CHAPTER 4.

Obesity-Induced Lysine Acetylation Increases Cardiac Fatty Acid Oxidation and Impairs Insulin Signaling

My role in this work involved performing all experiments (except those noted below), as well as the writing of the manuscript. Cory Wagg perfused the mouse heart, and Sowndramalingam Sankaralingam performed perfusion data analysis. Amy Barr conducted the MRI for mice body fat composition.

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CHAPTER 4.

Acetylation of Regulatory Proteins in the Heart Increases Fatty Acid Oxidation and Impairs Insulin Signaling

Abstract

Aims: Lysine acetylation has emerged as a novel post-translational pathway that regulates the activities of enzymes involved in both fatty acids and glucose metabolism. We examined whether lysine acetylation controls heart glucose and fatty acid β-oxidation in high fat diet (HFD) obese and SIRT3 KO mice.

Methods and results: C57BL/6 mice were placed on either a HFD (60% fat) or low fat diet (LFD) (4% fat) for 16- or 18-wk. Cardiac fatty acid β-oxidation rates were significantly increased in HFD vs LFD mice (845±76 vs 551±87 nmol.g dry wt-1.min-1, $p<0.05$). Activities of the fatty acid β-oxidation enzymes, long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were increased in hearts from HFD vs LFD mice, and were correlated with LCAD and β-HAD hyperacetylation. Cardiac protein hyperacetylation in HFD fed mice was associated with a decrease in SIRT3 expression, while expression of the mitochondrial acetylase, GCN5L1, did not change. Interestingly, SIRT3 deletion in mice also led to an increase in cardiac fatty acid β-oxidation compared to WT mice (422±29 vs 291±17 nmol.g dry wt-1.min-1, $p<0.05$).

Cardiac lysine acetylation was increased in SIRT3 KO mice compared to WT, including increased acetylation and activity of LCAD and β-HAD. Although the HFD and SIRT3 deletion decreased glucose oxidation, pyruvate dehydrogenase (PDH) acetylation was unaltered. However, the HFD did increase Akt acetylation, while decreasing its phosphorylation and activity.

Conclusions: We conclude that increased lysine acetylation enhances cardiac fatty acid β-oxidation, and impairs myocardial insulin sensitivity.

4.1 INTRODUCTION

Fatty acids β-oxidation is a major energy source for the adult heart (2). However, the presence of obesity, diabetes, and heart failure result in an increased reliance of the heart on fatty acid β-oxidation as a source of energy, which can decrease cardiac efficiency and compromise cardiac function (89). The reasons for the increase in cardiac fatty acid β-oxidation under these conditions are not completely understood, but do involve increases in circulating fatty acids, alterations in transcriptional control of fatty acid oxidative enzymes, and the development of cardiac insulin resistance that decreases glucose oxidation (58). Given the large number of patients with obesity and diabetes, a better understanding of processes that regulate cardiac energy metabolism could lead to novel therapeutic approaches to treat cardiovascular complications associated with these conditions by optimizing cardiac energy metabolism.

Alterations in post-translational control of fatty acid and glucose oxidation are another potential mechanism contributing to the obesity-induced shift in energy metabolism in the heart. Lysine acetylation has recently emerged as a novel important post-translational modification, which can modify the activity of a number of enzymes involved in fatty acid and glucose metabolism (31,94,98,159,165,243). This post-translational modification is controlled, in part, by sirtuins (SIRTs), which are nicotinamide adenine dinucleotide (NAD^+) dependent deacetylases

localized in distinct subcellular compartments (200,208). Three of the SIRT isoforms (SIRT1, SIRT6, and SIRT7) are localized in the nucleus (178), where they can play an important role in regulating acetylation of nuclear proteins involved in transcriptional regulation of genes involved in cardiac energy metabolism. This includes the acetylation of peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α), a transcriptional regulator of genes that encode enzymes involved in fatty acid β-oxidation. Previous studies showed that acetylation of PGC-1α by the nuclear acetyltransferase general control of amino acid synthesis 5 (GCN5), results in an inhibition of PGC-1α transcriptional activity (94,197). Interestingly, SIRT6 can deacetylate and activate GCN5 thereby increasing PGC-1α acetylation (193). On the other hand, SIRT1 deacetylates and activates PGC-1α (244,245).

In mitochondria, SIRT3 appears to be the major deacetylase (140,190) and targets numerous enzymes involved in energy metabolism, including key enzymes involved in fatty acid β-oxidation such as long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) (98,174,179,190,243). However, conflicting information has emerged regarding the role of deacetylation in controlling fatty acid oxidation. Hirschey *et al* proposed that deacetylation of LCAD by SIRT3 accelerates fatty acid β-oxidation in the liver (179,243). Furthermore, chemical acetylation of LCAD on lysines 318/322 reduced enzymatic activity, while deacetylation with recombinant SIRT3 restored

catalytic activity (171). In contrast, Zhao *et al* showed that acetylation of β-HAD results in an activation of enzyme activity in muscle cells (98). Mitochondria from hindlimb muscles of fasted mice were also shown to have an increased acetylation that was associated with an increase in fatty acid β-oxidation rates (194). Diaphragm muscle of SIRT3 knockout mice also showed hyperacetylation and increased fatty acid β-oxidation rates (194). Furthermore, LCAD is hyperacetylated in obese dam offsprings concomitant with a significant decrease in SIRT3 expression and activity (191). Therefore, further studies are needed to clarify the impact of acetylation on fatty acid oxidation.

GCN5-like 1 (GCN5L1) was recently identified as an essential component of the mitochondrial acetyltransferase machinery (196). However, little is known about its role in regulating acetylation levels of mitochondrial proteins in obesity. Obesity and type 2 diabetes have been shown to be associated with cardiac dysfunction and impaired insulin signaling (192,206). In the heart, insulin stimulated glucose oxidation is dramatically decreased in high fat diet (HFD)-induced obesity (201). Disruption of cardiac insulin signaling by inhibiting protein phosphorylation along the insulin/Akt (protein kinase B)/ glycogen synthase kinase 3-β (GSK3β) axis can lead to a reduced cardiac glucose use (201,246). However, in addition to inhibition of Akt phosphorylation, acetylation may play important role in the initiation of insulin signaling. A recent study by Gupta *et al* identified lysine acetylation as an important regulator of Akt

activity during cardiac hypertrophy (211). Despite this, the role of lysine acetylation in regulating cardiac insulin signaling in obesity has not yet been defined. The aim of this study is to identify what role lysine acetylation has in regulating cardiac fatty acid and glucose oxidation in obese and SIRT3 KO mice heart. In addition, we determined what effect lysine acetylation has on cardiac insulin signaling.

4.2 MATERIALS AND METHODS

Mice received care and were treated according to the guidelines of the Canadian Council on Animal Care, and all procedures performed on animals were approved by the University of Alberta Health Sciences Animal Welfare Committee. GTT and MRI were conducted to determine levels of blood glucose and body composition of fat, respectively. Hearts isolated from pentobarbital euthanized mice were perfused as isolated working hearts with Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 5 mM [U-^{14}C]glucose, 0.8 mM [9,10-^{3}H]palmitate prebound to 3% albumin, in the presence or absence of 100 μU/mL insulin, as described in details in **chapter 2**. At the end of the perfusion, hearts were immediately frozen in liquid N_2 and processed for acetylation status. Protocols for assessment of β-HAD activity, LCAD activity, immunoprecipitation, immunoblotting, and statistical analysis are described in details in **chapter 2** of this thesis.

4.3 RESULTS

4.3.1 Characteristics of HFD obese mice:

Body weight and whole body fat mass, as measured by MRI in a wake mice, were significantly increased after 18 wk of HFD feeding compared to LFD fed mice (Table 4-1). Heart weight normalized to tibia length was also increased in mice fed a HFD. Blood glucose levels in overnight-fasted mice, as assessed by OGTT, were higher in HFD obese mice compared to mice on a LFD (Table 4-1).

4.3.2 Cardiac lysine acetylation in HFD obese mice:

Overall myocardial acetylation levels were increased in obese HFD fed mice compared to LFD fed mice (Figure 4-1A). Mitochondrial proteins acetylation were also markedly increased in hearts isolated from HFD fed mice (Figure 4-1B). The effect of a HFD on the expression of cardiac sirtuins (SIRTs, lysine deacetylases), and GCN5L1 (mitochondrial acetyltransferase) are shown in Figure 4-1. There were no changes in SIRT1 (Figure 4-1C) and GCN5L1 expression (Figure 4-1G); however the level of SIRT3 protein expression was significantly decreased in HFD obese mice (Figure 4-1D). In contrast, the level of SIRT6 expression was significantly increased in HFD obese mice (Figure 4-1E), while the expression of SIRT2 was significantly decreased (Figure 4-1F).

4.3.3 Cardiac palmitate oxidation rates in HFD obese mice:

To examine what effect obesity has on cardiac energy metabolic rates; glucose and palmitate oxidation rates were measured in isolated working hearts from mice subjected to either a HFD or a LFD for an 18-wk period. A significant increase in palmitate oxidation rates (Table 4-2) were seen in mice subjected to a HFD, compared to LFD controls. In contrast, rates of glucose oxidation were significantly lower in hearts from HFD obese mice vs LFD mice. Because cardiac work is an important determinant of oxidative metabolism, rates of palmitate and glucose oxidation were also normalized to cardiac work. The increase in palmitate oxidation and decrease in glucose oxidation were confirmed (Table 4-2). Since all hearts were perfused under identical conditions, the data suggests that molecular changes at the level of the heart were responsible for the shift from glucose oxidation to fatty acid oxidation in hearts from HFD obese mice.

4.3.4 Acetylation of fatty acid β-oxidation enzymes in hearts from HFD obese mice:

Because cardiac fatty acid β-oxidation rates were significantly higher in HFD obese mice, we examined whether the expression levels, activity, and acetylation state of fatty acid oxidative enzymes were altered in hearts from HFD obese mice. β-Hydroxyacyl CoA dehydrogenase (β-HAD) and long chain acyl CoA dehydrogenase (LCAD) are two enzymes

of fatty acid β-oxidation pathway that have been shown to be under acetylation control. We therefore assessed the level of acetylation of these enzymes in hearts from obese HFD fed mice. The levels of β-HAD protein were not changed in mice on a HFD or LFD (Figure 4-2A). However, the levels of β-HAD acetylation were significantly increased in hearts of HFD obese mice (Figure 4-2B) accompanied by a significant increase in β-HAD activity (Figure 4-2C).

Similar to what was observed with β-HAD, the level of LCAD protein expression was not different between hearts of HFD obese and LFD mice (Figure 4-3A). However, a significant increase in cardiac LCAD acetylation was observed in HFD obese mice (Figure 4-3B) accompanied by a significant increase in LCAD activity (Figure 4-3C). We also observed a strong positive correlation between LCAD acetylation and rate of fatty acid β-oxidation in the HFD obese and LFD mice (Figure 4-3D).

4.3.5 PGC-1α acetylation in hearts from HFD obese mice:

PGC-1α is an important transcriptional nuclear regulator of mitochondrial biogenesis and oxidative metabolism, and is also under acetylation control (173). We therefore determined if alterations in expression and acetylation of PGC-1α could contribute to the increase in fatty acid oxidation observed in the hearts of obese HFD obese mice. The amount of PCG-1α protein was significantly decreased in HFD obese mice compared to LFD controls (Figure 4-4A). Concomitantly, an increase in

overall acetylation of PGC-1α was observed, resulting in a marked increase in the Ac-PGC-1α/PGC-1α ratio (Figure 4-4B). Since acetylation of PGC-1α is believed to decrease PGC-1α transcriptional activity (173,209), the decrease in PGC-1α expression and increased acetylation are unlikely to explain the increase in fatty acid oxidation seen in the hearts from the HFD obese mice. Indeed, not only was overall expression of β-HAD or LCAD not altered (Figures 4-2A and 3B), neither was the expression of peroxisome proliferator-activated receptors alpha (PPARα) (Figure 4-4C), which is involved in the transcriptional control of fatty acid oxidative enzymes. Expression of the tricarboxylic acid (TCA cycle) enzyme, citrate synthase (CS) (Figure 4-4D) and the electron transport chain enzyme, Complex I (Figure 4-4E), were also unaffected. Combined, this suggests that the increase in fatty acid oxidation seen in hearts of HFD obese mice are not due to increases in mitochondrial biogenesis, but rather direct changes in acetylation control of fatty acid oxidation enzymes.

4.3.6 Role of acetylation in impaired cardiac insulin signaling in HFD obese mice:

As shown in Table 4-2, hearts from HFD obese mice showed a decrease in glucose oxidation that accompanied the increase in fatty acid oxidation. The ability of insulin to stimulate glucose oxidation in hearts from HFD obese mice was also markedly impaired compared to hearts from mice fed a LFD (Figure 4-5A). Pyruvate dehydrogenase (PDH), the

rate-limiting enzyme for glucose oxidation has recently been shown to be under acetylation control (59,191,192,201). We therefore determined if PDH expression and acetylation status were altered in hearts from obese HFD fed mice. Neither PDH expression (Figure 4-5B), nor the ratios of PDH acetylation/PDH expression were found to be different between hearts from obese HFD vs LFD mice (Figure 4-5C).

Akt is involved in insulin signaling, and can be modulated by phosphorylation and acetylation. SIRT1-mediated deacetylation of Akt, and its upstream activator, phosphoinositide dependent kinase 1 (PDK1) promotes Akt activation (164). Immunoblot analysis showed a decreased cardiac expression of Akt in obese HFD mice (Figure 4-6A). In contrast, the level of Akt acetylation was significantly increased in hearts from obese HFD mice (Figure 4-6B). This increase in acetylation was accompanied by a significant decrease in Akt phosphorylation (Figure 4-5C), suggesting that acetylation may contribute to the impaired insulin signaling seen in hearts from obese HFD mice (Figure 4-5A). GSK3β is a Ser/Thr protein kinase that phosphorylates and inactivates glycogen synthase (42). GSK3β itself is regulated by phosphorylation, with phosphorylation at Ser9 by Akt being inhibitory (250). GSK3β protein expression remained similar in hearts of HFD obese and LFD mice (Figure 4-6D). However, the level of GSK3β phosphorylation on Ser9 was significantly reduced, similar to what was seen with Akt phosphorylation in HFD obese mice (Figure 4-6D), and is consistent with the decrease in p-

Akt seen in hearts from HFD obese mice (Figure 4-6C). These findings are consistent with the insulin resistance observed in hearts from HFD obese mice.

4.3.7 Regulation of fatty acid β-oxidation enzymes by mitochondrial deacetylation:

Because SIRT3 protein levels were decreased in hearts from HFD-induced obese mice (Figure 4-1C), and SIRT3 has been implicated in deacetylation of fatty acid oxidative enzymes, we examined the effect of SIRT3 deletion on cardiac fatty acid β-oxidation and fatty acid oxidative enzyme acetylation status. Isolated working hearts from SIRT3 KO mice showed no differences in cardiac function compared to WT mice (Table 4-3). However, cardiac energy metabolism was significantly shifted, with a significant increase in fatty acid β-oxidation (Table 4-3, Figure 4-7B), and a decrease in glucose oxidation (Table 4-3). The increase in fatty acid oxidation and decrease in glucose oxidation remained when rates were normalized for cardiac work (Table 4-3). The increase in fatty acid β-oxidation in SIRT KO mouse hearts was accompanied by a significant increase in overall acetylation of cardiac proteins, when compared to WT mice (Figure 4-7A). We also measured the expression, acetylation and activity of fatty acid β-oxidation enzymes β-HAD and LCAD. While no changes in protein expression of β-HAD was observed, acetylation of β-HAD was significantly increased in hearts from SIRT3 KO mice in

comparison to WT mice (Figure 4-7C), which was accompanied by increase in β-HAD activity (Figure 4-7D). Similar to β-HAD, acetylation of LCAD was significantly increased in SIRT3 KO hearts without changes in protein expression (Figure 4-7E) and was associated with an increase in LCAD activity (Figure 4-7F).

4.3.8 Role of acetylation in regulation of cardiac glucose oxidation in SIRT3 KO mice:

Recent studies have implicated SIRT3 in deacetylation and inhibition of pyruvate dehydrogenase (PDH), the rate-limiting enzymes for glucose oxidation (201). In SIRT3 KO mouse hearts, insulin stimulated glucose oxidation was markedly impaired compared to hearts from WT mice (Figure 4-8A). We therefore determined if PDH acetylation was altered in hearts from SIRT3 KO mice. Neither PDH expression (Figure 4-8B), nor the ratios of PDH acetylation/PDH expression were different between hearts from SIRT3 KO versus WT mice (Figure 4-8C).

Table 4-1: Metabolic parameters of C57BL/6 mice fed a HFD for 18 weeks.

Heart weight (HW) normalized to tibia length (TL); Whole body fat mass as assessed by MRI; Oral GTT in overnight-fasted mice; Values represent mean±SEM. (n=5). *P<0.05, significantly different from LFD.

	LFD	HFD
Body weight (g)	31.2±1.2	48.3±1.51*
HW/TL (g)	8.4±0.4	10.4±0.5*
Fat mass (g)	5.8±0.32	18.3±0.61*
Percent Fat mass/ BW ratio	18±0.6	38.2±0.86*
GTT (A.U.C)	49.7±2.6	69.7±2.9*

Table 4-1

Figure 4-1: Overall cardiac protein acetylation and deacetylase expression in mice fed a high fat diet (HFD) for 16 wk.

A) Total protein acetylation in non-perfused hearts from LFD and HFD mice. **B)** Mitochondrial proteins acetylation in hearts from LFD and HFD mice. Mitochondria were isolated in the presence or absence of 500 nM TSA, and 10 mM nicotinamide. Protein expression of SIRT1 **(B)**, SIRT3 **(C)**, SIRT6 **(D)**, SIRT2 **(E)**, and GCN5L1 **(F)** in hearts from mice fed either a low fat diet (LFD) or HFD. Values represent mean ±SEM. (n=5/group). *P<0.05, significantly different from LFD.

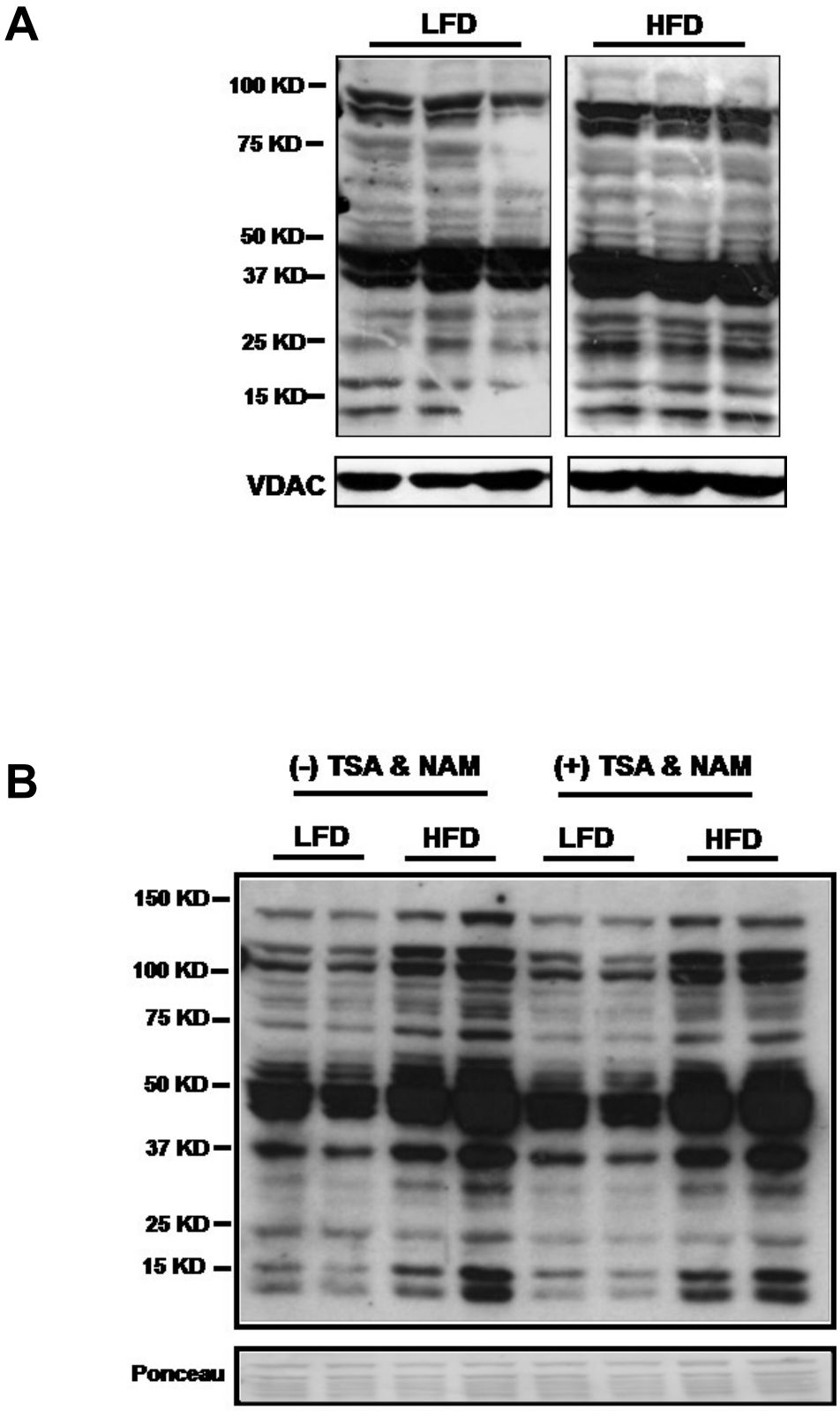
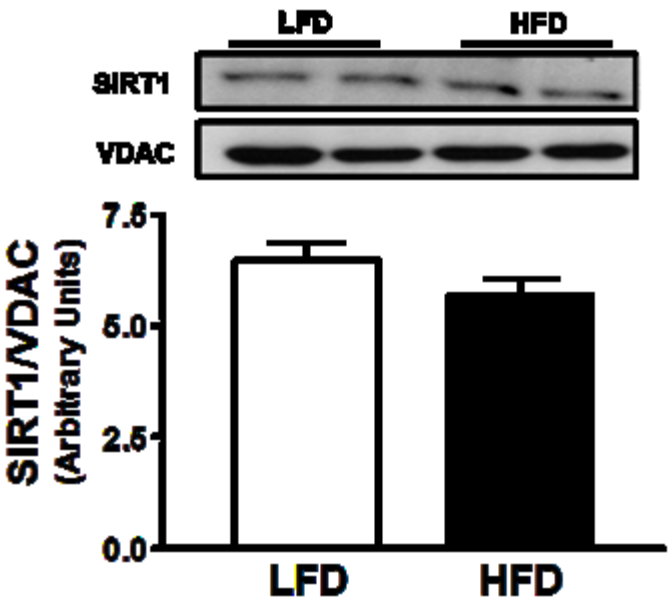

A
LFD
HFD
100 KD
75 KD
50 KD
37 KD
25 KD
15 KD
VDAC
B
(-) TSA & NAM
(+) TSA & NAM
LFD
HFD
LFD
HFD
150 KD
100 KD
75 KD
50 KD
37 KD
25 KD
15 KD
Ponceau

Figure 4-1

C


LFD
HFD
SIRT1
VDAC
SIRT1/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

D

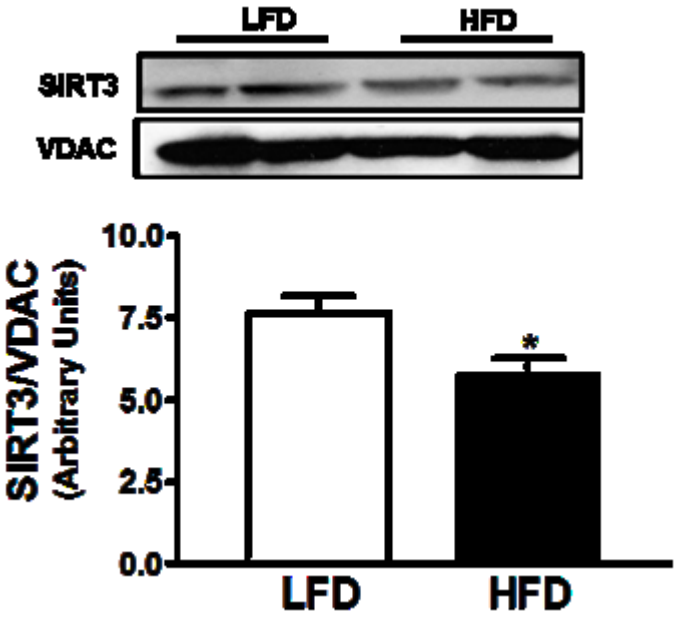
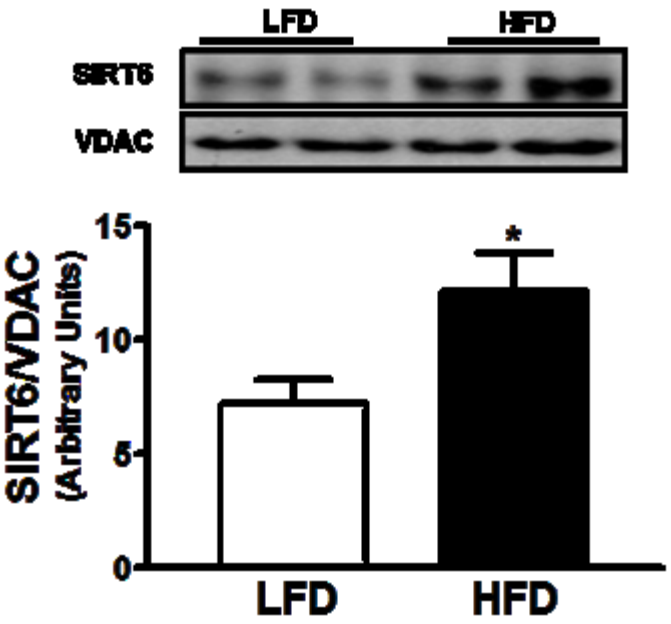

LFD
HFD
SIRT3
VDAC
SIRT3/VDAC
(Arbitrary Units)
10.0
7.5
5.0
2.5
0.0
*
LFD
HFD

Figure 4-1

E


LFD
HFD
SIRT6
VDAC
SIRT6/VDAC
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

F

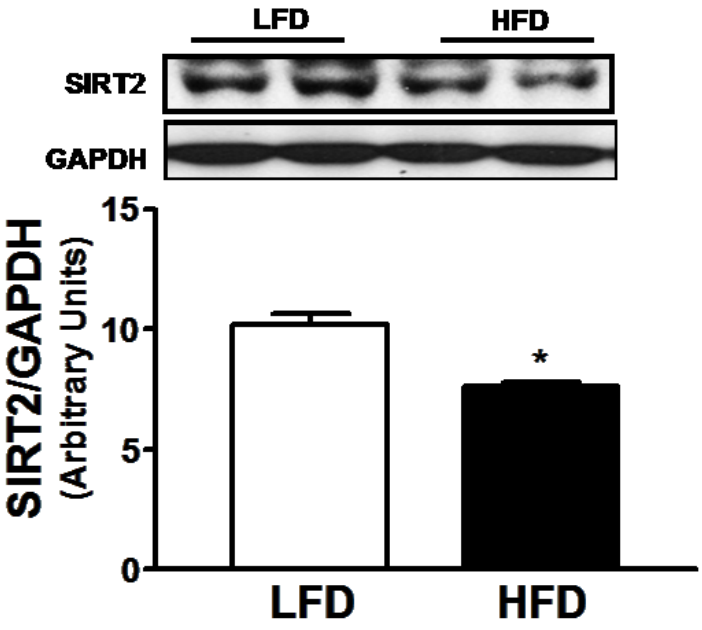

LFD
HFD
SIRT2
GAPDH
SIRT2/GAPDH
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

Figure 4-1

G

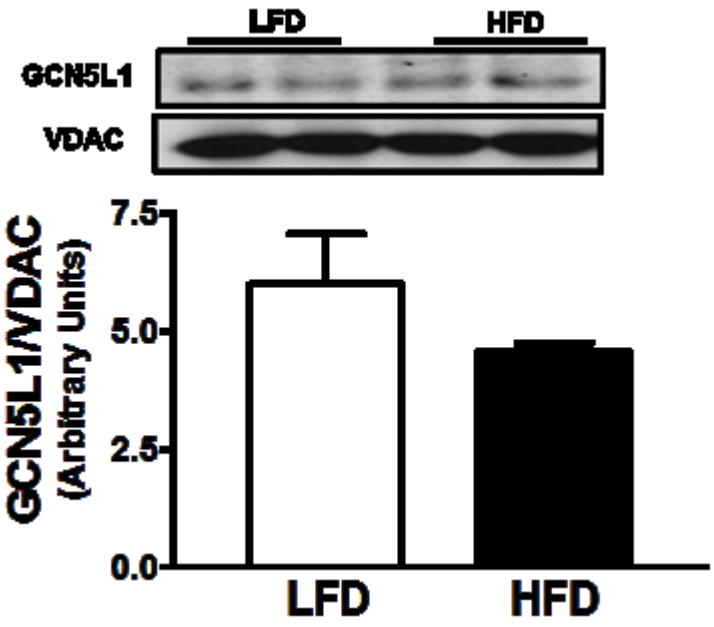

LFD
HFD
GCN5L1
VDAC
GCN5L1/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

Figure 4-1

Table 4-2: Effect of chronic HFD diet feeding on metabolic rates of palmitate and glucose oxidations in isolated working mice hearts

Isolated working hearts were perfused for a 60 min period in the presence of insulin. Rates of glucose and palmitate oxidation were determined as described in methods. Values represent mean±SEM; (n=8). *P<0.05, significantly different from LFD.

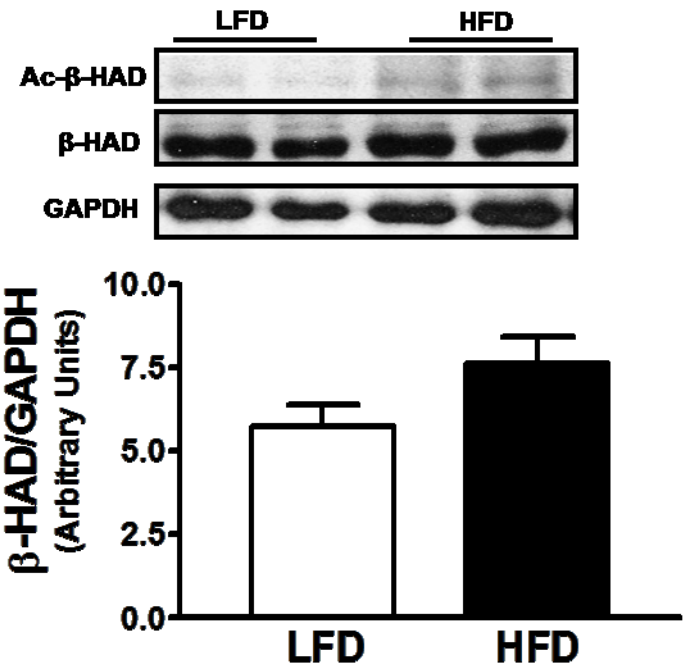
	LFD	HFD
	Absolute rates, nmol. g dry $wt^{-1}.min^{-1}$	
Palmitate Oxidation	551±87	845±76*
Glucose Oxidation	1235±160	635±146*
	Rates normalized for work differences, nmol. g dry wt^{-1}.mjoules	
Palmitate Oxidation	207±35	350±57*
Glucose Oxidation	463±55	263±65*

Table 4-2

Figure 4-2: Protein expression, acetylation, and activity of β-hydroxylacyl CoA dehydrogenase (β-HAD) in hearts from obese mice on a HFD.

A) β-HAD expression. **B)** Level of β-HAD acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for β-HAD. **C)** β-HAD enzyme activity. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

A


LFD
HFD
Ac-β-HAD
β-HAD
GAPDH
β-HAD/GAPDH
(Arbitrary Units)
10.0
7.5
5.0
2.5
0.0
LFD
HFD

B

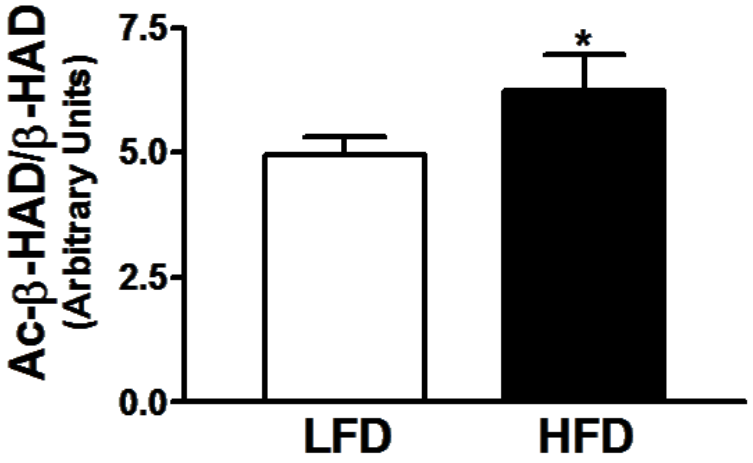

Ac-β-HAD/β-HAD
(Arbitrary Units)
7.5
5.0
2.5
0.0
*
LFD
HFD

Figure 4-2

C

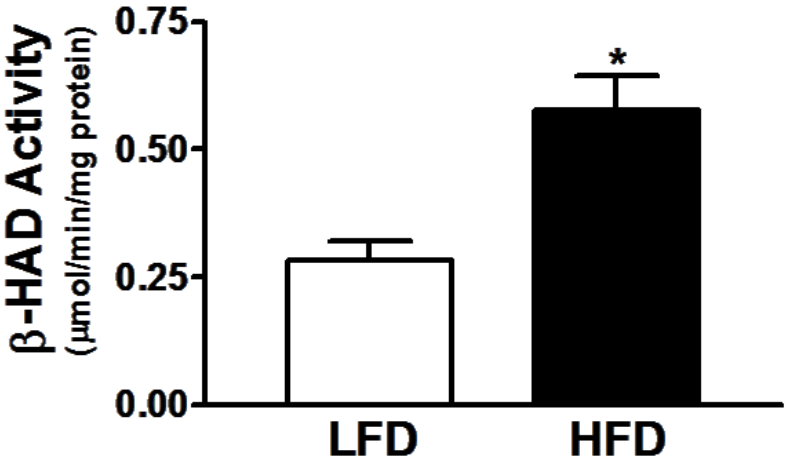
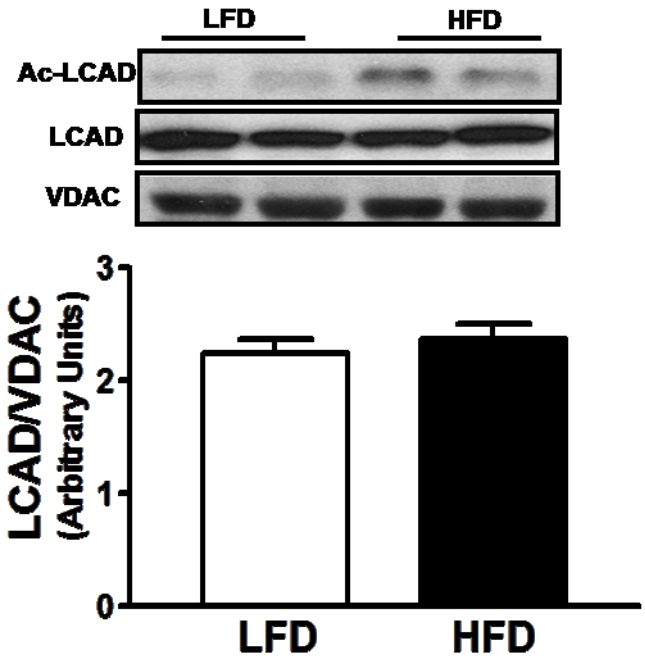


Figure 4-2

Figure 4-3: Level of long chain acyl CoA dehydrogenase (LCAD) acetylation and its correlation to rates of palmitate oxidation.

A) LCAD expression. **B)** Level of LCAD acetylation. Total lysate from isolated working hearts were immunoprecipitated with anti-acetyl-lysine antibodies and then immunoblotted with antibodies specific for LCAD. **C)** LCAD activity. **D)** Correlation curve between palmitate oxidation and LCAD acetylation. Values represent mean ± SEM. (n=5/group). *$P<0.05$, significantly different from LFD.

A


LFD
HFD
Ac-LCAD
LCAD
VDAC
LCAD/VDAC
(Arbitrary Units)
3
2
1
0
LFD
HFD

B

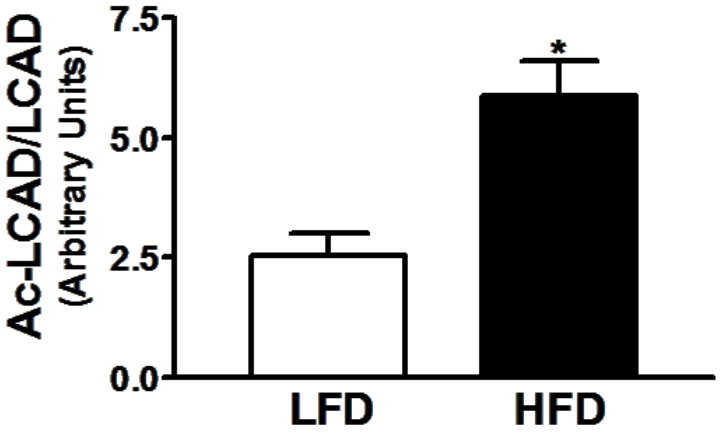
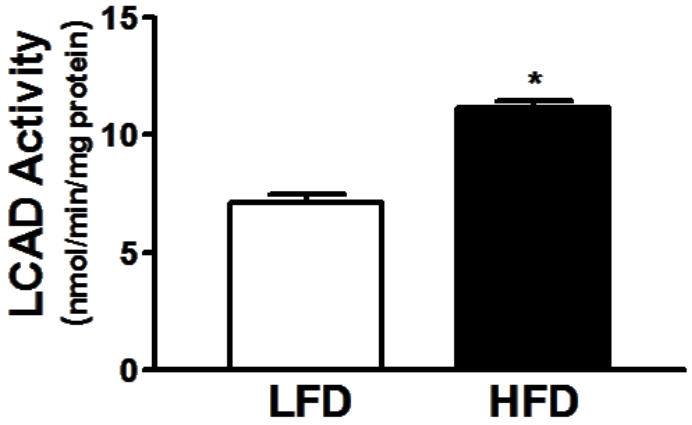

Ac-LCAD/LCAD
(Arbitrary Units)
7.5
5.0
2.5
0.0
*
LFD
HFD

Figure 4-3

C


LCAD Activity
(nmol/min/mg protein)
15
10
5
0
*
LFD
HFD

D

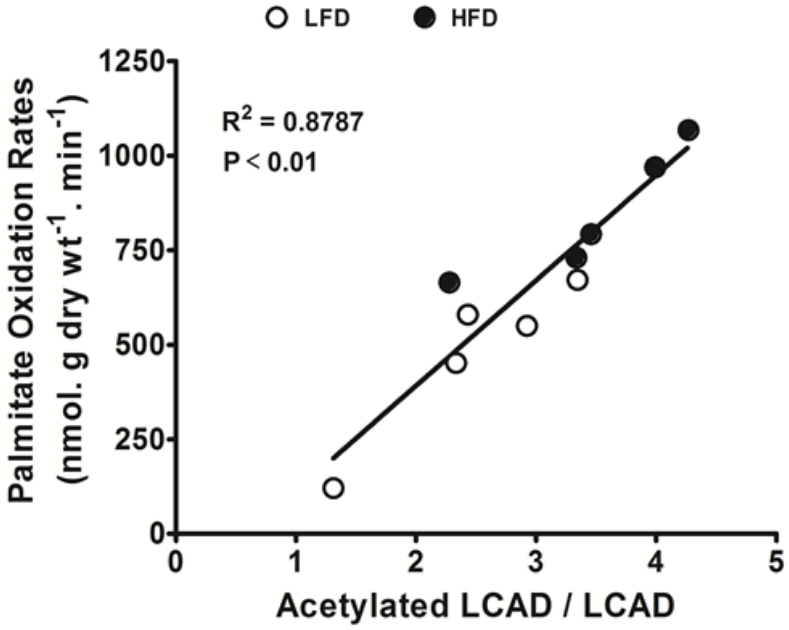
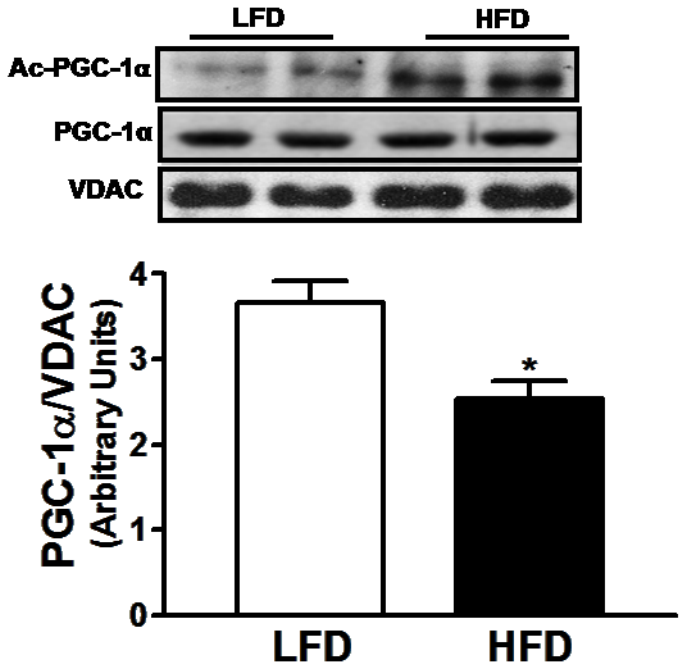

LFD
HFD
Palmitate Oxidation Rates
(nmol. g dry wt-1. min-1)
1250
1000
750
500
250
0
R2 = 0.8787
P < 0.01
0
1
2
3
4
5
Acetylated LCAD / LCAD

Figure 4-3

Figure 4-4: Level of PGC-1α acetylation and its downstream target protein expression in non-perfused hearts from obese mice on a HFD.

A) PGC-1α expression. **B)** Level of PGC-1α acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PGC-1α. Protein expression of PPARα **(C)**, CS **(D)**, and complex I **(E)** in hearts from mice fed either a LFD or HFD. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

A


LFD
HFD
Ac-PGC-1α
PGC-1α
VDAC
PGC-1α/VDAC
(Arbitrary Units)
0
1
2
3
4
*
LFD
HFD

B

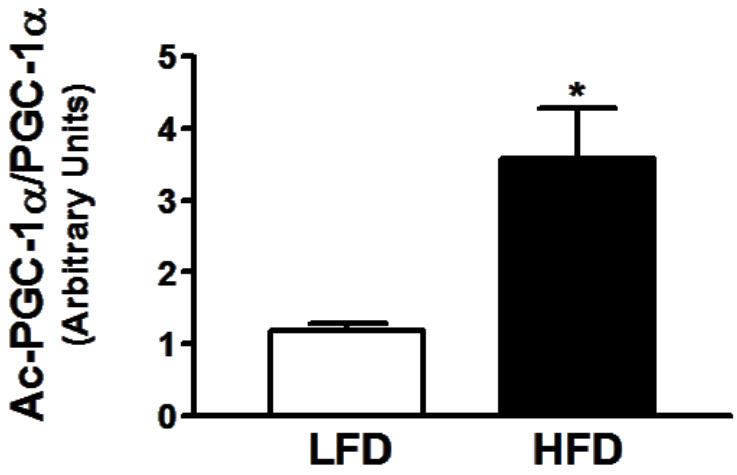
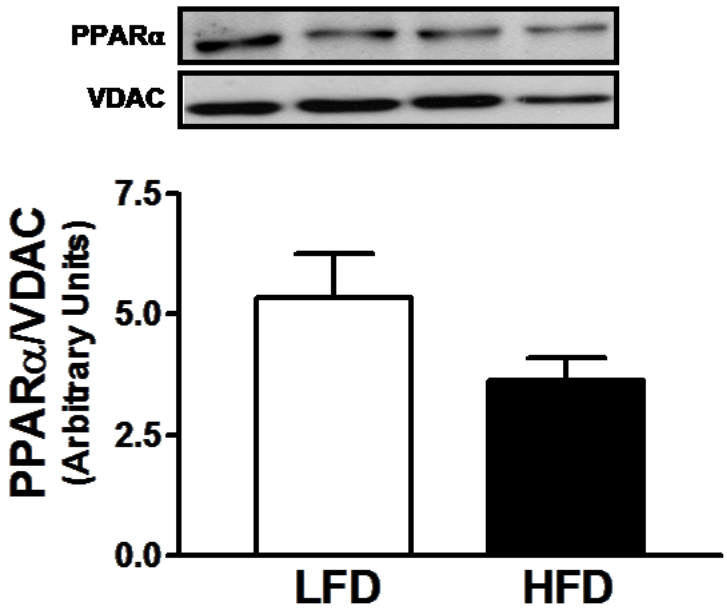

Ac-PGC-1α/PGC-1α
(Arbitrary Units)
0
1
2
3
4
5
*
LFD
HFD

Figure 4-4

C


PPARα
VDAC
PPARα/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

D

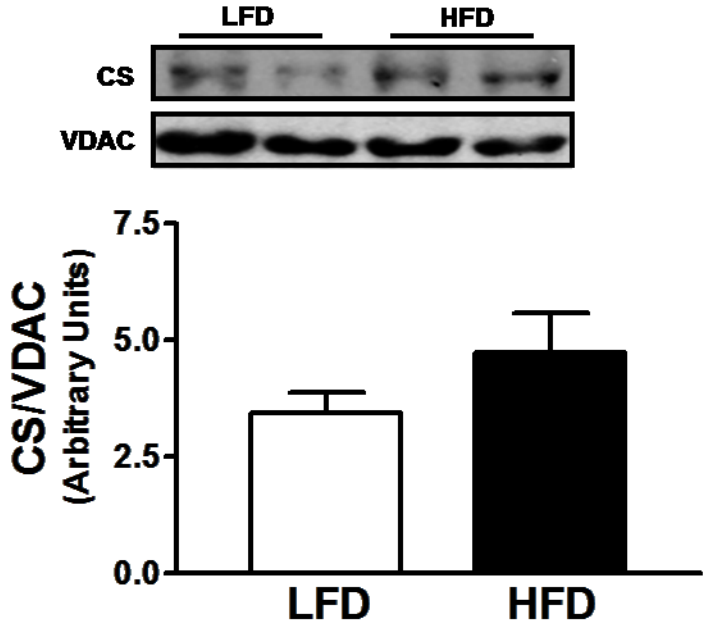

LFD
HFD
CS
VDAC
CS/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

Figure 4-4

E

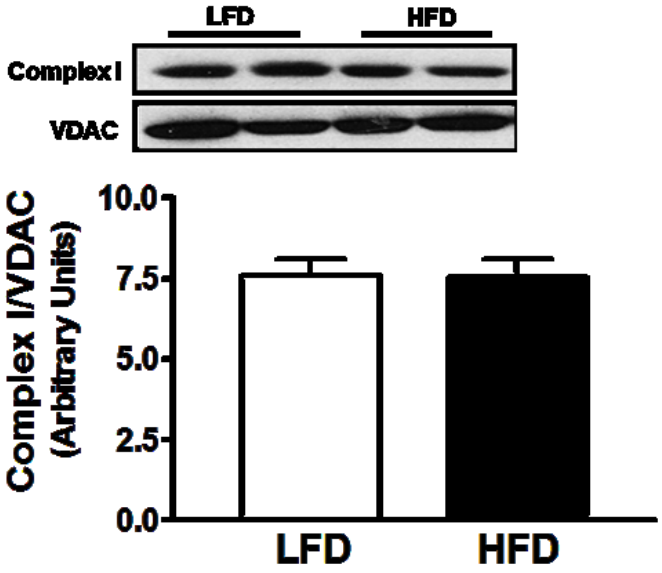
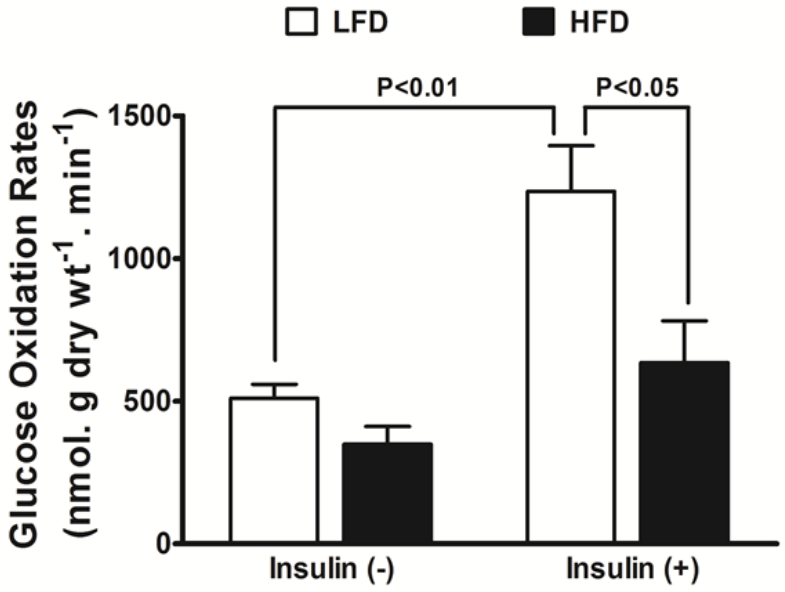

LFD
HFD
Complex I
VDAC
Complex I/VDAC
(Arbitrary Units)
10.0
7.5
5.0
2.5
0.0
LFD
HFD

Figure 4-4

Figure 4-5: Glucose oxidation rates and PDH acetylation levels in hearts from obese mice on a HFD.

A) Glucose oxidation rates in hearts from mice fed either a LFD or HFD, in the presence (+) or absence (-) of insulin. **B)** PDH expression. **C)** Level of PDH acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-lysine antibodies and then immunoblotted with antibodies specific for PDH. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

A


LFD
HFD
P<0.01
P<0.05
Glucose Oxidation Rates
(nmol. g dry wt-1. min-1)
1500
1000
500
0
Insulin (-)
Insulin (+)

B

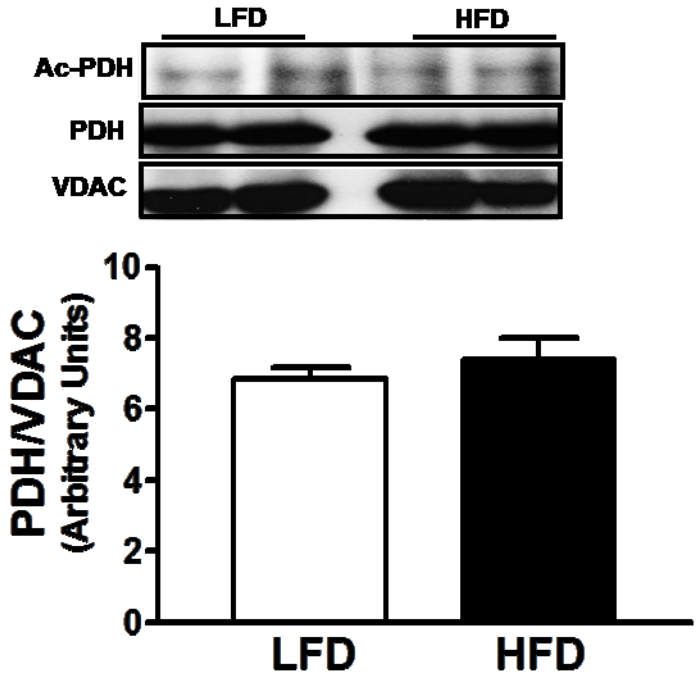

LFD
HFD
Ac-PDH
PDH
VDAC
PDH/VDAC
(Arbitrary Units)
10
8
6
4
2
0
LFD
HFD

Figure 4-5

C

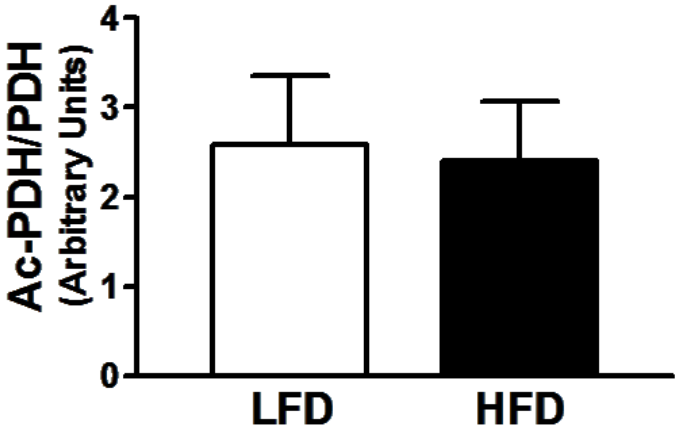
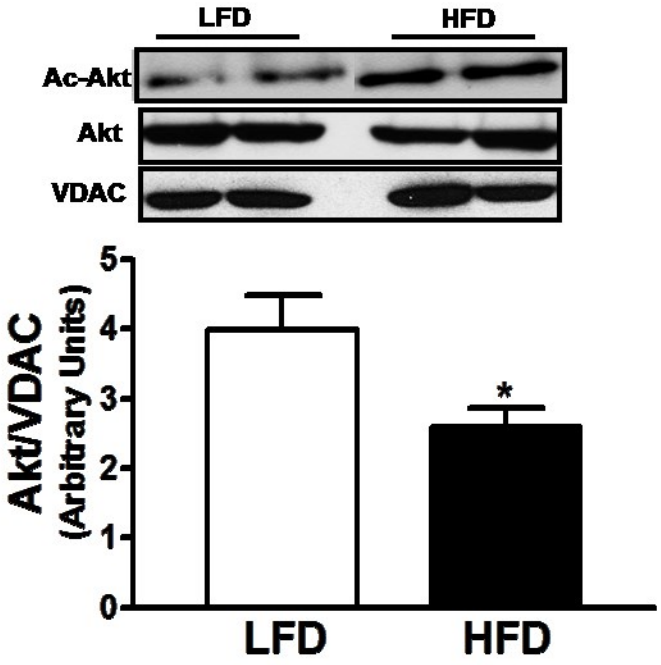


Figure 4-5

Figure 4-6: Effect of chronic HFD feeding on Akt acetylation and activity.

A) Akt expression. **B)** Level of Akt acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for Akt. **C)** Level of Akt phosphorylation at Ser407 residue. **D)** Level of GSK3β phosphorylation at Ser9 residue. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

A


LFD
HFD
Ac-Akt
Akt
VDAC
Akt/VDAC
(Arbitrary Units)
5
4
3
2
1
0
*
LFD
HFD

B

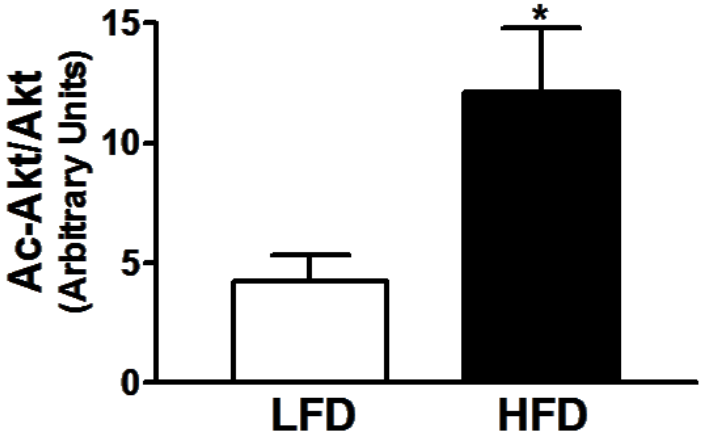
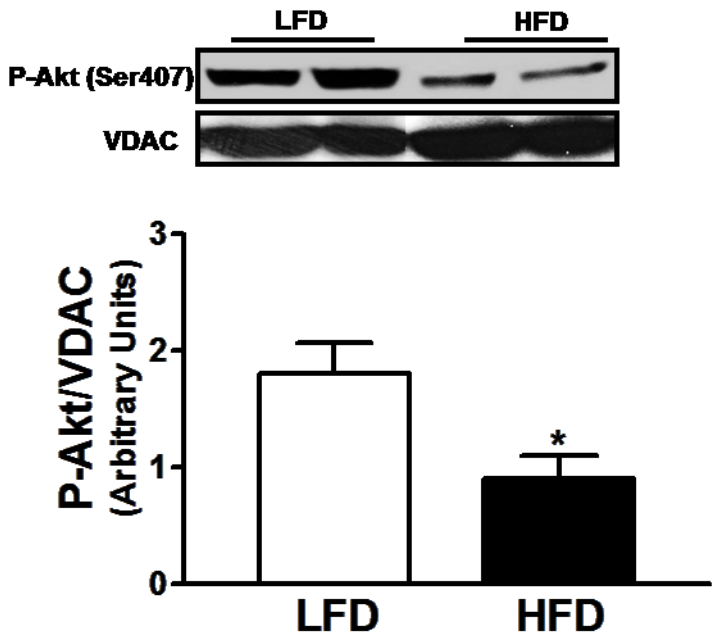

Ac-Akt/Akt
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

Figure 4-6

C


LFD
HFD
P-Akt (Ser407)
VDAC
P-Akt/VDAC
(Arbitrary Units)
3
2
1
0
*
LFD
HFD

D

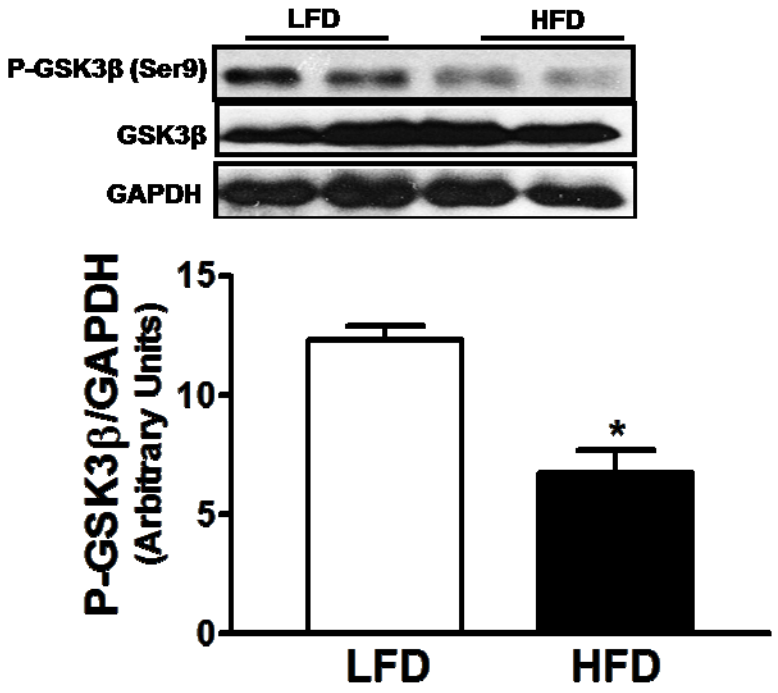

LFD
HFD
P-GSK3β (Ser9)
GSK3β
GAPDH
P-GSK3β/GAPDH
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

Figure 4-6

Table 4-3: Effects of SIRT3 deletion on cardiac function and cardiac metabolic rates

Isolated working hearts from wild type (WT) and SIRT3 KO mice were perfused for a 60 min period for measurement of cardiac function, palmitate oxidation, and glucose oxidation. Rates of glucose and palmitate oxidation were determined as described in experimental methods. Values represent mean±SEM. *$P<0.05$, significantly different from WT.

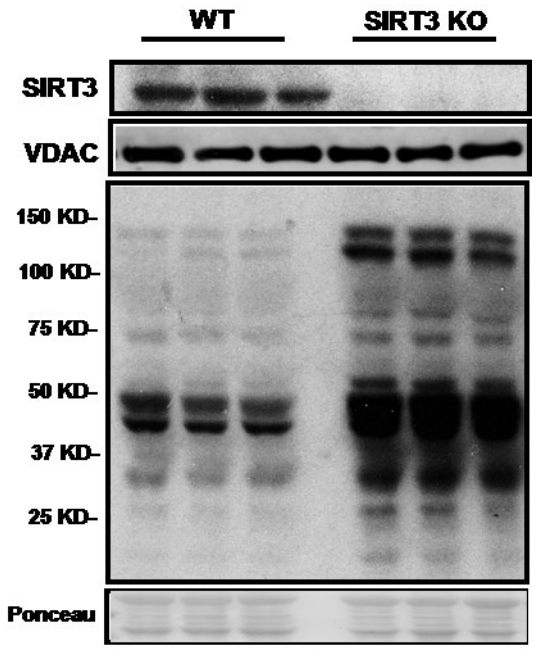
	WT n=8	SIRT3 KO n=8
	Cardiac function	
Heart rate (beat/min)	313±4	272±15
Cardiac output (ml/min)	9.6±0.4	8.7±0.5
Cardiac work (joules/min/g dry wt)	1.8±0.1	1.8±0.1
	Oxidative rates *(nmol. g dry wt^{-1}.min^{-1})*	
Palmitate Oxidation	291±17	422±29*
Glucose Oxidation	1983±174	1262±121*
	Oxidative rates normalized for work *(nmol. g dry wt^{-1}.mjoules)*	
Palmitate Oxidation	158±16	240±32*
Glucose Oxidation	1100±99	712±62*

Table 4-3

Figure 4-7: Effects of SIRT3 deletion on cardiac fatty acid β-oxidation.

A) Total protein acetylation in isolated working hearts from WT and SIRT3 KO mice. **B)** Palmitate oxidation rates. **C)** Acetylated β-HAD (Ac-β-HAD) normalized to its level of expression. **D)** β-HAD enzyme activity. **E)** Acetylated LCAD (Ac-LCAD) normalized to its level of expression. **F)** LCAD activity. Values represent mean ± SEM. (n=7/group). *P<0.05, significantly different from WT.

A


WT
SIRT3 KO
SIRT3
VDAC
150 KD-
100 KD-
75 KD-
50 KD-
37 KD-
25 KD-
Ponceau

B

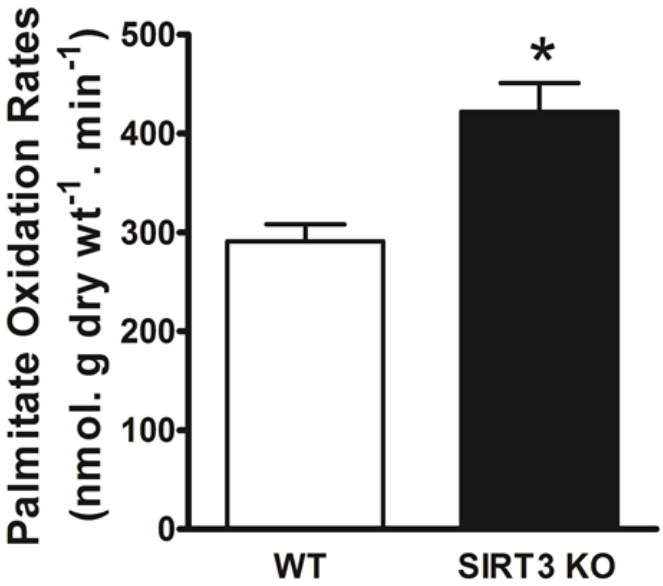
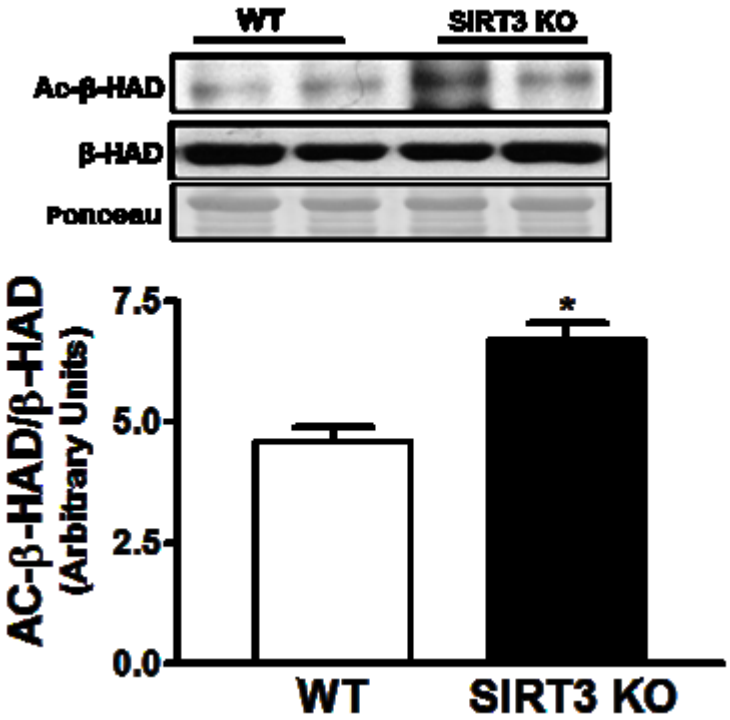

Palmitate Oxidation Rates
(nmol. g dry wt-1. min-1)
500
400
300
200
100
0
*
WT
SIRT3 KO

Figure 4-7

C


WT
SIRT3 KO
Ac-β-HAD
β-HAD
Ponceau
AC-β-HAD/β-HAD
(Arbitrary Units)
7.5
5.0
2.5
0.0
*
WT
SIRT3 KO

D

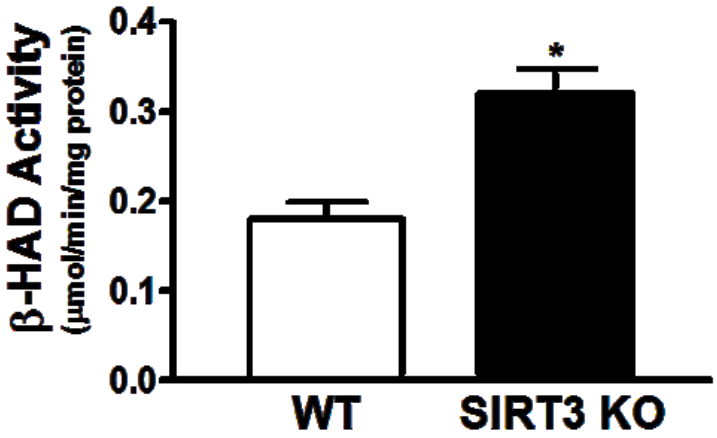
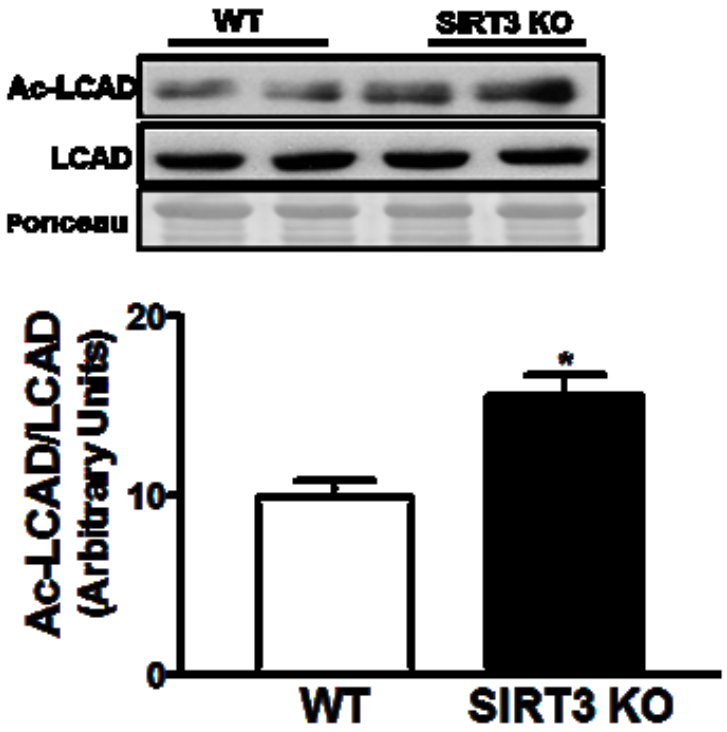

β-HAD Activity
(µmol/min/mg protein)
0.4
0.3
0.2
0.1
0.0
*
WT
SIRT3 KO

Figure 4-7

E


WT
SIRT3 KO
Ac-LCAD
LCAD
Ponceau
Ac-LCAD/LCAD
(Arbitrary Units)
20
10
0
*
WT
SIRT3 KO

F

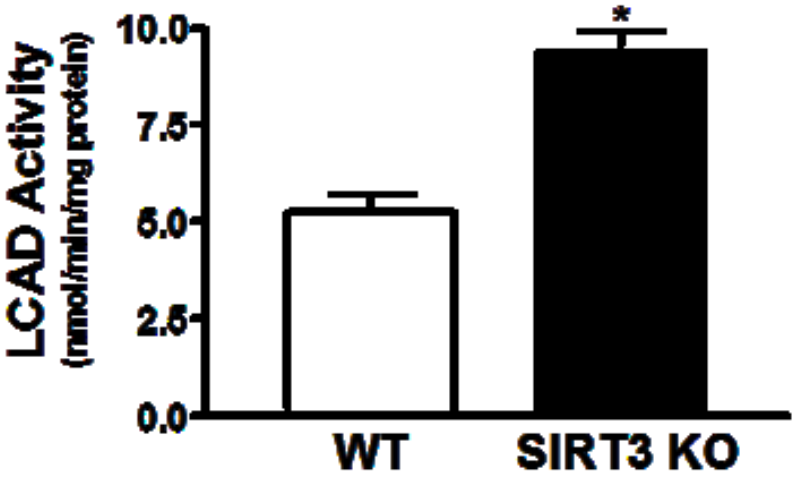
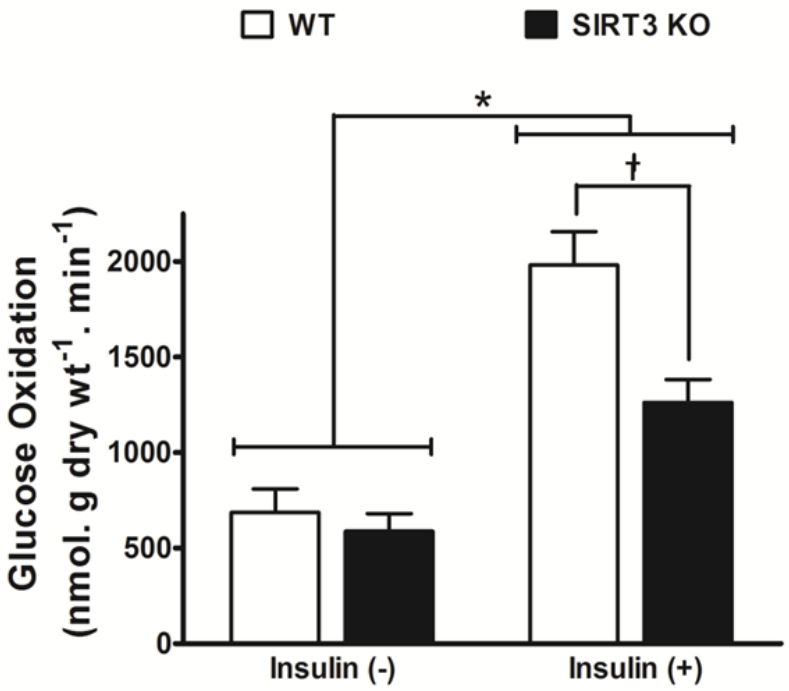

LCAD Activity
(nmol/min/mg protein)
10.0
7.5
5.0
2.5
0.0
*
WT
SIRT3 KO

Figure 4-7

Figure 4-8: Glucose oxidation rates and pyruvate dehydrogenase (PDH) acetylation levels in hearts from SIRT3 deficient mice.

A) Glucose oxidation rates in isolated working hearts from WT or SIRT3 KO mice, in the presence (+) or absence (-) of insulin. **B)** PDH expression. **C)** Level of PDH acetylation. Total lysate from perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PDH. Values represent mean ± SEM. (n=7/group). *P<0.05, significantly different from same group in response to inulin.

A


WT
SIRT3 KO
*
†
Glucose Oxidation
(nmol. g dry wt-1 . min-1)
2000
1500
1000
500
0
Insulin (-)
Insulin (+)

B

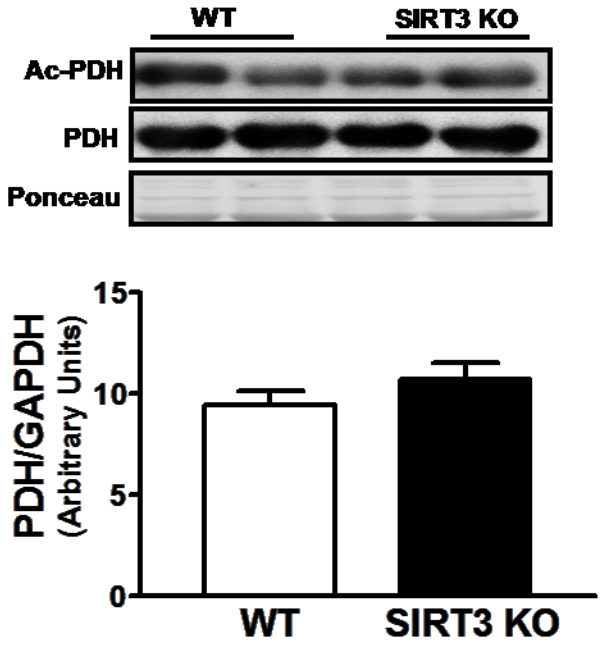

WT
SIRT3 KO
Ac-PDH
PDH
Ponceau
PDH/GAPDH
(Arbitrary Units)
15
10
5
0
WT
SIRT3 KO

Figure 4-8

C

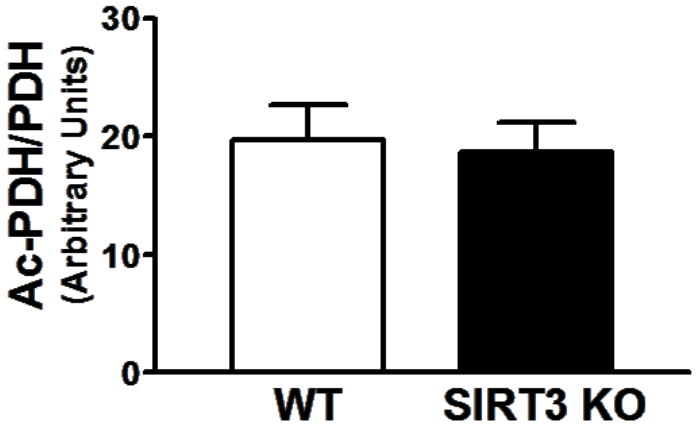


Figure 4-8

Figure 4-9: Model for the role of SIRT3 in regulation of cardiac energy metabolism.

A) In normal condition, cardiac SIRT3 expression is abundant and leads to deacetylation of the fatty acid β-oxidation enzymes, β-HAD and LCAD. This is associated with phosphorylation of Akt and GSK3β resulting in enhanced insulin sensitivity. **B)** In contrast, decreased cardiac SIRT3 expression by high fat feeding or genetic deletion leads to hyperacetylation and activation of β-HAD and LCAD which results in increased fatty acid β-oxidation. Increased acetylation of Akt inhibits its ability to phosphorylate GSK3β and impairs insulin sensitivity.

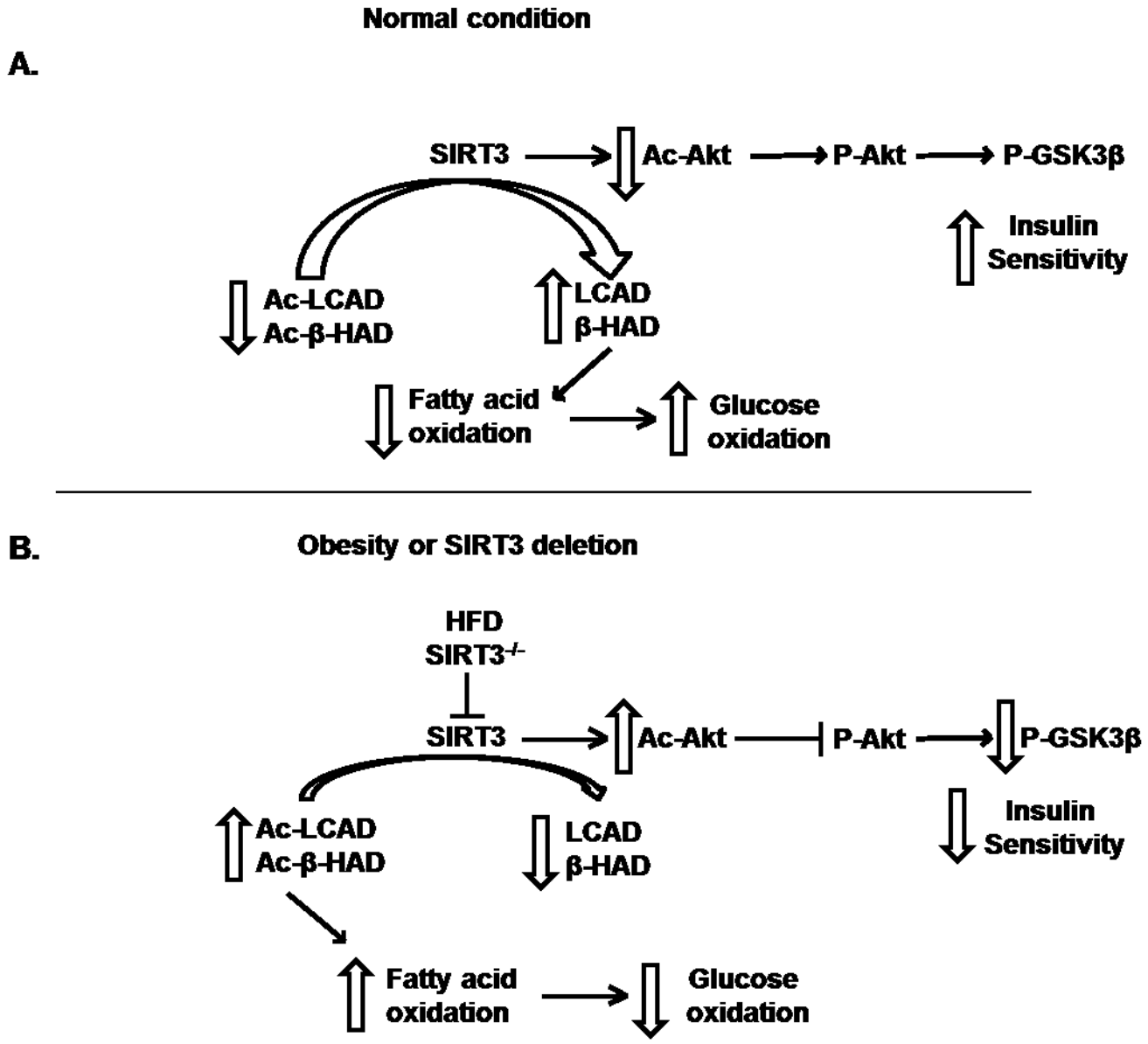

Normal condition
A.
SIRT3
Ac-Akt
P-Akt
P-GSK3β
Insulin Sensitivity
Ac-LCAD
Ac-β-HAD
LCAD
β-HAD
Fatty acid oxidation
Glucose oxidation
B.
Obesity or SIRT3 deletion
HFD
SIRT3-/-
SIRT3
Ac-Akt
P-Akt
P-GSK3β
Insulin Sensitivity
Ac-LCAD
Ac-β-HAD
LCAD
β-HAD
Fatty acid oxidation
Glucose oxidation

Figure 4-9

4.4 DISCUSSION

This study identified a number of key roles for lysine acetylation in the control of cardiac energy metabolism and insulin signaling. First, obesity is associated with an overall increase in acetylation of cardiac proteins, with a concomitant decrease in SIRT3 expression. Second, the increase in fatty acid oxidation rates observed in hearts from obese mice is accompanied by an increased acetylation and activity of the fatty acid oxidation enzymes LCAD and β-HAD, demonstrating that the acetylation of these enzymes increases rather than decreases flux through the fatty acid oxidative pathway. Third, the marked cardiac insulin resistance observed in hearts from obese mice is associated with an increased acetylation and decreased phosphorylation/activity of Akt. Fourth, SIRT3 deletion increases cardiac fatty acid oxidation, which is accompanied by a significant decrease in glucose oxidation. Finally, SIRT3 deletion induces hyperacetylation and activation of the fatty acid β-oxidation enzymes, β-HAD and LCAD. Collectively, these data demonstrate that lysine acetylation has an important role in regulating fatty acid β-oxidation in the heart and that increased acetylation of fatty acid oxidative enzymes contributes to the excessive reliance of the heart on fatty acid β-oxidation in obesity.

Post-translational lysine acetylation has emerged as a potentially important mechanism for controlling many energetic pathways (165,200).

Lysine acetylation occurs on a large number of enzymes involved in mitochondrial metabolism and glycolysis (191,192). Mitochondrial metabolism, is regulated by lysine acetylation, with SIRT3 being an important enzyme involved in the mitochondrial deacetylation process (194). A key role for SIRT3 in mitochondrial electron transport chain activity and TCA cycle activity is evident. For instance, complex I and complex II in the respiratory chain are hyperacetylated and inactivated in SIRT3$^{-/-}$ mice (204,205). In addition, SIRT3 deacetylates and activates TCA cycle enzymes, such as isocitrate dehydrogenase, during caloric restriction (199). Another important target of SIRT3 is the fatty acid β-oxidation pathway, with both LCAD and β-HAD being activated by acetylation. However, conflicting results have been reported that this post translational modification inhibits enzyme activity (196,200,206). The increase in overall acetylation and decrease in SIRT3 we observed in obese mice hearts is also consistent with other studies. Chronic HFD feeding and diabetes, both of which are associated with high fatty acid oxidation rates (61,88,207,243), lead to a decrease in SIRT3 protein expression (11, 24, 41) a result also seen in our study. Overall protein acetylation is also increased in livers from mice fed a HFD (196,197). Recently, lysines 318/322 located near the active site of LCAD were identified as a SIRT3 target (206). Interestingly, we show that LCAD is hyperacetylated and activated in hearts from HFD fed mice. Because fatty acid β-oxidation is enhanced in hearts from mice subjected to HFD, our

result suggest that acetylation of LCAD correlates positively with an increase, rather than a decrease, in fatty acid oxidation. In addition, the acetylation and activity of β-HAD, another enzyme of the fatty acid β-oxidation pathway is also enhanced in HFD obese mice. Collectively, these results suggest a stimulatory effect of lysine acetylation on enzymes involved in fatty acid β-oxidation.

Elevation in circulating levels of free fatty acids and triacylglycerol seen in obesity contributes to an increase in myocardial fatty acid uptake and fatty acid β-oxidation, which can potentially lead to the accumulation of lipid metabolites that perturbs insulin signaling (89). Furthermore, high fatty acid β-oxidation rates can decrease cardiac efficiency, and therefore could have important consequences in insulin-resistant hearts, as well as in the setting of obesity where the overall rates of fatty acid utilization are markedly increased (58,98,243). Our data implicates post-translational regulation of fatty acid oxidative enzymes as a contributing factor to the high fatty acid β-oxidation rates in obesity. As a result, decreasing acetylation status of fatty acid oxidative enzymes has potential therapeutic approach to decreasing fatty acid oxidation in obesity and diabetes. In support of this, we demonstrate that increasing acetylation via deletion of SIRT3 does increase fatty acid β-oxidation in the heart at the expense of glucose oxidation. While this was not associated with any functional deficit in the heart, it is possible that in times of stress, this increase in fatty acid oxidation may contribute to cardiac pathology. It is clear that

excessive fatty acid β-oxidation, which inhibits glucose oxidation, can contribute to cardiovascular disease and induce cardiac insulin resistance (1,2,59,88,89,207). In support of this, previous studies have shown that increasing SIRT3 can decrease cardiac hypertrophy and protect hearts from oxidative stress injury (198,225).

GCN5L1 as a component of the mitochondrial acetyltransferase machinery plays an important role in modulating the acetylation levels of various mitochondrial enzymes. In our study, GCN5L1 protein expression was unaltered by the type of diet. This result suggests that decreased activity of the mitochondrial deacetylase, SIRT3, is primarily responsible for the increased mitochondrial enzyme acetylation under HFD feeding conditions, including LCAD and β-HAD acetylation (159,194,200). Our data provides evidence that HFD feeding decreases SIRT3 expression and enhances acetylation and activity of enzymes involved in fatty acid oxidation.

Previous studies have shown that acetylation of PGC-1α represses its ability to function as a transcriptional coactivator (140,171,178). We found that PGC-1α was robustly acetylated in obese HFD fed mice. This enhanced acetylation of PGC-1α could have potentially contributed to impaired mitochondrial biogenesis and dysregulation of mitochondrial function seen in obesity (173,251). While SIRT1 is a key activator of PGC-1α (171), we did not find any changes in SIRT1 expression between HFD and LFD mice experimental groups. Previous study has reported that

SIRT1 is not required for PGC-1α deacetylation and importantly that GCN5 acetyltransferase activity is the key regulator of PGC-1α acetylation (252). Interestingly, we found a significant increase in SIRT6 expression in obese HFD mice. Although not detected in the current study, recent results show that SIRT6 can deacetylate and activate GCN5 thereby increasing PGC-1α acetylation (179). Thus; this raises the possibility that SIRT6 can deacetylate and activate the nuclear acetyltransferase GCN5 and hence increase PGC-1α acetylation in hearts from HFD fed mice. Interestingly, SIRT6 is able to remove long chain fatty acyl groups from lysine residues (253). Thus, SIRT6-mediated deacylation of enzymes involved in metabolism may represent a potential post-translational regulation of enzyme activities and this process warrants further investigation.

As shown in this study, and as reported previously (1,61,207,88,254), glucose oxidation rates are decreased in hearts from obese mice. Recent studies have shown that the rate-limiting enzyme for glucose oxidation, PDH, is also under acetylation control. We therefore determined whether PDH acetylation was altered in hearts of obese HFD fed mice. We found that the acetylation level of PDH was not altered by a HFD diet. Despite this finding, glucose oxidation rates were markedly decreased in mice fed a HFD compared to LFD fed mice. These results suggest that mechanisms other than PDH acetylation primarily regulate PDH activity under HFD feeding conditions. The inverse relation between

fatty acid and glucose oxidation as described by Randle *et al* at 1963, could be the main determinant for the low glucose oxidation seen in hearts from HFD fed mice. Increases in fatty acid β-oxidation will directly inhibit glucose oxidation in hearts from HFD mice. Moreover, up-regulation of pyruvate dehydrogenase kinase 4 results in hyper-phosphorylation and inactivation of PDH in HFD fed mice (207).

HFD induced obesity contributes to the development of myocardial insulin resistance and dysfunction (61,88). Previous studies by us and others suggest that accumulation of lipid intermediates, such as diacylglycerol and ceramide, enhance the activity of protein kinases that can phosphorylate and inactivate Akt (61,249,255). In this study, we also provide data potentially linking alterations in acetylation to Akt inactivation in hearts from HFD fed mice. The level of Akt acetylation was increased in hearts from HFD obese mice. Recent finding showed that deacetylation of Akt is a prerequisite for its activation, and that activation of SIRT1 will enhance insulin signaling in obesity and diabetes (164-166,244). In addition, SIRT2 overexpression leads to deacetylation and activation of Akt in hepatic cells (256). Hyperacetylation blocked Akt phosphorylation in hearts from HFD mice. To our knowledge, this is the first report demonstrating that HFD induces Akt acetylation and inactivation, which can subsequently lead in part to cardiac insulin resistance.

Cardiac fatty acid β-oxidation rates were significantly increased in SIRT3 KO compared to WT. These results are consistent with a recent

study that reported an increase in palmitate oxidation in skeletal muscles of SIRT3 KO mice compared to WT mice (201). This study suggested that hyperacetylation and inactivation of PDH induces a switch in skeletal muscle energy substrate utilization from glucose to fatty acids (201). However, we did not observe changes in acetylation of PDH. On the other hand, our study suggests that deletion of SIRT3 leads to hyperacetylation and activation of β-HAD and LCAD, which increases cardiac fatty acid oxidation and that occurred primarily at the expense of glucose oxidation (Randle cycle). In support of this, deletion of SIRT4 (which is another mitochondrial deacetylase) was recently shown to increase lipid oxidation in liver and skeletal muscles (216,217). These results challenged the notion that acetylation is always a signal of inhibition.

Our study implicates alterations in enzyme acetylation as having important effects on cardiac energy metabolism. A limitation of our study is that we did not use isolated heart mitochondria to assess acetylation level of enzymes localized in mitochondria, such as β-HAD, LCAD, and PDH. However, mitochondrial isolation takes several hours to be completed. During this time, changes in acetylation/deacetylation of proteins may occur. Unlike phosphatase and kinase inhibitors that can be added during mitochondrial isolation, acetylase inhibitors are not yet available. Therefore, we chose to perform our experiments on whole heart lysates, to limit isolation time. It is important to emphasize, however, that our results

from frozen heart tissue mimicked what was previously found in mitochondrial preparations of liver tissue (196,197,200).

In summary, a decrease in SIRT3 seen following chronic HFD feeding or SIRT3 deletion enhances acetylation of the fatty acid oxidation enzymes, β-HAD and LCAD (Figure 9). This induces a switch in cardiac energy substrate utilization from glucose to fatty acid oxidation. Our results offer new insights into the role of acetylation in regulating cardiac energy metabolism.