Acetylation of mitochondrial proteins by GCN5L1 promotes enhanced fatty acid oxidation in the heart

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ABSTRACT

Lysine acetylation is a reversible post-translational modification, and is particularly important in the regulation of mitochondrial metabolic enzymes. Acetylation uses acetyl-CoA derived from fuel metabolism as a co-factor, thereby linking nutrition to metabolic activity. In this study, we investigated how mitochondrial acetylation status in the heart is controlled by food intake, and how these changes affect mitochondrial metabolism. We found that there was a significant increase in cardiac mitochondrial protein acetylation in mice fed a long-term high fat diet, and that this change correlated with an increase in the abundance of the mitochondrial acetyltransferase-related protein GCN5L1. We show that the acetylation status of several mitochondrial fatty acid oxidation enzymes (LCAD, SCAD and HADHA) and a pyruvate oxidation enzyme (PDH) was significantly upregulated in high fat diet mice, and that the increase in LCAD and SCAD acetylation correlated with increased enzymatic activity. Finally, we demonstrate that the acetylation of mitochondrial fatty acid oxidation proteins is decreased following GCN5L1 knockdown, and that reduced acetylation leads to diminished fatty acid oxidation in cultured H9C2 cells. These data indicate that lysine acetylation promotes fatty acid oxidation in the heart, and that this modification is regulated in part by the activity of GCN5L1.

KEYWORDS

Mitochondria; Acetylation; GCN5L1; SIRT3; Fatty Acid Oxidation; High Fat Diet; Heart
Recent research shows that acetylation of mitochondrial fatty acid oxidation enzymes has greatly contrasting effects on their activity in different tissues. Here we provide new evidence that acetylation of cardiac mitochondrial fatty acid oxidation enzymes by GCN5L1 significantly upregulates their activity in diet-induced obese mice.
INTRODUCTION

High energetic demand requires that the heart continuously generates ATP to sustain cardiac contractile function (23, 25). The heart uses several fuel substrates for energy metabolism (22, 24), with fatty acid oxidation (FAO) accounting for 50-70% of the total ATP generated (6, 20, 23). While healthy hearts have the flexibility to use various substrates, a shift towards increased fatty acid utilization is a key feature of obesity and diabetes (21). Elevated levels of circulating free fatty acids and triacylglycerol, combined with alterations in FAO enzyme abundance or activity, promote cardiomyocyte lipid accumulation, cardiac dysfunction and heart failure (7, 9, 16, 40). As the prevalence of obesity and diabetes continues to increase, it is imperative that we examine the processes that regulate cardiac energy metabolism under these conditions.

Lysine acetylation, a reversible post-translational modification, has recently been identified as a novel regulator of mitochondrial bioenergetic function. A growing body of literature has found that the majority of mitochondrial metabolic enzymes are acetylated (37, 41), and the regulation of FAO enzyme activity by acetylation appears to be particularly important (2, 26, 29, 39, 41). Mitochondrial protein acetylation is significantly increased in various tissues during high fat diet feeding (14, 17), and fatty acids are the main source of the acetyl-CoA used as a co-factor for lysine acetylation (28). These data imply that there is an intrinsic link between nutritional inputs, lysine acetylation and metabolic activity. Taken together, changes in the acetylation status of cardiac FAO enzymes may greatly impact their activity, and could be a key cellular mechanism that drives metabolic dysfunction in the hearts of obese individuals.

The regulation of mitochondrial lysine acetylation has predominantly focused on the activity of SIRT3, the major mitochondrial deacetylase enzyme (13, 14, 33). SIRT3 has been shown to target a number of different regulators of energy metabolism, including the FAO enzyme long-chain acyl-CoA
dehydrogenase (LCAD) (13, 14), and the catalytic subunits of pyruvate dehydrogenase (PDHA) (15). In
many tissues the deacetylation of mitochondrial enzymes promotes their enzymatic function (4, 5, 14); however there are contrasting reports in the heart (1, 8) which highlights the need for further study. While there have been numerous reports on the regulation of mitochondrial lysine deacetylation, few studies have addressed the counteracting process of lysine acetylation. In particular, the function of the recently discovered mitochondrial acetyltransferase protein GCN5L1(35) in cardiac energy metabolism has yet to be intensively explored.

GCN5L1 is localized in the mitochondrial matrix, and has been shown to counter the activity of the mitochondrial deacetylase protein SIRT3 (35). Deletion of GCN5L1 blunts mitochondrial protein acetylation and bioenergetics, which promotes mitochondrial dysfunction and cellular energy depletion in mouse embryonic fibroblasts (34). In the current study, we examined the effect of long-term high fat diet on the regulation of GCN5L1 and lysine acetylation in the heart. We show that nutrient excess promotes increased mitochondrial lysine acetylation, which correlates with upregulated expression of GCN5L1 at the expense of SIRT3. High fat feeding led to the hyperacetylation of mitochondrial FAO proteins, which was associated with increased enzymatic activity in in vitro assays. Finally, we found that knockdown of GCN5L1 reduced both the acetylation and activity of FAO enzymes, which culminated in a reduction in mitochondrial bioenergetic output in cardiac-derived H9C2 cells.
MATERIALS AND METHODS

Animal Care and Use

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the University of Pittsburgh animal facility. Animals were housed under standard conditions with ad libitum access to water and food, and maintained on a constant 12h light/12h dark cycle. Animals were fed either a standard chow diet (67% carbohydrate, 13% fat, 20% protein, 3.6 Kcal/g; No. 01351, Harlan Teklad, Madison, WI) or a high fat chow (40% carbohydrate, 41% fat, 19% protein, 4.4 Kcal/g; No. 96001, Harlan Teklad) for 24 weeks. At the end of 24 weeks, animals were euthanized and cardiac tissues excised for analysis. Experiments were conducted in compliance with National Institutes of Health guidelines, and followed procedures approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Protein Isolation

For whole cardiac protein lysate, tissues were minced and lysed in CHAPS buffer on ice for ~2 hours. Homogenates were spun at 10,000 g, and the supernatants collected for western blotting or co-immunoprecipitation experiments. For mitochondrial assays, tissues were lysed in detergent-free buffer and mitochondrial proteins were isolated using the QProteome Mitochondrial Isolation Kit (Qiagen) using the manufacturer’s protocol. For activity assays, muscle homogenates were prepared in a modified Chappell-Perry Medium A buffer (120 mM KCl, 20mM Hepes, 5 mM MgCl₂, 1 mM EGTA, pH~7.2) supplemented with 5 mg/ml fat-free bovine serum albumin. After centrifugation at 10,000 g, supernatants were used to assay enzyme activity.
Western Blotting and Co-Immunoprecipitation

Protein lysates were prepared in LDS sample buffer, separated using Bolt SDS/PAGE 4-12% or 12% Bis-Tris gels, and transferred to nitrocellulose membranes (All Life Technologies). Protein expression was analyzed using the following primary antibodies: rabbit SIRT3, rabbit acetyl-lysine (Ac-K), rabbit glutamate dehydrogenase (GDH), rabbit pyruvate dehydrogenase (PDH) and rabbit cytochrome c oxidase subunit 4 (COX IV) from Cell Signaling Technologies; rabbit phospho-PDH (Ser 293) from Novus; rabbit LCAD, rabbit SCAD and rabbit HADHA from Proteintech; GCN5L1 as reported previously (35). Fluorescent anti-mouse or anti-rabbit secondary antibodies (red, 700 nm; green, 800 nm) from LiCor were used to detect expression levels. For co-immunoprecipitation experiments, protein lysates were harvested in CHAPS buffer, and equal amounts of total protein were incubated overnight at 4 ºC with the relevant antibody or an IgG control. Immunocaptured proteins were isolated using ProteinG agarose beads (Cell Signaling Technology), washed multiple times with CHAPS buffer and then eluted in LDS sample buffer at 95 ºC. Samples were separated on 12% Bis-Tris Bolt gels and probed with appropriate antibodies. Protein densitometry was measured using Image J software (National Institutes of Health, Bethesda, MD). Protein loading was further confirmed using GDH or COX IV loading controls where appropriate.

Cellular Assays

Acyl-coenzyme A dehydrogenase assays were performed following the protocol by Verity et al. (36). Palmitoyl-CoA (60 µM) and butyryl-CoA (60 µM) were used as substrates for long chain acyl-coenzyme A dehydrogenase (LCAD) and short chain acyl-coenzyme A dehydrogenase (SCAD) activity, respectively. Briefly, 25 µg of protein was incubated with 0.1M potassium phosphate buffer, 50 µM 2,6-dichlorophenolindophenol, 2 mM phenazine ethosulfate, 0.2 mM N-ethylmaleide, 0.4 mM potassium
cyanide and 0.1% Triton X-100 at 37 °C for 4 minutes. The reaction was initiated with 60 µM palmitoyl-CoA or 60 µM butyryl-CoA, and the rate of absorbance change was measured at 600 nm over 5 min. Activities were converted to nanomoles of substrate oxidized/min/mg protein against a standard curve. LDH release assays (MAK066 kit from Sigma) and Crystal Violet staining were performed on H9C2 cells after exposure to 50 mM H2O2 for 30 min.

RNA Isolation and qRT-PCR
For qRT-PCR, mRNA was isolated using a total RNA extraction kit (Qiagen), and cDNA was produced using a first-strand synthesis kit (Invitrogen). Transcript levels were measured using validated gene-specific primers (Qiagen). Each experiment was carried out at least three times, and representative results are shown. Gapdh was used as the internal control.

GCN5L1 Knockdown Stable Cell Lines
H9C2 cells were purchased from ATCC (Manassas, VA). Cells were transduced (MOI = 2) with Mission lentiviral shRNA particles targeting GCN5L1 (two different shRNAs either used individually [KD1, KD2] or as a pool [KD3]) or a scrambled control sequence. Transduced cells were selected using 1.25 µg/ml puromycin (determined following a kill-curve experiment), and cultured in this concentration for several passages. Stable cell lines were verified by qRT-PCR and western blot for gene knock down efficiency.

Mitochondrial Bioenergetics Measurements
Oxygen Consumption Rate (OCR) was measured in GCN5L1 control and knockdown stable H9C2 cell lines using the Seahorse XFe96 system (Seahorse Bioscience). Cells (5 x 10³ cells/well) in each
condition were plated (n = 8) and allowed to attach overnight in DMEM. On the assay day, media was changed to unbuffered DMEM media supplemented with 5 mM glucose, 2 mM glutamine and 0.5 mM carnitine, and the plate incubated in a non-CO₂ incubator (37 °C; 15 min) before running. Basal OCR in each well was measured, followed by serial treatment with palmitate (200 µM), etomoxir (40 µM), FCCP (7.5 µM) and rotenone (2 µM). After completion, viability was assessed by Crystal Violet staining, and OCR normalized to cell number.

**Statistics**

Means ± SEM were calculated for all data sets. Data were analyzed using two-tailed student’s t-tests, one-way ANOVA or two-way ANOVA (with Tukey post-hoc tests) as appropriate. $P < 0.05$ was considered significant.
RESULTS

Mitochondrial Fuel Metabolism Gene Expression is Modulated by HFD

Significant increases in heart mass were observed in animals following 24 weeks of HFD (Table 1; *$P < 0.05$), suggesting a negative impact on overall cardiac health. To begin to understand how HFD may regulate cardiac fuel metabolism, we first assessed the expression levels of cardiac fatty acid metabolism genes. Animals fed a HFD showed a general rise in FAO gene expression, with significant increases in long-chain acyl-coenzyme A dehydrogenase (Acadl), carnitine palmitoyltransferase 1b (Cpt1b), and the fatty acid translocase Cd36 (Figure 1C-E). The increase in FAO gene expression corresponded with enhanced expression of the pyruvate dehydrogenase negative regulator Pdk4, indicating a shift from pyruvate oxidation towards FAO in HFD animals (Figure 1F). While the expression of the transcriptional coactivator Ppargc1a was downregulated in HFD mice, there was a significant increase in the main FAO-related transcription factor Ppara (Figure 1G, H). In summary, the transcriptional profile of cardiac tissue in HFD mice suggests an increase in FAO at the expense of other fuel substrates, in line with previous reports (1, 7, 10).

HFD Increases Lysine Acetylation of Cardiac Mitochondrial Proteins

HFD has been linked to increased mitochondrial protein lysine acetylation, a post-translational modification that has the capacity to modulate the activity of numerous metabolic enzymes (Reviewed in (41)). To further investigate how HFD controls mitochondrial acetylation in the heart, we first examined the expression of two mitochondrial acetylation regulators, GCN5L1 and SIRT3. Transcriptional analyses of Gcn5l1, a mitochondrial acetyltransferase-related protein (35), demonstrated a significant increase in gene expression in the hearts of mice following HFD. (Figure 2A). This
elevation was matched by a significant increase in *Sirt3* mRNA levels (a mitochondrial-localized deacetylase protein (32, 33)) under the same conditions (*Figure 2A, B*). Interestingly, while GCN5L1 gene and protein expression levels were coordinately upregulated, SIRT3 protein expression was significantly reduced under conditions where its mRNA abundance was elevated (*Figure 2C-F*). SIRT3 is a nuclear-encoded protein that is imported into the mitochondrial matrix following the removal of an N-terminal localization sequence (32). We therefore checked to see whether the reduced abundance of the mitochondrial form of SIRT3 (~28 kDa) was caused by a decrease in production of the full-length cytosolic form (~40 kDa). Western blot analysis demonstrated that there was no significant difference in the pre-import form of SIRT3 between chow and HFD animals (*Figure 2G, H*), suggesting that the reduced abundance of mitochondrial SIRT3 in HFD mouse hearts is not caused by a defect in SIRT3 translation.

As exposure to a long-term HFD raises both circulating fatty acid and glucose levels, we next aimed to determine which metabolic substrate was responsible for the elevated gene expression of *Gcn5l1* and *Sirt3*. We cultured rat cardiac-derived H9C2 cells in media containing either low glucose (5 mM), high glucose (25 mM) or palmitate (200 μM) for 4 h, then measured the relative expression of each gene by qRT-PCR. Exposure to palmitate led to a significant increase in both *Gcn5l1* and *Sirt3* gene expression (*Figure 2I, J*), which tallied with the elevated expression seen in HFD mice (*Figure 2A, B*). In contrast, exposure to a high glucose concentration had opposite effects on these genes, with *Gcn5l1* expression showing a significant increase (*P* = 0.03), and *Sirt3* trending towards decreased expression (*P* = 0.05) (*Figure 2I, J*). These data suggest that the increase in *Gcn5l1* expression is caused by a non-substrate specific increase in available nutrients, whereas *Sirt3* expression is controlled by substrate type.
Finally, our overall protein expression data from these acetylation regulators suggested a shift towards a pro-acetylation phenotype. To test this, we isolated mitochondria from chow and HFD mice, and examined the acetylation status of mitochondrial proteins using a pan-acetylation antibody. This confirmed that there was indeed an increase in this modification in the heart following HFD (Figure 3).

**Hyperacetylated FAO Proteins from HFD Mice Show Increased Enzymatic Activity**

The role of acetylation in regulating metabolic enzyme activity has been extensively studied in several tissues, particularly the liver and skeletal muscle (13-15, 17, 18, 28, 41). However, the role of this modification in the heart remains to be fully elucidated. In an attempt to understand the effects of increased cardiac lysine acetylation on FAO, we examined the acetylation status and activity of mitochondrial fuel substrate oxidation enzymes. While there was no significant difference in the cardiac protein abundance of short- and long-chain acyl-CoA dehydrogenases (SCAD and LCAD, respectively) between chow and HFD animals, there was an increase in the acetylation of these two enzymes (Figure 4A, B). Examination of a further FAO enzyme (hydroxyacyl-CoA dehydrogenase; HADHA), and the alpha subunit of pyruvate dehydrogenase (PDH), showed a similar increase in acetylation (Figure C, D). In the case of the latter, acetylation of PDH has been shown to inhibit its activity, which could further exacerbate the switch to a pro-FAO metabolic phenotype in the obese heart. We next examined whether HFD had any effect on FAO enzyme activity, and found that both SCAD and LCAD oxidation rates were significantly upregulated in samples obtained from overfed mice (Figure 5A, B). We performed a regression analysis between LCAD acetylation status and enzyme activity, and found that increased acetylation positively correlated with elevated LCAD activity ($r^2 = 0.6875; P = 0.01$) (Figure 5C). These data indicate that a HFD leads to increased FAO enzyme acetylation, and that this modification correlates with increased FAO activity in the heart.
Reductions in GCN5L1 Expression Limit Cellular FAO Activity

The observed differences in FAO enzymatic activities and mitochondrial protein acetylation, coupled with a significant increase in GCN5L1 protein content in obese mice, led us to further examine the regulatory role of GCN5L1 in cardiac mitochondrial bioenergetics. To aid these studies, we created three stable GCN5L1 knockdown H9C2 cell lines using different lentiviral-delivered shRNAs. GCN5L1 mRNA levels and protein content were significantly decreased in two lines (KD1 and KD3) (Figure 6A) which were then selected for further characterization. We first tested whether loss of GCN5L1 had any effect on overall cellular fitness, and found that GCN5L1 depletion in H9C2 cells had no effect on viability at baseline or following exposure to reactive oxygen species (Figure 6B, C). We next analyzed whether loss of GCN5L1 had an effect on mitochondrial bioenergetic function using the Seahorse XF system (Figure 6D). Both GCN5L1 knockdown cell lines had significantly impaired baseline respiration, and there was a significant decrease in maximal respiration in KD1, which was matched with a trend towards reduced uncoupled respiration in KD3 (Figure 6E, F), which complements similar findings seen previously in GCN5L1 knockout MEF cells (34). In addition, both GCN5L1 knockdown H9C2 cell lines displayed significantly attenuated decreases in FAO in response to treatment with the CPT1 inhibitor etomoxir (Figure 6G), indicating that these cells predominantly use glucose-based respiratory substrates at the expense of FAO.

GCN5L1 Knockdown Decreases Acetylation and Enzymatic Activity of FAO Proteins

Finally, we attempted to investigate whether there was a mechanistic link between GCN5L1-regulated mitochondrial protein acetylation and FAO rates in H9C2 cells. Using our immunoprecipitation-based acetylation assay, we found a large decrease in the acetylation status of both SCAD and LCAD in
GCN5L1 knockdown cells relative to the control (Figure 7 A, B); although no change in acetylation level was seen in HADHA and PDH using the same methodology (data not shown). We then assessed the activity of SCAD and LCAD in vitro, and found that loss of GCN5L1 significantly reduced the oxidation capacity of both dehydrogenases (Figure 7 C, D). From these studies, we conclude that GCN5L1 promotes the acetylation of mitochondrial FAO proteins, and that this modification is strongly associated with increased FAO activity in cardiac cells.
DISCUSSION

Mitochondrial protein acetylation is controlled by the activity of opposing acetyltransferase and deacetylase enzymes in response to changing nutritional conditions. To further understand the importance of this post-translational modification *in vivo*, we examined the regulatory role played by lysine acetylation on cardiac bioenergetics during extended periods of excess dietary fatty acids. Our studies demonstrate that increased acetylation status of cardiac mitochondrial proteins following high fat feeding correlates with increased GCN5L1 expression and decreased SIRT3 abundance. Furthermore, we show that prolonged exposure to a high fat diet led to increased acetylation of mitochondrial FAO enzymes, which was linked to an increase in their enzymatic activity. Finally, we found that knockdown of GCN5L1 in our stable H9C2 cell lines decreased the acetylation and activity of SCAD and LCAD, which resulted in a significant reduction in cellular FAO rates. These findings suggest that GCN5L1 activity may promote cardiac bioenergetic output through the acetylation and activation of multiple mitochondrial FAO enzymes.

In our HFD-induced obesity model, increased expression of *Ppara*, *Acadl*, *Cpt1b*, *Cd36* and *Pdk4* suggest an increased reliance on FAO, at the expense of pyruvate oxidation. Similar increases in FAO have been observed in human subjects undergoing seven days of high fat feeding (31), as well as in rats with three weeks of HFD (10). This shift towards increased utilization of fatty acids for ATP production is commonly observed in obesity and diabetes, and may be exacerbated by cardiac insulin resistance and decreased glucose uptake (21). As excess fat conditions persist, changes in cardiac substrate handling may lead to elevated levels of circulating free fatty acids and triacylglycerol, culminating in myocardial lipid accumulation and decreased cardiac output (7, 40). Identification of the
mechanisms involved in regulating this metabolic perturbation are therefore of interest in both basic and
translational research settings.

Lysine acetylation is a reversible post-translational modification that controls numerous cellular
processes and energetic pathways (2, 26, 29, 41). Proteomic studies have shown that enzymes involved
in almost every major metabolic pathway are acetylated (37, 41). Furthermore, studies assessing
acetylation dynamics in several tissues, including liver (17), skeletal muscle (19) and heart (1), have
shown that multiple mitochondrial proteins are hyperacetylated. Our study is in agreement with these
findings, and demonstrates that there is a significant increase in cardiac mitochondrial protein
acetylation following extended high fat exposure (Figure 3). While numerous studies have shown that
SIRT3 abundance and activity are regulated by prevailing nutrient conditions (13, 17), there have been
few reports of the effect of diet on GCN5L1. Livers from fasted mice show a significant decrease in
GCN5L1 abundance (38), while there have been conflicting reports of the effect of nutrient excess on
GCN5L1 expression in the heart in both non-surgical (1) and surgical investigations of cardiac
metabolism (30). Our current findings are in direct contrast to a previous publication by Alrob et al.,
which showed that GCN5L1 expression was not significantly changed in the heart following HFD (1).
Here we show that GCN5L1 is significantly upregulated at both the gene and protein level in HFD-
exposed mice, and that high concentrations of both glucose and palmitate can upregulate Gcn5l1
expression in H9C2 cells. The contrasting findings may also explain why we found a significant increase
in PDH acetylation not observed previously (1). The reasons behind the diverse study outcomes are
currently unclear, although may be related to simple differences in diet composition (41% vs. 60% fat)
or study length (16-18 weeks vs. 24 weeks).

In addition to our findings on GCN5L1 regulation, we observed an interesting discrepancy
between SIRT3 gene and protein expression in our HFD animals. Numerous studies have shown that
SIRT3 protein abundance is decreased in response to a HFD, however there is little published information on how Sirt3 gene expression is affected by nutrient excess. We show here that Sirt3 is significantly upregulated in both HFD mouse hearts and in palmitate-treated H9C2 cells, while its protein abundance is reduced in vivo under the same conditions (Figure 2). The mechanism by which the mitochondrial form of SIRT3 is reduced – despite unchanged levels of the pre-import cytosolic form – remains to be determined, but may be linked to increased SIRT3 degradation under high fat conditions. Overall, we conclude that regulators of both mitochondrial lysine acetylation and deacetylation are closely controlled by nutrient availability, and that this system has evolved to regulate mitochondrial bioenergetics in response to changing nutritional and physiological conditions.

We previously showed that GCN5L1 modulates the acetylation of electron transport chain proteins (35), and its loss negatively impacts mitochondrial bioenergetic output (34). Here we focused on the effect of GCN5L1 on FAO enzymes SCAD and LCAD, which oxidize short and long chain fatty acids respectively. LCAD function is particularly important in maintaining cardiac bioenergetic output, and loss of LCAD activity has been shown to result in an elevated reliance on glucose oxidation (3). Conversely, increased activity of LCAD has been associated with increased FAO in the heart (1). We found that SCAD and LCAD are hyperacetylated following a long-term HFD, and have increased enzymatic activity in in vitro assays. As these changes correlated with an increase in GCN5L1 abundance, we investigated whether this protein had a functional effect on FAO enzyme activity. Knockdown of GCN5L1 in our stable cardiac cell line resulted in decreased acetylation of SCAD and LCAD, and significantly reduced the enzymatic activity of LCAD. Furthermore, we show for the first time that reductions in GCN5L1 abundance lead to a significant decrease in cellular FAO rates. These findings suggest that GCN5L1 function is central to the regulation of cardiac mitochondrial bioenergetics, and is indispensable for the regulation of acetylation and enzymatic activity of FAO
proteins. Future studies, examining the impact of GCN5L1 loss on fatty acid metabolism and bioenergetics in vivo, should help to further elucidate the importance of lysine acetylation in cardiac metabolism.

The outcomes of the present study are in broad agreement with a very recent publication, which showed that reduced GCN5L1 expression led to a significant decrease in the in vitro activity of LCAD and β-HAD (11, 12). Importantly, this study also showed that GCN5L1 abundance increased during early development, which closely correlated with the switch from a glycolytic to a FAO metabolic profile in the heart (11, 12). This indicates that an increase in the acetylation of cardiac FAO enzymes plays a functional role in upregulating their activity, and corroborates several recent studies in the heart (1, 30) which have shown clear links between upregulated FAO enzyme acetylation and increased cardiac fatty acid utilization.

However, it must be noted that in the majority of tissues examined (particularly the liver), an increase in the acetylation of FAO enzymes has been linked to a decrease in their activity. For example, hyperacetylation of Lcad, caused by loss of SIRT3, leads to a decrease in enzymatic activity and hepatic lipid accumulation (5, 13). This discrepancy in the impact of acetylation on FAO in different tissues clearly requires further investigation. One potential explanation is that different lysine residues on the same protein (e.g. LCAD) are acetylated under the same HFD conditions in different tissues, which may increase or decrease its enzymatic activity in response to the contrasting metabolic needs of each organ (e.g. liver vs. heart). Proteomic studies have shown differing acetylation patterns in different organs from the same mouse (reviewed in (27)), and it may be that each tissue uses acetylation to regulate metabolic activity differently, even on a protein-by-protein basis. Given that the heart is particularly reliant on FAO (21), and that fatty acids are the main source of acetyl-CoA for acetylation (28), it may be logical that increases in fatty acid availability in the heart would not immediately downregulate FAO
via acetylation, as it does in other tissues. Future comparative studies, investigating the sites of lysine acetylation on the same protein in different tissues, should help to answer some of these questions.

In conclusion, our findings in this study suggest that the regulation of lysine acetylation in mitochondria plays a major role in controlling cardiac bioenergetic output, and that the utilization of fatty acids in the heart is enhanced when increased nutrient availability promotes the acetylation of FAO enzymes (Figure 8). Further work on the function of GCN5L1 and SIRT3 in this context will likely yield important information on cardiac bioenergetics, and may in the future uncover new therapeutic targets in cardiac metabolism under various disease states.
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AUTHOR CONTRIBUTIONS

D.T. and I.S. conceived and designed research; D.T., J.R.M. and I.S. analyzed data and prepared figures; D.T. and I.S. drafted and revised the manuscript; D.T., M.Z., J.R.M., D.A.G. and M.W.S. performed experiments; R.M.O. and S.S. provided critical resources and expertise; all authors approved final version of manuscript.
REFERENCES


Table 1 – Characteristic features of Chow and HFD mice. Body weight, heart weight, and heart
weight/tibia length are significantly increased in high fat diet animals. Values are means ± SEM. *P <
0.05 chow fed vs. high fat diet using two-way student’s t-test.

Figure 1 – Expression of cardiac fuel metabolism genes. High fat diet promotes an enhanced fatty
acid oxidation phenotype in the heart. (A, B) No significant difference was observed in Scad or Mcad
levels. (C-G) Significant increases in mRNA content of Lcad, Cpt1b, Cd36, Pdk4 and Ppara were
observed with high fat feeding, while (H) Pparc1a was significantly decreased. Values are expressed
as means ± SEM. P value significance is as shown in the graphs using two-way student’s t-test, n = 4 per
group.

Figure 2 – Expression of mitochondrial acetylation regulators in cardiac mitochondria. High fat
diet leads to a pro-acetylation phenotype in cardiac mitochondrial proteins. (A, B) Mitochondrial
acetyltransferase Gcn5l1 and deacetylase Sirt3 mRNA expression is significantly increased in cardiac
mitochondria from chronic high fat diet fed mice. (C-E) While GCN5L1 protein level is significantly
increased in HFD animals, SIRT3 protein abundance is significantly decreased. Western blots for
GCN5L1 and SIRT3 were obtained from the same gel, and therefore the same GDH loading control was
used for each blot. (G) There is no significant difference in the abundance of the 40 kDa pre-import,
cytosolic form of SIRT3 in chow and HFD mice. (H) In contrast, there is a significant decrease in the 28
kDa mitochondrial form of the enzyme in HFD animals. (I, J) Gcn5l1 gene expression in H9C2 cells
was significantly increased after 4h of high glucose (25 mM) and palmitate (200 μM) exposure in basal
DMEM relative to low glucose (5 mM), while Sirt3 expression was only elevated in response to palmitate. Values are expressed as means ± SEM. $P$ value significance is as shown in the graphs using two-way student’s $t$-test, $n = 4$ per group.

**Figure 3 - Global mitochondrial lysine acetylation in chow and HFD hearts.** Overall cardiac mitochondrial protein acetylation is significantly increased, with no changes observed in protein content of COX IV. Values are expressed as means ± SEM. $P$ value significance is as shown in the graphs using two-way student’s $t$-test, $n = 4$ per group.

**Figure 4 – Impact of HFD on metabolic enzyme acetylation status.** High fat diet leads to increased FAO enzyme acetylation, which contributes to upregulated fatty acid utilization in diet-induced obese mice. (A-C) Acetylated lysine pull down showed an increase in the acetylation levels of the FAO proteins SCAD, LCAD and HADHA, and (D) the pyruvate oxidation enzyme PDH, from HFD cardiac tissue relative to chow-fed controls. Values are expressed as means ± SEM. $P$ value significance is as shown in the graphs using two-way student’s $t$-test, $n = 4$ per group.

**Figure 5 - Impact of HFD-related acetylation on in vitro FAO enzyme activity.** (A, B) Enzymatic activity of SCAD and LCAD was significantly elevated in cardiac tissues from HFD mice. (C) Regression analysis showed a significant correlation between LCAD acetylation status and enzymatic activity from all tested mice. Values are expressed as means ± SEM. $P$ value significance is as shown in the graphs using two-way student’s $t$-test, $n = 4$ per group.
Figure 6 – Regulation of cellular respiration and FAO by GCN5L1. GCN5L1 is a key regulator of FAO in cardiac cells. (A) mRNA and protein levels of three stable GCN5L1 knockdown H9C2 cell lines created using different lentiviral-derived shRNAs. GDH was used as loading control. Significant decreases in both mRNA and protein content were observed in two lines (KD1 and KD3). (B, C) There was no significant difference in either cell death (LDH release) or cell viability (Crystal Violet staining) in GCN5L1 knockdown cells relative to the control when exposed to 50 mM H₂O₂ for 30 mins. (D) Mitochondrial bioenergetics profile of GCN5L1 control, KD1 and KD3 cells. (E-G) There was a significant decrease in basal respiration, maximal uncoupled respiration (for KD1) and fatty acid oxidation in GCN5L1 knockdown cells relative to the control. Values are expressed as means ± SEM. P value significance is as shown in the graphs, n = 4 per group for GCN5L1 mRNA and protein levels, n = 8 per group for respiration measurements. One-way ANOVA was used for Figure 6A, E-G, two-way ANOVA was used for Figure 6B-C, Tukey post-hoc testing was performed in each case.

Figure 7 – Impact of GCN5L1 knockdown on cardiac FAO enzyme acetylation and activity.

GCN5L1 promotes the acetylation and activity of cardiac FAO proteins. (A, B) Our immunoprecipitation-based acetylation assay showed a large decrease in the acetylation status of SCAD and LCAD proteins in GCN5L1 knockdown cells, relative to the control. (C, D) This decrease correlated with reduced enzymatic activities of SCAD and LCAD in vitro. Values are expressed as means ± SEM. P value significance is as shown in the graphs using two-way student’s t-test, n = 4 per group.

Figure 8 – Model of lysine acetylation and FAO in cardiac metabolism. Under normal conditions, a balance between lysine acetylation and deacetylation allows mitochondria to maintain bioenergetic
output under changing physiological conditions. In obese mice, increased acetylation of mitochondrial proteins drives a pro-FAO metabolic phenotype, thereby reducing substrate flexibility in the heart.
<table>
<thead>
<tr>
<th></th>
<th>Chow (n = 8)</th>
<th>HFD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>33.90 +/- 0.62</td>
<td>48.75 +/- 1.05*</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>124.9 +/- 2.23</td>
<td>137.7 +/- 1.35*</td>
</tr>
<tr>
<td>Tibia Length (mm)</td>
<td>11.63 +/- 0.18</td>
<td>11.50 +/- 0.19</td>
</tr>
<tr>
<td>Heart/Tibia (mg/mm)</td>
<td>10.75 +/- 0.21</td>
<td>11.99 +/- 0.21*</td>
</tr>
</tbody>
</table>
Chow Diet

SIRT3  \[\rightarrow\]  GCN5L1
\[\rightarrow\]
Balanced Mitochondrial Acetylation
\[\rightarrow\]
Maintain Fuel Substrate Flexibility

High Fat Diet

SIRT3  \[\rightarrow\]  GCN5L1
\[\rightarrow\]
Increased Mitochondrial Acetylation
\[\rightarrow\]
Shift Towards Upregulated FAO