Obesity-induced lysine acetylation increases cardiac fatty acid oxidation and impairs insulin signalling

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1. Introduction

Fatty acid β-oxidation is a major energy source for the adult heart.¹ However, the presence of obesity, diabetes, and heart failure result in an increased reliance of the heart on fatty acid oxidation as a source of energy, which can decrease cardiac efficiency and compromise cardiac function.² 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.³ 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 oxidation and impairs insulin signalling.⁴–⁹ This post-translational modification is controlled, in part, by sirtuins (SIRTs), which are nicotinamide adenine dinucleotide-dependent deacetylases that function as longevity genes and have recently emerged as regulators of aging and disease. These enzymes function as a series of related proteins that influence a number of biological processes including cell growth, proliferation, and death. Lysine acetylation increases cardiac fatty acid oxidation in response to high-fat feeding is controlled, in part, by sirtuins (SIRTs), which are nicotinamide adenine dinucleotide-dependent deacetylases that function as longevity genes and have recently emerged as regulators of aging and disease. These enzymes function as a series of related proteins that influence a number of biological processes including cell growth, proliferation, and death.

Keywords

Lysine acetylation  •  Long-chain acyl-CoA dehydrogenase  •  B-hydroxyacyl-CoA dehydrogenase  •  Sirtuin 3  •  Glucose oxidation  •  Akt  •  Obesity
adenine dinucleotide (NAD\(^+\))-dependent deacetylases localized in distinct subcellular compartments.\(^{10,11}\) Three of the SIRT isoforms (SIRT1, SIRT6, and SIRT7) are localized in the nucleus,\(^{12}\) 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-1\(\alpha\)), a transcriptional regulator of genes that encode enzymes involved in fatty acid oxidation. Previous studies showed that acetylation of PGC-1\(\alpha\) by the nuclear acetyltransferase general control of amino acid synthesis 5 (GCN5) results in an inhibition of PGC-1\(\alpha\) transcriptional activity.\(^{7,13}\) Interestingly, SIRT6 can deacetylate and activate GCN5, thereby increasing PGC-1\(\alpha\) acetylation.\(^{14}\) On the other hand, SIRT1 deacetylates and activates PGC-1\(\alpha\).\(^{15,16}\)

In mitochondria, SIRT3 appears to be the major deacetylase\(^{17,18}\) and targets numerous enzymes involved in energy metabolism, including key enzymes involved in fatty acid oxidation such as long-chain acyl-CoA dehydrogenase (LCAD) and \(\beta\)-hydroxyacyl-CoA dehydrogenase (\(\beta\)-HAD).\(^{4,5,18,20}\) However, conflicting information has emerged regarding the role of deacetylation in controlling fatty acid oxidation. Hirschey et al.\(^{5,19}\) proposed that deacetylation of LCAD by SIRT3 accelerates fatty acid oxidation in the liver. Furthermore, chemical acetylation of LCAD on lysines 318/322 reduced enzymatic activity, while deacetylation with recombinant SIRT3 restored catalytic activity.\(^{21}\) In contrast, Zhao et al.\(^{6}\) showed that acetylation of \(\beta\)-HAD results in an activation of enzyme activity in muscle cells. Mitochondria from hindlimb muscles of fasted mice were also shown to have an increased acetylation that was associated with an increase in fatty acid oxidation rates.\(^{22}\) Diaphragm muscle of SIRT3 knockout (KO) mice also showed hyperacetylation and increased fatty acid oxidation rates.\(^{22}\) Furthermore, LCAD is hyperacetylated in obese dam offsprings concomitant with a significant decrease in SIRT3 expression and activity.\(^{23}\) 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.\(^{24}\) 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 signalling.\(^{25,26}\) In the heart, insulin-stimulated glucose oxidation is dramatically decreased in high-fat diet (HFD)-induced obesity\(^{27}\) Disruption of cardiac insulin signalling by inhibiting protein phosphorylation along the insulin/Akt (protein kinase B)/glycogen synthase kinase 3-\(\beta\) (GSK3\(\beta\)) axis can lead to a reduced cardiac glucose use.\(^{27,28}\) However, in addition to inhibition of Akt phosphorylation, acetylation may play an important role in the initiation of insulin signalling. A recent study by Gupta and colleagues\(^{29}\) identified lysine acetylation as an important regulator of Akt activity during cardiac hypertrophy. Despite this, the role of lysine acetylation in regulating cardiac insulin signalling in obesity has not yet been defined. The aim of this study was 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 signalling.

2. Methods

2.1 Animals

Mice received care and treatment 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. C57BL/6 male mice at 8 weeks of age were fed either a HFD (60% calories from fat, Research Diets, Inc., New Brunswick, NJ, USA) or a low-fat diet (LFD; 12% calories from fat, PiccoLab Laboratory Rodent Diet, 500D) for a 16- or 18-week period. Wild-type (WT, 129/Sv) and SIRT3 KO mice aged 8 weeks were obtained from Jackson Laboratories (Bar Harbor, ME, USA) from colonies maintained by Dr Mahesh Gupta (University of Chicago) or Dr Evangelos Michelakis (University of Alberta). WT and SIRT3 KO animals were fed a diet in which 12% calories were obtained from fat.

2.2 Oral glucose tolerance test

Following an overnight fast, mice were subjected to an oral gavage with glucose (2 g/kg body weight).\(^{30}\) Glucose levels were then assessed at times 0, 30, 60, and 120 min from tail vein blood samples using Accu-check Aviva glucometer.

2.3 Magnetic resonance imaging

Body composition of fat mass in mice was analysed after 18 weeks of the feeding protocol using the EchoMRI QMNR 4-in-1 Whole Body Composition Analyzer, Echo Medical Systems.

2.4 Isolated working heart perfusions

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-14C]glucose, and 0.8 mM [9,10-3H] palmitate prebound to 3% albumin, in the presence or absence of 100 \(\mu\)M of insulin, as described previously.\(^{31}\) To measure rates of glucose and palmitate oxidation, we simultaneously collected \(^{14}\)CO\(_2\) and \(^{3}\)H\(_2\)O produced from the oxidation of [U-14C]glucose and [9,10-3H]palmitate, respectively.

2.5 Immunoblot analysis

Proteins (25 \(\mu\)g/lane) were separated by SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk for 1 h and probed with specific primary acetyl-lysine (Millipore) antibodies. SIRT1, SIRT3, SIRT6, and LCAD antibodies were purchased from Abcam (Cambridge, MA, USA). Akt, P-Akt, PGC-1\(\alpha\), PDH, GSK3\(\beta\), and P-GSK3\(\beta\) were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody to GCN5L1 was generously provided by Dr M. Sack (NIH, Bethesda, MD, USA). After washing three times for 10 min with Tween-TBS, membranes were incubated with appropriate secondary antibodies for 1 h. After washing, an enhanced chemiluminescence system was used for detection.

2.6 Immunoprecipitation

Twenty-five milligrams of frozen ventricular tissue were homogenized for 30 s with a Polytron homogenizer in a homogenization buffer containing 0.05 M Tris–HCl, 10% glycerol, 1 mM EDTA, 0.02% Brij-35, and 1 mM dithiothreitol in the presence of phosphatases and proteases inhibitors (Sigma). Homogenized tissues were then cleared by centrifugation at 800 g for 10 min. One hundred microliter of total heart lysate protein was pre-cleared with 50 \(\mu\)L of protein A-agarose beads and used for immunoprecipitation. Lysates were rotated with acetyl-K antibodies (3 \(\mu\)g/100 \(\mu\)g lysate protein) overnight at 4°C. About 50 \(\mu\)L of protein A-agarose beads were added to each sample and incubated on a rotator for 4 h at 4°C. After 4 h, beads were washed three times and centrifuged at 16 000 g for 5 min. Bound proteins were boiled in a sample preparation buffer for 5 min and analysed.\(^{32}\)

2.7 Assessment of \(\beta\)-hydroxyacyl CoA dehydrogenase (\(\beta\)-HAD) activity

\(\beta\)-HAD activity was assayed in total heart lysates prepared from frozen heart tissues. Heart lysates were pipetted into a 96-well plate. Each well was
brought to a final volume of 190 μL with 160 μL of 50 mM imidazole (pH 7.4) and 20 μL of 1.5 mM NADH. The reaction was initiated by the addition of 10 μL of 2 mM acetoacetyl-CoA and the absorbance at a 340 nm wavelength was followed for 5 min using a spectrophotometric kinetic plate reader, as described previously.31

2.8 Assessment of LCAD activity

LCAD activity was assayed based on the method described by Lehman et al.32 In brief, 20 μg of total heart lysate was added to potassium phosphate buffer containing 200 μM ferrocenium hexafluorophosphate (Fc⁺PF6⁻), N-ethylmaleimide (500 μM), and EDTA (0.1 mM) at pH 7.2. The reaction was initiated by the addition of palmitoyl-CoA (50 μM) and the absorbance at 300 nm wavelength was followed for 2 min using a spectrophotometer kinetic plate reader.

2.9 Statistical analysis

Data are represented as means ± SEM. Comparisons between two groups were performed using the unpaired Student’s t-test. Multiple comparisons were performed by two-way ANOVA followed by Bonferroni’s multiple comparison test whenever differences were detected. Differences of P < 0.05 were considered significant.

3. Results

3.1 Characteristics of HFD obese mice

Body weight and whole body fat mass, as measured by magnetic resonance imaging in wake mice, were significantly increased after 18 weeks of HFD feeding compared with LFD-fed mice (see Supplementary material online, Table S1). Heart weight normalized to tibia length was also increased in mice fed a HFD. Blood glucose levels in overnight-fasted mice, as assessed by oral glucose tolerance test, were higher in HFD obese mice compared with mice on a LFD (see Supplementary material online, Table S1).

3.2 Cardiac lysine acetylation in HFD obese mice

Overall myocardial acetylation levels were increased in obese HFD-fed mice compared with LFD-fed mice (Figure 1A). The effect of a HFD on the expression of cardiac SIRTs (lysine deacetylases) and GCN5L1 (mitochondrial acetyltransferase) is shown in Figure 1. There were no changes in SIRT1 (Figure 1B) and GCN5L1 expression (Figure 1E); however, the level of SIRT3 protein expression was significantly decreased in HFD obese mouse (Figure 1C). In contrast, the level of SIRT6 expression was significantly increased in HFD obese mouse (Figure 1D), whereas the expression of SIRT2 was significantly decreased (Figure 1F).

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-week period. A significant increase in palmitate oxidation rates (Table 1) was seen in mice subjected to a HFD, compared with 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 1). Since all hearts were perfused under identical conditions, the data suggest 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.

3.4 Acetylation of fatty acid oxidation enzymes in hearts from HFD obese mice

Because cardiac fatty acid 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. The levels of β-HAD protein were not changed in mice on a HFD or a LFD (Figure 2A). However, the levels of β-HAD acetylation were significantly increased in hearts of HFD obese mice (Figure 2B and C) accompanied by a significant increase in β-HAD activity (Figure 2D).

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 3A). However, a significant increase in cardiac LCAD acetylation was observed in HFD obese mouse (Figure 3B and C) accompanied by a significant increase in LCAD activity (Figure 3D). We also observed a strong positive correlation between LCAD acetylation and rate of fatty acid oxidation in the HFD obese and LFD mice (Figure 3E).

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.34 We therefore determined if alterations in the expression and acetylation of PGC-1α could contribute to the increase in fatty acid oxidation observed in the hearts of HFD obese mice. The amount of PGC-1α protein was significantly decreased in HFD obese mice compared with LFD controls (Figure 4A). Concomitantly, an increase in overall acetylation of PGC-1α was observed (Figure 4B), resulting in a marked increase in the acetylated PGC-1α (Ac-PGC-1α)/PGC-1α ratio (Figure 4C). Since acetylation of PGC-1α is believed to decrease PGC-1α transcriptional activity,34,35 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 2A and 3B), neither was the expression of peroxisome proliferator-activated receptors alpha (PPARα; Figure 4D), which is involved in the transcriptional control of fatty acid oxidative enzymes. Expression of the tricarboxylic acid (TCA cycle) enzyme, citrate synthase (CS; Figure 4E), and the electron transport chain enzyme, Complex I (Figure 4F), were also unaffected.

3.6 Role of acetylation in impaired cardiac insulin signalling in HFD obese mice

As summarized in Table 1, 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 with hearts from mice fed a LFD (see Supplementary material online, Figure S1A). Pyruvate dehydrogenase (PDH), the rate-limiting enzyme for glucose oxidation, has recently been shown to be under acetylation control.36,20,22,39 We therefore determined whether PDH expression...
and acetylation status were altered in hearts from obese HFD-fed mice. Neither PDH expression (see Supplementary material online, Figure S1B) and PDH acetylation (see Supplementary material online, Figure S1C) nor the ratios of PDH acetylation/PDH expression were found to be different between hearts from obese HFD vs. LFD mice (see Supplementary material online, Figure S1D).

Akt is involved in insulin signalling, and can be activated by Ser/Thr phosphorylation and inhibited by acetylation. Since Akt is known to be regulated by phosphorylation and more recently by acetylation, we investigated what effect the HFH had on Akt expression, phosphorylation, and acetylation. Immunoblot analysis showed a decreased cardiac expression of Akt in HFD obese mice (Figure 5A). In contrast, the level of Akt acetylation was significantly increased in hearts from HFD obese mice (Figure 5B and C). This increase in acetylation was accompanied by a significant decrease in Akt phosphorylation (Figure 5D), suggesting that acetylation is inhibiting Akt activity by

Figure 1  Overall cardiac protein acetylation and deacetylase expression in mice fed a HFD for 16 weeks. (A) Total protein acetylation in non-perfused hearts from LFD and HFD mice. Protein expression of SIRT1 (B), SIRT3 (C), SIRT6 (D), GCN5L1 (E), and SIRT2 (F) in hearts from mice fed either a LFD or a HFD. Values represent mean ± SEM. (n = 5/group). *P < 0.05, significantly different from LFD.
blocking its phosphorylation. GSK3β is a Ser/Thr protein kinase that phosphorylates and inactivates glycogen synthase. GSK3β itself is regulated by phosphorylation, with phosphorylation at Ser9 by Akt being inhibitory. GSK3β protein expression remained similar in hearts of HFD obese and LFD mice (Figure 5E). However, the level of GSK3β phosphorylation on Ser9 was significantly reduced, similar to what was seen with Akt phosphorylation in HFD obese mice (Figure 5F), and is consistent with the decrease in p-Akt seen in hearts from HFD obese mice. These findings are consistent with the insulin resistance observed in hearts from HFD obese mice.

### 3.7 Regulation of fatty acid oxidation enzymes by mitochondrial deacetylation

Because SIRT3 protein levels were decreased in hearts from HFD-induced obese mice (Figure 1C), and SIRT3 has been implicated in deacetylation of fatty acid oxidative enzymes, we examined the effect of SIRT3 deletion on cardiac fatty acid oxidation and fatty acid oxidative enzyme acetylation status. Isolated working hearts from SIRT3 KO mice showed no differences in cardiac function compared with WT mice (Table 2). However, cardiac energy metabolism was significantly shifted, with a significant increase in fatty acid oxidation (Table 2) and a decrease in glucose oxidation (Table 2). The increase in fatty acid oxidation and decrease in glucose oxidation remained when rates were normalized for cardiac work (Table 2).

The increase in fatty acid oxidation in SIRT KO mouse hearts was accompanied by a significant increase in overall acetylation of cardiac proteins, when compared with WT mice (Figure 6A and B). Despite the increase in fatty acid oxidation seen in SIRT3 KO mice, we found no changes in either SIRT2 or SIRT6 expression in hearts from SIRT3 KO mice compared with WT mice (Figure 6C and D). Phosphorylation of

### Table 1 Effect of chronic HFD diet feeding on metabolic rates of palmitate and glucose oxidations in isolated working mice hearts

<table>
<thead>
<tr>
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<th>LFD</th>
<th>HFD</th>
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<tr>
<td><strong>Cardiac function</strong></td>
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<td>Heart rate (beats/min)</td>
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<td>275 ± 10</td>
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<td>Cardiac output (mL/min)</td>
<td>11.5 ± 0.7</td>
<td>10.3 ± 1.1</td>
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<td>Cardiac work (L/min/g dry wt)</td>
<td>2.5 ± 0.2</td>
<td>2.3 ± 0.3</td>
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<tr>
<td><strong>Oxidative rates (nmol/g dry wt min)</strong></td>
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<tr>
<td>Palmitate oxidation</td>
<td>551 ± 87</td>
<td>845 ± 76*</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>1235 ± 160</td>
<td>635 ± 146*</td>
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<td>Oxidative rates normalized for work (nmol/g dry wt/mJ)</td>
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<tr>
<td>Palmitate oxidation</td>
<td>207 ± 35</td>
<td>350 ± 57*</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>463 ± 55</td>
<td>263 ± 65*</td>
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</table>

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 the section ‘Methods’. Values represent mean ± SEM; (n = 5).

*P < 0.05, significantly different from LFD.

### Figure 2

Protein expression, acetylation, and activity of β-hydroxyacyl CoA dehydrogenase (β-HAD) in hearts from obese mice on a HFD. (A) β-HAD expression. (B) Level of β-HAD acetylation. Total lysates from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for β-HAD. (C) Acetylated β-HAD (Ac-β-HAD) normalized to its level of expression. (D) β-HAD enzyme activity. Values represent mean ± SEM. (n = 5/group). *P < 0.05, significantly different from LFD.
Akt was also not changed in hearts from SIRT3 KO mice compared with WT mice (Figure 6E). We also measured the expression, acetylation, and activity of the fatty acid oxidation enzymes β-HAD and LCAD. In WT mice, β-HAD activity was slightly lower (Figure 7B) compared with LFD mice (Figure 2D), as was LCAD activity (compare Figure 7D with Figure 3D). This may be due to the different background strains of these two groups of mice. In support of this, we found that baseline fatty acid oxidation rates were higher in C57Bl/6J mice (Table 1) compared with rates seen in 129/V mouse hearts (Table 2). While no changes in protein expression of β-HAD were observed between WT and SIRT3 KO mouse hearts, acetylation of β-HAD was significantly increased in hearts from SIRT3 KO mice in comparison with WT mice (Figure 7A), which was accompanied by an increase in β-HAD activity (Figure 7B). Similar to β-HAD, acetylation of LCAD was significantly increased in SIRT3 KO hearts without changes in protein expression (Figure 7C) and was associated with an increase in LCAD activity (Figure 7D).

**Figure 3** Level of LCAD acetylation and its correlation to rates of palmitate oxidation. (A) LCAD expression. (B) Level of LCAD acetylation. Total lysates from isolated working hearts were immunoprecipitated with anti-acetyl-lysine antibodies and then immunoblotted with antibodies specific for LCAD. (C) Acetylated LCAD (Ac-LCAD) normalized to its level of expression. (D) LCAD activity. (E) Correlation curve between palmitate oxidation and LCAD acetylation. Values represent mean ± SEM. (n = 5/group). *P < 0.05, significantly different from LFD.
3.8 Role of acetylation in regulation of cardiac glucose oxidation in SIRT3 KO mice

In SIRT3 KO mouse hearts, insulin-stimulated glucose oxidation was markedly impaired compared with hearts from WT mice (see Supplementary material online, Figure S2A). We therefore determined whether PDH acetylation was altered in hearts from SIRT3 KO mice. Neither PDH expression (see Supplementary material online, Figure S2B) and PDH acetylation (see Supplementary material online, Figure S2C) nor the ratios of PDH acetylation/PDH expression were different between hearts from SIRT3 KO vs. WT mice (see Supplementary material online, Figure S2D).

Figure 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 lysates from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PGC-1α. (C) Ac-PGC-1α normalized to its level of expression. Protein expression of PPARα (D), CS (E), and complex I (F) in hearts from mice fed either a LFD or a HFD. Values represent mean ± SEM. (n = 5/group). *P < 0.05, significantly different from LFD.
4. Discussion

This study identified a number of key roles for lysine acetylation in the control of cardiac energy metabolism and insulin signalling. First, obesity is associated with an overall increase in acetylation of cardiac proteins, with a concomitant decrease in SIRT3 expression. Secondly, 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. Thirdly, the marked cardiac insulin resistance observed in hearts from obese mice is associated with an increased acetylation and decreased phosphorylation/activity of Akt. Fourthly, 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 oxidation enzymes, β-HAD and LCAD. Collectively, these data demonstrate that lysine acetylation has an important role in...
regulating fatty acid oxidation in the heart, and that increased acetylation of fatty acid oxidative enzymes contributes to the excessive reliance of the heart on fatty acid oxidation in obesity.

Post-translation lysine acetylation has emerged as a potentially important mechanism for controlling many energetic pathways.4,5 Lysine acetylation occurs on a large number of enzymes involved in mitochondrial metabolism and glycolysis.18,20 Mitochondrial metabolism is regulated by lysine acetylation, with SIRT3 being an important enzyme involved in the mitochondrial deacylation process.17 A key role for SIRT3 in mitochondrial electron transport chain activity and TCA cycle activity is evident. For instance, Complexes I and II in the respiratory chain are hyperacetylated and inactivated in SIRT3−/− mice.37,38 In addition, SIRT3 deacetylates and activates TCA cycle enzymes, such as isocitrate dehydrogenase, during caloric restriction.39 Another important target of SIRT3 is the fatty acid 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.5,19,21 The increase in overall acetylation and decrease in SIRT3 we observed in obese mouse hearts is also consistent with other studies. Chronic HFD feeding and diabetes, both of which are associated with high fatty acid oxidation rates,27,31,40 lead to a decrease in SIRT3 protein expression,6,19,35 a result also seen in our study. Overall protein acetylation is also increased in hearts from fed mice fed a HFD.8,19 Recently, lysines 318/322 located near the active site of LCAD were identified as a SIRT3 target.21 Interestingly, we show that LCAD is hyperacetylated and activated in hearts from HFD-fed mice. Because fatty acid oxidation is enhanced in hearts from mice subjected to a HFD, our results 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 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 oxidation.

High fatty acid oxidation rates can decrease cardiac efficiency and therefore could have important consequences in insulin-resistant hearts.1−3 Our data implicate post-translational regulation of fatty acid oxidative enzymes as a contributing factor to the high fatty acid oxidation rates in obesity. As a result, decreasing acetylation status of fatty acid oxidative enzymes has a potential therapeutic approach to decrease fatty acid oxidation in obesity and diabetes. In support of this, we demonstrate that increasing acetylation via deletion of SIRT3 does increase fatty acid 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. Importantly, previous studies have shown that increasing SIRT3 can decrease cardiac hypertrophy and protect hearts from oxidative stress injury.29,41

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.4,5,17

Previous studies have shown that acetylation of PGC-1α represses its ability to function as a transcriptional coactivator.7,12,16 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.34,35 Surprisingly, we find that PGC-1α expression (and increased acetylation) is dissociated from the regulation of fatty acid oxidation in the current study. This suggests that the increase in fatty acid oxidation seen in hearts of HFD obese mice is not only due to increases in mitochondrial biogenesis, but rather direct changes in acetylation control of fatty acid oxidation enzymes.

While SIRT1 is a key activator of PGC-1α,16 we did not find any changes in SIRT1 expression between HFD and LFD mice experimental groups. Previous studies have reported that SIRT1 is not required for PGC-1α deacetylation, and importantly that GCN5 acetyltransferase activity is the key regulator of PGC-1α acetylation.44 Interestingly, we found a significant increase in SIRT6 expression in HFD obese mice. Although not detected in the current study, recent results show that SIRT6 can deacetylate and activate GCN5 thereby increasing PGC-1α acetylation.14 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.44 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,37,31,40 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. Despite this finding, glucose oxidation rates were markedly decreased in mice fed a HFD compared with 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. in 1963,28 could be the main determinant for the low glucose oxidation seen in hearts from HFD-fed mice. Increases in fatty acid oxidation will directly inhibit glucose oxidation in hearts from HFD mice. Moreover,

### Table 2 Effects of SIRT3 deletion on cardiac function and cardiac metabolic rates

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<tr>
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<td>272 ± 15</td>
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<td>Cardiac output (mL/min)</td>
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<td>Oxidative rates (nmol/g dry wt min)</td>
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<tr>
<td>Palmitate oxidation</td>
<td>291 ± 17</td>
<td>422 ± 29*</td>
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<tr>
<td>Glucose oxidation</td>
<td>1983 ± 172</td>
<td>1262 ± 121*</td>
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<tr>
<td>Oxidative rates normalized for work (nmol/g dry wt/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitate oxidation</td>
<td>158 ± 16</td>
<td>240 ± 32*</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>1100 ± 99</td>
<td>712 ± 62*</td>
</tr>
</tbody>
</table>

Isolated working hearts from WT and SIRT3 KO mice were perfused for a 60-min period for the measurement of cardiac function, palmitate oxidation, and glucose oxidation. Values represent mean ± SEM.

*P < 0.05, significantly different from WT.
up-regulation of PDH kinase 4 results in hyperphosphorylation and inactivation of PDH in HFD-fed mice.31

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.27,28 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 findings showed that deacetylation of Akt is a prerequisite for its activation, and that activation of SIRT1 will enhance insulin signalling in obesity.

Figure 6 Overall cardiac protein acetylation and deacetylase expression in WT and SIRT3 KO mice. (A) Total protein acetylation in isolated working hearts from WT and SIRT3 KO mice. (B) Palmitate oxidation rates. (C) SIRT2 protein expression. (D) SIRT6 protein levels. (E) Levels of Akt phosphorylation. Values represent mean ± SEM. (n = 7/group). *P < 0.05, significantly different from WT.
In addition, SIRT2 overexpression leads to deacetylation and activation of Akt in hepatic cells. 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 oxidation rates were significantly increased in SIRT3 KO mice compared with WT mice. These results are consistent with a recent study that reported an increase in palmitate oxidation rates in skeletal muscles of SIRT3 KO mice compared with WT mice. This study suggested that hyperacetylation and inactivation of PDH induces a switch in skeletal muscle energy substrate utilization from glucose to fatty acids. 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 occurs primarily at the expense of glucose oxidation (Randle cycle). Since there were no changes in either SIRT2 or SIRT6 expression in SIRT3 KO hearts, and the fact that SIRT2 is cytoplasmic and SIRT6 is nuclear in localization, we suggest that acetylation changes in fatty acid oxidation rates are primarily due to changes in SIRT3 expression.

Support for a role of decreased SIRT3 expression increasing fatty acid oxidation, rather than decreasing fatty acid oxidation, can be found in studies on mice with a SIRT4 deletion. Deletion of SIRT4 (which is another mitochondrial deacetylase) was recently shown to increase lipid oxidation in liver and skeletal muscles. 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.
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. This is associated with phosphorylation of Akt and GSK3β resulting in enhanced insulin sensitivity. (B) In contrast, decreased cardiac SIRT3 expression by high feeding fat or genetic deletion leads to hyperacetylation and activation of β-HAD and LCAD, which result in increased fatty acid β-oxidation. Increased acetylation of Akt inhibits its ability to phosphorylate GSK3β and impairs insulin sensitivity.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

**Conflict of interest:** Sowndramalingam Sankaralingam is a fellow of the Alberta Innovates Health Solutions (AIHS). Richard Lehner and Gary D. Lopaschuk are scientists of the AIHS. Natasha Fillmore holds AIHS Studentship Award.

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