**). Statistics were run with a N-size of 24.

Effects of high fat feeding on fasting acetylation levels in skeletal muscle.

Table 2 displays the effects of a chronic high fat diet (obesity) on acetylation levels in carbohydrate and fatty acid metabolism, tricarboxylic acid cycle (TCA), and electron transport chain (ETC) metabolic proteins in a fasted state. Acetylation levels of glycolytic metabolic proteins associated with carbohydrate metabolism were significantly lower with obesity, whereas proteins associated with fatty acid metabolism, TCA cycle, and ETC were significantly higher.

Glycolysis. Four glycolytic proteins showed significantly lower levels of acetylation in our obese mice compared to our healthy mice: fructose bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. Interestingly, these enzymes are at, or just beyond the splitting stage (aldolase) of the glycolytic pathway, whereas none of the well-established rate-limiting enzymes of glycolysis appear to be acetylated, e.g., hexokinase, phosphofructokinase, and pyruvate kinase. Triosephosphate isomerase is the reversible reaction between glycerol-3-phosphate and dihydroxyacetone phosphate; the interconversion between these two intermediates is important for glyceroneogenesis and electron transfer via the glycerol-3-phosphate shuttle. Glyceraldehyde-3-phosphate dehydrogenase is a redox reaction, and phosphoglycerate kinase is an ATP-producing enzymatic reaction. The physiological relevance of acetylation of these enzymes has yet to be determined, and moreover,

the ramification of lower levels of acetylation in the context of obesity deserves further investigation.

Beta Oxidation. Acetylation levels of very long-chain specific acyl-CoA dehydrogenase (VLCAD), long-chain specific acyl-CoA dehydrogenase (LCAD), and medium chain-specific acyl- CoA dehydrogenase (MCAD) were significantly higher in obese mice relative to controls. These three acyl-CoA dehydrogenases are involved in the beginning step of beta oxidation, where there is an addition of a double bond between the α and β carbon of the acyl CoA, producing $FADH_2$ in the process. Dehydrogenation between carbon 2 and 3 of the fatty acid is followed by hydration, dehydrogenation, and cleavage. These steps are carried out by a protein complex comprising the remaining three out of four enzymatic reactions of the β-oxidation pathway: trifunctional enzyme subunit alpha and trifunctional enzyme subunit beta (HADHA & HADHB). HADHA and HADHB represent the proteins hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/enoyl-CoA hydratase alpha & beta and are the 2^{nd}, 3^{rd}, and 4^{th} steps of β-oxidation. Significantly higher levels of acetylation of HADHA and HADHB were also observed in the high fat fed, obese mice. Along with higher levels of acetylation of HADHA/ HADHB are greater levels of acetylation for individual proteins of the trifunctional protein complex; hydroxyacyl CoA dehydrogenase and 3-ketoacyl CoA thiolase. The hydrolysis of fatty acyl-CoAs to their constituent free fatty acids and CoAs is performed by acyl CoA thioesterases. Acyl CoA thioesterase 2 was also found to be hyper-acetylated in the obese mice compared to lean mice. As was, 2,4-dienoyl-CoA reductase and enoyl CoA delta isomerase which are necessary for the oxidation of unsaturated fatty acids. Interestingly, the enzymes of beta oxidation exhibited the largest percentage of hyper-acetylation (~122%) in the high fat fed, obese mice, relative to lean controls, which is far greater than any other pathway examined in our data set

Pyruvate Oxidation and the Tricarboxylic Acid Cycle. Acetylation of the E3 component of pyruvate dehydrogenase, dihydrolipoyl dehydrogenase, was higher in an obese state. All enzymes of the TCA cycle were found to be significantly acetylated in the high fat fed, obese mice, relative of lean controls, except for α-ketoglutarate dehydrogenase. Of note, significant changes in acetylation were observed in the TCA cycle at the enzymatic steps where NADH, $FADH_2$, and GTP are produced. The conversion of α-ketoglutarate to succinyl-CoA via α-ketoglutarate dehydrogenase is a key regulatory point of the TCA cycle whereby once it proceeds it is irreversible. Not a single acetyl modification was detected by mass spectrometry of this regulatory protein in either lean or obese animals.

Electron Transport and ATP Synthase. As with fat metabolism and TCA cycle proteins, ETC proteins show elevated levels of acetylation in an obese state. Complex I, II, III, IV and ATP synthase (F (0)) all showed significantly higher levels of acetylation compared to lean, control mice.

Table 2

Acetylation in a Fasted State

Pathway	ID	% Difference	P-Value
CHO Metabolism	Triosephosphate Isomerase	-16.35	0.0234
	Glyceraldehyde-3-Phosphate Dehydrogenase	-32.69	< 0.0001
	Phosphoglycerate Kinase	-17.46	0.0007
	Fructose Bisphosphate Aldolase	-9.64	0.05
Fat Metabolism	Very Long Chain Specific Acyl CoA Dehydrogenase	77.37	0.0351
	Long Chain Specific Acyl CoA Dehydrogenase	131.50	< 0.0001
	Medium Chain Specific Acyl CoA Dehydrogenase	132.51	0.0256
	Enoyl CoA Delta Isomerase	101.86	0.0013
	2,4-Dienoyl CoA Reductase	188.83	0.0017
	Hydroxyacyl CoA Dehydrogenase	130.34	0.0004
	3-Ketoacyl CoA Thiolase	130.78	< 0.0001
	HADHA	121.08	< 0.0001

	HADHB	59.94	0.0031
	Acyl CoA Thioesterase 2	256.02	< 0.0001
	Acyl Carrier Protein	42.15	0.0018
	Acetyl CoA Acetyltransferase	107.88	< 0.0001
TCA Cycle	Dihydrolipoyl Dehydrogenase	36.21	< 0.0001
	Citrate Synthase	70.99	< 0.0001
	Aconitate Hydratase	28.96	< 0.0001
	Isocitrate Dehydrogenase	49.00	< 0.0001
	Succinyl CoA Ligase	50.23	0.0325
	Succinate Dehydrogenase	20.81	0.0131
	Fumarate Hydratase	50.41	< 0.0001
	Malate Dehydrogenase	21.67	0.0021
ETC	NADH Dehydrogenase	45.23	0.0001
	Cytochrome b-c1	33.63	< 0.0001
	Cytochrome C Oxidase	53.59	0.0148
	Electron Flavotransfer-Ubiquinone Oxidoreductase	53.59	< 0.0001
	ATP Synthase	48.00	< 0.0001
	ATP Synthase F(0)	38.98	< 0.0001
	ATP Synthase Coupling Factor 6	56.17	0.0042

TABLE 2: **Table 2** is a listing of proteins determined to be significantly different in acetylation using fasted lean animals as a baseline and set to 1. Percent difference was calculated as (((Fasted Obese/Fasted Lean)-1) 100). Negative values represent a decrease in acetylation. P-values calculated by t-test using GraphPad Prism software.

Effects of high fat feeding on skeletal muscle acetylation during postprandial metabolism.

The effects of feeding on acetylation. **Table 3** shows the metabolic proteins, in lean and obese mice, that were affected by a high fat meal challenge. Very few of the same proteins were affected in both lean and obese animals in the postprandial period. Long chain specific acyl CoA dehydrogenase, isocitrate dehydrogenase, and cytochrome C oxidase were acetylated in both lean and obese mice, but were affected differently. Long chain acyl CoA dehydrogenase and cytochrome C oxidase were significantly increased in lean animals but decreased in obese animals. Isocitrate dehydrogenase, however, was increased in both lean and obese animals but to

a much greater extent in lean animals with an 11% increase compared to a 3.9% increase in obese animals.

Glycolysis. We observed a significant decrease in acetylation of two glycolytic proteins, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase 1, in the lean mice with no change in the obese mice. Acetylation of enolase (beta) was significantly reduced by feeding in the obese animals with no observed changes in the lean animals.

Beta Oxidation. Acetylation of fat metabolizing proteins was increased, on average by 8%, in our lean animals in response to a feeding. **Table 3** illustrates that all the β-oxidation pathway underwent significant acetylation changes in the fasted to fed transition. Acyl CoA dehydrogenase, enoyl CoA hydratase, HADHA, and acetyl CoA acetyltransferase (thiolase) exhibited significant increases in acetylation; this encompasses all the redox reactions for fat oxidation of long chain fatty acids. Varied signals were observed in fat oxidation pathways of obese animals. Long chain-specific acyl CoA dehydrogenase and 2,4-dienoyl CoA reductase (both are redox reactions) showed decreases in acetylation where acyl CoA thioesterase 13 was increased. As presented in **Table 3**, the fat metabolism data in obese animals was the start of a trend witnessed throughout the remaining mitochondrial metabolic pathways where we no longer observed a uniform change in acetylation, but rather a mixed set of results with both increasing and decreasing acetylation values in the pathway.

The Tricarboxylic Acid Cycle. In control animals, citrate synthase, aconitate hydratase, and isocitrate dehydrogenase were modulated by acetylation in the postprandial period. While aconitate hydratase is a reversible reaction, citrate synthase and isocitrate dehydrogenase are not, and are rate-limiting steps in the TCA cycle. Pyruvate dehydrogenase, the last step of glycolysis and a bridge between cytosolic glycolysis and mitochondrial TCA cycle, shows a decrease in

acetylation in our obese mice. Pyruvate dehydrogenase is a heavily regulated checkpoint because it is an irreversible conversion of pyruvate to acetyl CoA. While isocitrate dehydrogenase acetylation was elevated in both lean and obese animals.

Electron Transport Chain and ATP Synthase. The ETC also showed significant increases in acetylation of proteins in the postprandial period (**Table 3**). Acetylation of Complex I, III, and ATP synthase were significantly increased in the postprandial period in our lean animals. As with the TCA cycle and β-oxidation pathway, the electron transport chain exhibits mixed acetylomic signals with both significant increases and decreases in obese animals in a fed state. Acetylation of complex II was significantly increased while Complex III and ATP synthase F (0) were decreased in the obese animals in the postprandial period.

Table 3

Acetylation in the Postprandial Period

Pathway	Enzyme	Lean (% difference from fasted)	Obese (% difference from fasted)
CHO Metabolism	Glyceraldehyde-3-Phosphate Dehydrogenase	-32.55	*NC*
	Phosphoglycerate Kinase 1	-20.97	*NC*
	Enolase (Beta)	*NC*	-9.70
	Pyruvate Dehydrogenase	*NC*	-6.37
Fat Metabolism	Acyl CoA Thioesterase 13	*NC*	10.71
	Long Chain Specific Acyl CoA Dehydrogenase	9.00#	-6.78#
	HADHA	8.45	*NC*
	Enoyl CoA Hydratase	4.71	*NC*
	Acetyl CoA Acetyltransferase	13.48	*NC*
	Enoyl CoA Delta Isomerase 1	4.49	*NC*
	2,4-Dienoyl CoA Reductase	*NC*	-5.21
TCA Cycle	Citrate Synthase	11.15	*NC*
	Aconitate Hydratase	9.01	*NC*
	Isocitrate Dehydrogenase	11.06#	3.96#
ETC	NADH Dehydrogenase	6.96	*NC*
	Cytochrome b-c1	*NC*	5.94
	Cytochrome C Oxidase	5.49#	-2.41#

	ATP Synthase	4.95	*NC*
	ATP Synthase (F0)	*NC*	3.63
	Electron Flavotransfer Ubiquinone Oxidoreductase	5.46	NC
	ATP Synthase Coupling Factor 6	*NC*	-5.37

TABLE 3: Table 3 is the difference between fasted and fed states presented in both lean and obese animals. Percent values are presented if that protein was detected and significant where NC = No change in that protein for that animal model. Most proteins were not significant across both the lean and obese model under the fasted to fed transition. Negative values represent a decrease in acetylation values. # indicates difference between lean and obese animal models. All % values represent significance of $p<0.05$.

Discussion

Our findings show that obesity from overfeeding, specifically from a chronic high-fat diet, is associated with mitochondrial hyper-acetylation, and upon transition to fasted or fed states, only minimal changes to the metabolic acetylome occur. Our findings also show that in healthy mice acetylation is highly dynamic, and could potentially be an integral regulator of metabolism. We observed numerous differences in the acetylome between lean and obese mice in a fasted state with almost every metabolic protein from the TCA cycle, β-oxidation pathway, and ETC having significantly increased acetylation levels due to obesity. We found that our obese model was significantly less likely to exhibit alterations to the acetylome upon feeding compared to control mice, and that there was only a small number of proteins that were significant in both control and obese mice in the postprandial state. Furthermore, we show possible novel regulatory glycolytic mechanisms governing ATP production and NADH regeneration.

Fasted State Metabolism: Lean vs Obese

Glycolytic Regulation: Over the last decade and a half, the posttranslational modification acetylation has come to the forefront as a metabolic regulator. Lv et al. (2011) showed that the M2 isoform of pyruvate kinase's activity is decreased by acetylation, and Lundby et al (2012) that acetylation decreased glycerol-3-phosphate dehydrogenase activity[25, 26]. Acetylation of glycolytic proteins in skeletal muscle has shown to inhibit activity, if not lead to proteomic degradation[12, 27].

In all our animal models and experimental conditions (lean v. obese/ fasted v. fed), every glycolytic protein of measured statistical significance showed a decrease in acetylation levels. None of the commonly known major regulatory checkpoints (hexokinase, phosphofructokinase, and pyruvate kinase) were shown not to be acetylated with any significance, or at all, in a fasted state. However, our data suggest acetylation may play a previously undefined role in carbohydrate metabolism with an important redox reaction and ATP producing step showing significant decreases in acetylation because of obesity. We observed four glycolytic proteins that showed significant decreases in acetylation in obese mice: fructose bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase.

The glycerol-3-phosphate shuttle uses co-factors regenerated through glycolysis to transfer electrons from NADH in the cytosol to $FADH_2$ in the mitochondria. Triosephosphate isomerase converts dihydroxyacetone to glyceraldehyde-3-phosphate (and vice versa), which is then converted into glycerol-3-phosphate in a redox reaction utilizing NADH. Our data may indicate that significant decreases in acetylation of triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase could lead to increased enzyme activity, and

increased NADH regeneration, thereby increasing the shuttling of electrons into the mitochondria via the glycerol-3-phosphate shuttle. Whether this is occurring is uncertain.

Another possible consequence of the significantly decreased fasted state acetylation observed is obese fasted mice is the increased production of lactate. We observed significantly increased levels of serum lactate (**Table 1**) in our obese mice. While no significant differences in acetylation between lean and obese mice were observed in lactate dehydrogenase or pyruvate kinase there were significant decreases in glyceraldehyde-3-phosphate dehydrogenase which regenerates the co-factor NADH required for the redox conversion of pyruvate to lactate. A significant decrease in the acetylation of phosphoglycerate kinase, an ATP producing reaction, two enzymatic reactions upstream of the production of pyruvate kinase was observed, and may indicate an upregulation of the glycolytic process with no major regulatory checkpoints between it and pyruvate kinase. An increase of pyruvate production and conversion to lactate would account for the significantly increased fasted state serum levels of lactate levels observed in obese animals. This could be evidence of an alternate regulatory mechanism for energy production and substrate handling in glycolysis using acetylation as a mediator.

If either of the two previously presented scenarios is occurring is speculation, but could be a sign of metabolic inflexibility in obese animals in a fasted state. A metabolically flexible response in fasted state skeletal muscle would be increased oxidation of fatty acids from intramuscular stores or FFA released from adipose tissue. Instead, fasted obese mice have very significantly increased levels of serum insulin, glucose, and lactate accompanied with decreased levels of skeletal muscle glycolytic acetylation for NADH and ATP producing enzymatic reactions.

Fasted State Fatty Acid Oxidation: Obesity is known to cause hyper-acetylation of mitochondrial proteins[17, 28]. Studies have also described the effect of acetylation on the β-oxidation pathway, and these studies have been invaluable to interpreting our data as they present a clear model to acetylation's effects [2, 3, 29, 30]. Hirschey et al. (2011) used a SIRT3 KO mouse model fed a chronic high fat diet and showed that long-chain acyl CoA dehydrogenase activity was decreased in response to acetylation, and that SIRT3 KO models developed all the symptoms of metabolic syndrome such as diet-induced obesity, insulin resistance, and hyperglycemia[17]. Enoyl-CoA hydratase/ 3-hydroxyacyl CoA (EEHADH) represents two of the three enzymes in the β-oxidation pathway following acyl CoA dehydrogenase, and in Chang human liver cells cultured with deacetylase inhibitors, EEHADH shows increased activity[2].

Results from our fasted state mice show the largest increases in acetylation (average 122%) due to obesity. This includes very-long, long, and medium-chain acyl CoA dehydrogenases, and the proteins comprising the remaining steps of β-oxidation (hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/enoyl-CoA hydratase alpha & beta) for oxidizing saturated fatty acids. Our results appear antithetical with acyl CoA dehydrogenases showing increased acetylation which would result in decreased activity while the remaining stages of β-oxidation are exhibiting the opposite trend with increased acetylation culminating in an increase in enzymatic activity. Overall, fat oxidation is elevated in fasted obese animals relative to controls; this is supported from results presented in **Figure 1** which show increased complete fat oxidation (CO_2 Produced). Increased complete fat oxidation is an adaptive response seen in obese mice initially to cope with increased levels of fat intake [31-33]. Our data points to acetylation as playing a role in the continuation of elevated fat oxidation levels in response to obesity due to a high fat diet.

Postprandial Metabolism

Metabolic Flux in an Obese State: Our data indicate that compared to healthy animals in a fed state, obese animals show a decrease (33%) in changes to the acetylome of mitochondrial metabolic proteins- as can be seen in **Table 3**. This suggests a situation of dysfunction where the acetylome is forced to remain in a hyper-acetylated. The main mitochondrial deacetylase (SIRT3) has its activity inhibited because of chronic high fat feeding [17, 29]. Chronic excess fat also inhibits PGC-1α expression, the main activator of SIRT3, further exacerbating the levels of aberrant acetylation in obese animals[17]. Our control model fed a low-fat diet presented a uniform acetylation response in the transition from fasted to fed states, with decreasing acetylation of glycolytic proteins and increasing acetylation of proteins in the TCA cycle, β-oxidation pathway, and the ETC. Obese animals presented no such pattern with fluctuations of increasing and decreasing acetylation within all pathways- except glycolysis. It is important to note that with any postprandial decrease or "No Change" in mitochondrial acetylation occurring in obese mice, the level of acetylation is still significantly elevated above that of control mice.

We observed minimal overlap of significant acetylation changes in the fed state between lean and obese animals (**Table 3)**, with only three common proteins shared by both groups. This may indicate that at the cellular level obese animals are no longer capable of altering their acetylome in a way appropriate for metabolic regulatory control. In fact, the opposite may be the case where a hyper-acetylated state pushes substrates into β-oxidation while simultaneously decreased glycolytic acetylation creates competition for substrate oxidation thereby overwhelming both the TCA cycle and electron transport chain.

There was little change in glycolytic acetylation from the fasted to fed transition in obese animals, but what change that occurred is important. Beta enolase showed further decreases in

acetylation along with pyruvate dehydrogenase showing decreased acetylation. This would almost certainly push metabolites into the TCA cycle only to be countered by unchanged heightened levels of TCA cycle protein acetylation. The ETC and β-oxidation pathway remain mostly unchanged from the fasted state with slight increases in acetylation of Complex II, ATP synthase F(0), and a decrease in acetylation 2,4-dienoyl CoA reductase- all restraining their activity. Long chain acyl CoA dehydrogenase and cytochrome C oxidase both showed reductions in acetylation promoting the initial stage of fatty acid oxidation and up-regulating Complex III.

Taken together our data suggests an increase in carbohydrate and saturated fatty acid oxidation in combination with a retardation of the TCA cycle and partial inhibition of the electron transport chain in the postprandial period in obese mice. This could aide in the state of metabolic inflexibility linked with obesity and type II diabetes in which β-oxidation is observed to ramped up with no corresponding increase in TCA cycle activity as well as hyperglycemia caused by insulin resistance[10]. Our data follows this pattern where acetylation of β-oxidation enzymes promotes fat metabolism accompanied with a probable slowdown of the TCA cycle from increased acetylation. Whether in the fasted or fed state, glycolysis shows a steady state of function with decreased acetylation promoting enzyme activity.

Conclusions

In conclusion, our findings from these studies are that obesity because of a chronic high fat diet directly impacts the acetylome in mice, specifically the acetylation pattern of metabolic proteins compared with lean control animals. Furthermore, we have characterized the normal acetylomic profile of healthy control C57Bl6J mice. While there are significant differences between the metabolic profiles of mice and humans we believe that this data can be translatable across species.

Of the thirty-one metabolic proteins we measured to be significantly acetylated between lean and obese animals in a fasted state, 27 of those were mitochondrially located. All 27 of these proteins had significantly increased acetylation levels in fasted obese animals. The remaining non-mitochondrially located proteins had significantly decreased acetylation levels. The heightened level of acetylation in obese animals suggests a static situation in the mitochondria where obesity leads to persistent hyper-acetylation that is non-responsive to changing metabolic signals or substrate levels.

Our research also highlights the effects of a single high fat meal on acetylation in metabolic pathways of lean control animals and obese animals. The results indicate a fluid situation in healthy animals where acetylation changes in metabolic pathways aide in substrate selection and storage. Obese animals exhibit a perturbed acetylomic profile compared to lean animals in that significant changes to the acetylome do not follow a pattern of increasing or decreasing within a pathway but fluctuate with no apparent design.

Changes in acetylation in obese animals in response to a high fat meal was significantly decreased, 33% less in obese animals. This in conjunction with the hyper-acetylated state adds to the evidence that obese mice are incapable of responding to substrate influx, and where the substrate influx may not be static the acetylome pattern is unresponsive.

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CHAPTER 6: LIMITATIONS & FUTURE DIRECTIONS

Limitations

This study was performed examining the effects of a high fat diet on acetylation of skeletal muscle metabolism. Metabolic function may be very different for a diet high in carbohydrate (sugar) than that of a high fat diet. The caloric make-up of the high fat diet was very low (10%) in sugar (carbohydrate) and may not be an accurate reflection of a westernized diet.

This study was conducted during the day which is the normal sleep phase of the rodents and could disrupt the metabolic response. This study also tried to mimic a human high fat feeding and the rodents were given a large high fat meal all at once which is more than a mouse would normally eat at once.

Future Directions

The difference in metabolic response and acetylation between chronic high fat and high sugar diets as well as high fat and high sugar gavages should be examined to determine if there are varying responses to nutrient and acetyl CoA sources.

Sirtuins are responsible for most deacetylase activity involved in skeletal muscle metabolic pathways, as with many other tissues. However, there is considerable overlap in the function of sirtuin function for several sirtuins- SIRT4 and SIRT5. Sirtuin 4 and 5 are of particular interest as they are weak deacetylases and have been shown to exhibit a more prominent role as de-acyl transferases. Acylation, like acetylation, is a new area of research as a protein modification and as with acetylation could prove to be key in the regulation of metabolic

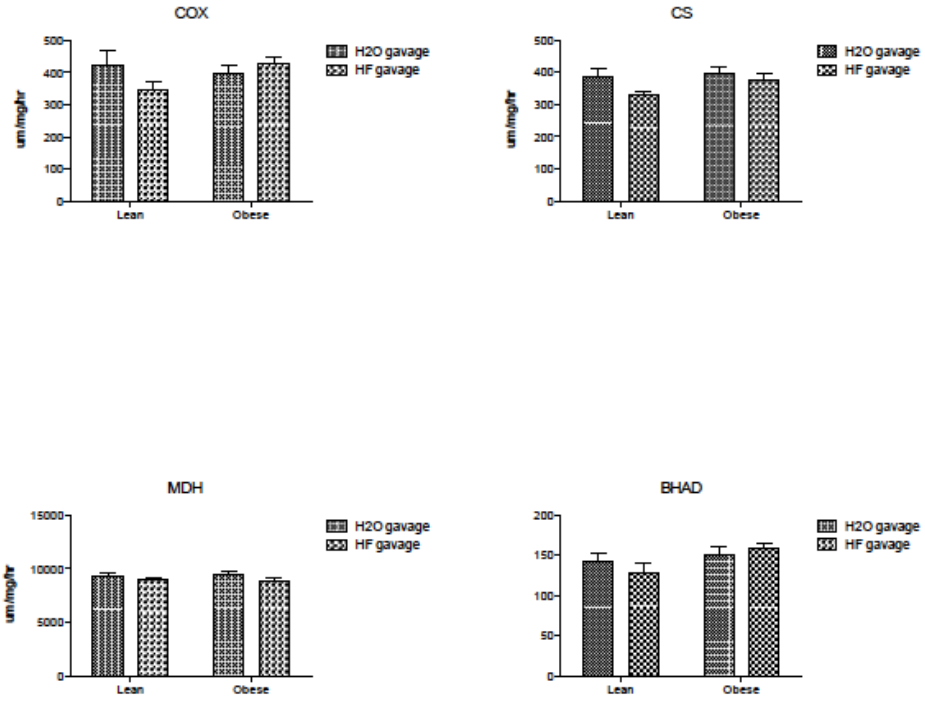
function. Sirtuins act on numerous protein modifications and the role these play in metabolism is vague.

Other modifications, such as O-GlcNAcylation, are also coming to the forefront of research as their roles in metabolism is starting to be viewed to be integral. Mass spectrometry could be used to further elucidate their roles in metabolic processes.

This study was performed during the mice normal nocturnal period, a study during their most active time could reveal even more variations in the acetylome for metabolic pathways.

Supplemental Figures

Enzyme analysis was run but no significant differences were detected by Two-way ANOVA.



COX- Cytochrome C Oxidase, CS- Citrate Synthase, MDH- Malate dehydrogenase, BHAD- Beta hydroxyacyl CoA Dehydrogenase.