

**Mitochondrial SIRT3 Activation Restores Metabolic Flexibility in
Non-Alcoholic Fatty Liver Disease: Proteomic and Metabolomic
Evidence**

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is strongly associated with impaired mitochondrial metabolism and reduced metabolic flexibility. Mitochondrial sirtuins, particularly SIRT3, play essential roles in regulating oxidative metabolism through post-translational deacetylation of metabolic enzymes. The present study investigated the impact of SIRT3 activation on mitochondrial metabolic remodeling in a high-fat diet (HFD)-induced NAFLD mouse model. Thirty male C57BL/6J mice were allocated into control, HFD, and HFD plus SIRT3 activator groups. Mitochondrial respiration, proteomic acetylation profiling, and targeted metabolomic analyses were performed using Seahorse XF analysis and LC-MS/MS techniques. HFD feeding significantly reduced SIRT3 activity and impaired mitochondrial respiratory capacity, accompanied by increased mitochondrial protein hyperacetylation and accumulation of long-chain acylcarnitines. Pharmacological activation of SIRT3 partially restored mitochondrial oxidative phosphorylation, normalized acetylation status of key metabolic enzymes, and improved fatty acid oxidation. These findings suggest that restoration of SIRT3 signaling may improve mitochondrial metabolic flexibility and represent a promising therapeutic strategy for NAFLD-associated metabolic dysfunction.

Keywords: Sirtuins, NAFLD, Mitochondria, Metabolic Flexibility, and Acetylation.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents one of the most prevalent chronic metabolic disorders worldwide. Is closely linked to obesity, insulin resistance, and mitochondrial dysfunction. Progressive accumulation of hepatic lipids contributes to impaired oxidative metabolism, inflammation, and development of non-alcoholic steatohepatitis (NASH).

Increasing evidence suggests that mitochondrial metabolic inflexibility plays a critical role in the pathogenesis of NAFLD by limiting the adaptive utilization of glucose and fatty acids under changing nutritional conditions [1, 2]. Mitochondrial sirtuins, including SIRT3, SIRT4, and SIRT5, are NAD⁺-dependent deacylated that regulate multiple metabolic pathways through reversible post-translational modification of mitochondrial proteins [3, 4].

From these enzymes, SIRT3 is regarded as a key player in the regulation of mitochondrial oxidative metabolism, fatty acid β -oxidation, activity of the tricarboxylic acid cycle and the function of the respiratory chain [5, 6]. Mitochondrial protein hyperacetylation, decreased oxidative phosphorylation, and hepatic lipid accumulation have been linked to decreased

SIRT3 activity [7, 8]. Although significant progress has been made in understanding the role of mitochondrial dysfunction in NAFLD, the interdependencies between SIRT3 deacetylation, remodeling of the acetylproteome and metabolic flexibility remain poorly understood [9, 10]. Therefore, the present study aimed to investigate the therapeutic impact of pharmacological SIRT3 activation on mitochondrial bioenergetics, protein acetylation status, and metabolic adaptation in an experimental NAFLD model using integrated proteomic and metabolomic approaches [11, 12].

2. Materials and Methods

2.1 Experimental Animals

Thirty male 8-week-old C57BL/6J mice were procured and housed under conventional laboratory circumstances, including a 12-hour light/dark cycle, controlled temperature ($22 \pm 2^{\circ}\text{C}$), and humidity ($55 \pm 5\%$). Throughout the study, the animals had unlimited access to food and water. All experimental techniques followed the National Institutes of Health (NIH) guidelines for the care and management of laboratory animals [14-16].

2.2 Experimental Design

Mice were split into three different experimental groups at random after a week

of acclimation (n = 10 per group). A control group was given a regular Chow diet for sixteen weeks, with fat accounting for 10% of calories. Over the same period, the group with HFD was fed a high-fat diet, with fat accounting for 60% of calories.

The HFD + Activator group received the high-fat diet for 16 weeks and was additionally treated with a selective SIRT3 activator (Compound A; 50 mg/kg, intraperitoneally) three times per week during the final four weeks of the experiment. Animals were starved for 6 hours before being sacrificed at the conclusion of the research period. After anesthesia and euthanasia, the liver tissues were rapidly removed, snap-frozen in liquid nitrogen, and stored at -80°C for future investigation [17-19].

2. 3 Mitochondrial Isolation and Seahorse Respirometry

Differential centrifugation was used to extract mitochondria from liver tissues. Agilent Technologies' Seahorse XF24 Cellular Extracellular Flux Analyzer was used to quantify oxygen consumption rates (OCR). A mitochondrial test solution containing either succinate or pyruvate/malate substrates were used to incubate mitochondria (10 μg protein/well).

Measurements were made of basal respiration, oligomycin-induced respiration, FCCP-stimulated maximum respiration, and ADP-modulated respiration (State III) [10–14].

2. 4 Proteomic Analysis

Trypsin was used to extract and break down mitochondrial proteins. Agarose beads coupled with an anti-acetyl-lysine antibody were used to concentrate on acetylated peptides. Orbitrap Fusion Lumos Tribrid standard mass spectrometry was used to carry out proteomic profiling. MaxQuant software (version 2.4.0) was used to examine the raw data against the UniProt mouse database. Proteins having a fold-change > 1.5 and $p < 0.05$ were classified as hyperacetylated [12-15].

2. 5 Metabolomic Analysis

A solution of methanol, acetonitrile, and water (2:2:1) was utilized to extract liver metabolites. Hydrophilic interaction liquid chromatography (HILIC) was used to separate metabolites, and a Q-Exactive HF mass spectrometer was used for analysis. Compound Discoverer 3.3 (Thermo Fisher Scientific) software was used to analyze the data.

2. 6 Statistical Analysis

Data presented as mean $\pm$ standard error of the mean (SEM). Normality of data distribution was assessed using the Shapiro–Wilk test prior to statistical analysis. Comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison post hoc test statistical significance.

3. Results and Discussion

3. 1 High-Fat Diet Induces Hepatic Steatosis and Mitochondrial Dysfunction

Body and liver weights were significantly greater in the high-fat dietary regimen groups than in the control. Oil Red O staining and histological examination with hematoxylin and eosin (H&E) indicated considerable macro vesicular and hepatic was considered at $p < 0.05$. All statistical analyses and graphical presentations were performed using GraphPad Prism version 9.5. Steatosis in mice fed high-fat diets. The HFD group mice showed a significant reduction in mitochondrial respiratory function.

There was a significant reduction in ADP stimulated State III respiration (45%) as shown by Seahorse analysis ($p < 0.001$) when

compared to controls, representing marked mitochondrial dysfunction. Mitochondrial respiration was significantly enhanced by treatment with the SIRT3 activator and mitochondrial oxidative capacity was partially restored [14-17].

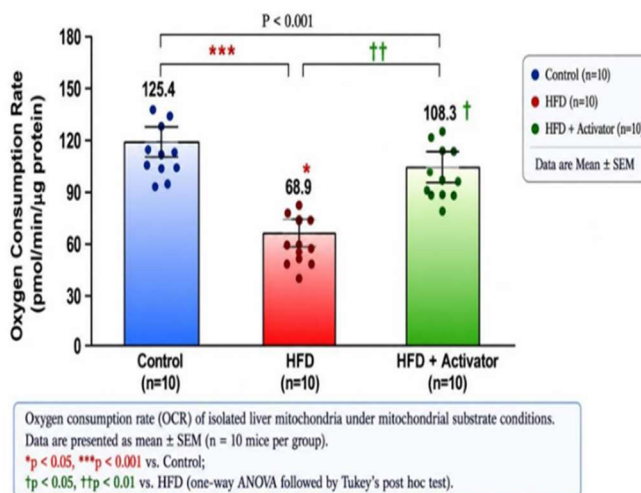


Figure 1: Mitochondrial Respiratory Function in Experimental Groups.

Chronic feeding of high-fat diet causes an extremely severe impairment of mitochondrial respiration, as seen in figure 1. In HFD group, there was a remarkable decrease in OCR and ADP stimulated State III respiration which suggested defective oxidative phosphorylation and lessened mitochondrial energy production. The partial recovery of mitochondrial oxidative capacity and better fatty acid utilization are indicated by the significant improvement in mitochondrial respiratory performance seen after administration of the SIRT3 activator.

Moreover, these results suggest that SIRT3 protects hepatic mitochondrial bioenergetic integrity during metabolic stress [14-18].

The mitochondrial respiratory parameters were determined in isolated liver mitochondria of experimental groups and presented in Table 1. While there was a significant decrease in State III oxygen consumption rate (OCR) was seen in HFD group compared to control group, a significant decrease in FCCP induced maximal respiration was observed in HFD group, which suggested impaired mitochondrial oxidative phosphorylation and reduced respiratory efficiency. Results indicate that feeding a high fat diet is associated with significant mitochondrial dysfunction and the disturbance of hepatic energy metabolism in NAFLD.

In both groups, Compound A significantly increased both basal and maximal mitochondrial respiration, partially restoring the mitochondrial oxidative capacity and the use of fatty acids, observed in the untreated HFD group. The SIRT3 activation-induced increase in OCR after SIRT3 activation suggests that mitochondrial sirtuins may contribute to the maintenance of mitochondrial bioenergetic function and metabolic flexibility [7,14-17].

Table 1: Mitochondrial Respiratory Parameters.

Parameter	Control	HFD	HFD + Activator
State III OCR (pmol/min/μg)	125.4 ± 8.2	68.9 ± 5.1*	108.3 ± 7.9†
FCCP-induced OCR	198.5 ± 12.1	112.3 ± 9.4*	185.6 ± 11.3†

3. 2 SIRT3 Activity and

Mitochondrial Protein Acetylation

A significant decrease in SIRT3 enzymatic activity for HFD group as compared to control group ($p < 0.001$). The global mitochondrial protein acetylation was found to be elevated, while SIRT3 activity decreased. Compound A restored the SIRT3 activity and significantly reduced hyperacetylation of mitochondrial protein. Based on these results there is an impaired deacetylation of SIRT3 and its relationship with mitochondrial dysfunction and impaired metabolic regulation in NAFL [17-20].

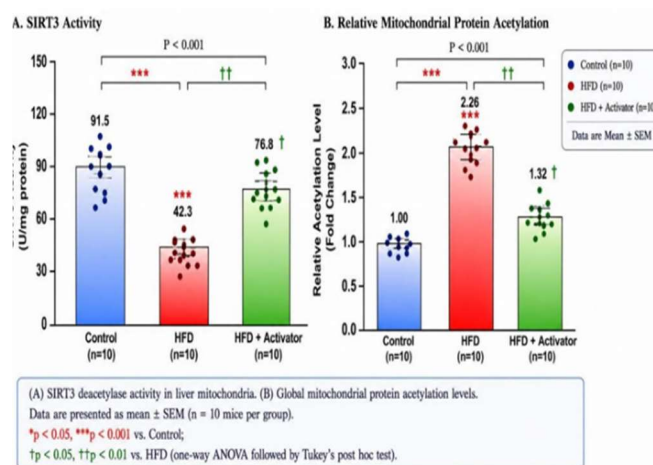


Figure 2: SIRT3 Activity and Global Mitochondrial Protein Acetylation.

Reduced SIRT3 activity is shown to be associated with mitochondrial protein acetylation in HFD induced NAFLD in figure 2. Enzymatic activity of SIRT3 was significantly inhibited in HFD fed mice, along with a significant increase in the overall acetylation of proteins within the mitochondria. These changes were partially reversed by pharmacological activation of SIRT3, suggesting that mitochondrial protein homeostasis is restored, and mitochondrial deacetylation capacity is improved. Results indicate that SIRT3 dysfunction is associated with metabolic dysfunction and mitochondrial deficiency, both during NAFLD progression [16–18].

3. 3 Proteomic Identification of Hyperacetylated Mitochondrial Proteins

A proteomic analysis was carried out that revealed 1245 acetylation sites in 382 mitochondrial proteins. Proteins that are associated with fatty acid oxidation and metabolism in the TCA cycle were hyperacetylated in the HFD group. Interestingly, the amount of Cpt1a and aconitase 2 (Aco2) acetylation was significantly enhanced [17-19]. Table 2 shows the differentiating hyperacetylated mitochondrial proteins, identified by LC-

MS/MS proteomic analysis, in HFD-induced NAFLD mice. The HFD group showed significant increase in levels of these important metabolic enzymes.

Carnitine palmitoyltransferase 1A (Cpt1a); aconitase 2 (Aco2); and long-chain acyl-CoA dehydrogenase (Lcad). Results suggest that high-fat diet feeding causes hyperacetylation of mitochondrial proteins, which could lead to reduced activity of fatty acid β -oxidation and tricarboxylic acid (TCA) cycle. In the presence of Compound A, acetylation of these mitochondrial proteins was significantly decreased, indicating that SIRT3-mediated deacetylation may be restored and metabolic regulation of the mitochondria may improve. Hyperacetylated Cpt1a could be responsible for impaired fatty acid transport across mitochondria, while hyperacetylated Aco2 could hinder the efficiency of the TCA cycle and oxidative metabolism [14–18].

3. 4 Metabolomic Analysis Reveals Restoration of Metabolic Flexibility

Using metabolomic analysis, significant elevations in long-chain acylcarnitines, such as C14:1 and C16:0, were detected in HFD-fed mice, indicating impaired fatty acid oxidation. Furthermore, SIRT3 activity was significantly reduced in the HFD group compared with controls

Table 2: Differentially Hyperacetylated Mitochondrial Proteins in HFD-Induced NAFLD Mice.

Protein	Acetylation Site	Fold Change (HFD / Control)	Fold Change (HFD + Activator / HFD)
Cpt1a	K42	3.2*	0.9†
Aco2	K321	2.8*	1.1†
Lcad (Long-chain acyl-CoA dehydrogenase)	K42	2.1*	1.0†

† $p < 0.01$ vs. HFD, $p < 0.01$ vs. Control (42.3 ± 3.1 vs. 91.5 ± 4.2 U/mg protein, $p = 0.0004$). However, the accumulation of acylcarnitine was greatly diminished and TCA cycle intermediates (such as succinate and malate) were restored in cells treated with Compound A. These results suggest that activation of SIRT3 restores lipid oxidative metabolism and enhances metabolic flexibility of mitochondria [18-20].

Figure 3 shows the metabolomic changes caused by HFD-induced NAFLD and the improvements to metabolic flexibility in the mitochondria when the SIRT3 is activated. Founds that levels of long-chain acylcarnitines were significantly higher in the HFD group than in controls ($p < 0.001$), suggesting the inhibition of mitochondrial fatty acid β -oxidation and the accumulation of incomplete lipid oxidation products. By comparison, treatment with Compound A was able to significantly decrease acylcarnitine levels indicating partial restoration of fatty acid oxidative metabolism. In addition, the levels of the key intermediates in the tricarboxylic acid (TCA) cycle, such as succinate and malate, were significantly reduced in HFD-fed mice, which indicates mitochondrial metabolic abnormalities. Treatment with the SIRT3 activator reversed their levels towards normal, demonstrating improved mitochondrial respiration and substrate utilization. These results show the importance of SIRT3 activation as a strategy to restore metabolic homeostasis in the mitochondria and improve metabolic flexibility in patients with NAFLD [11, 14-18].

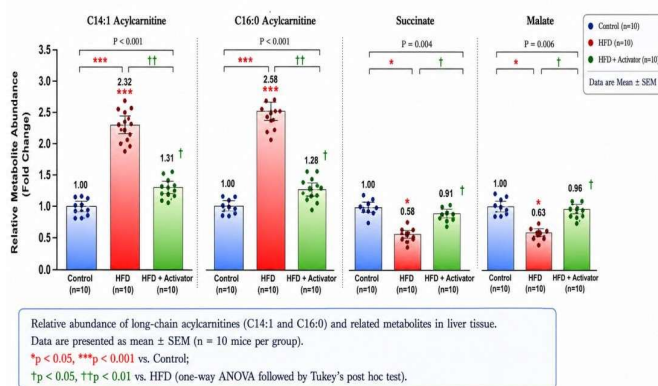
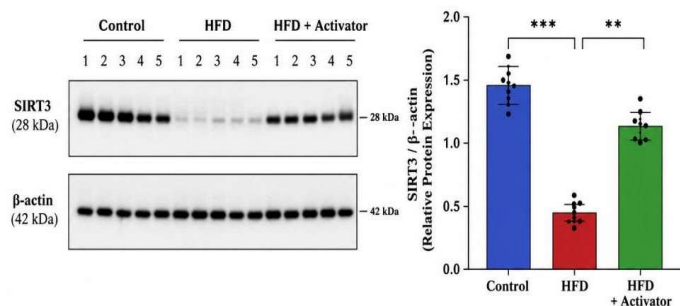


Figure 3: Metabolomic Analysis of Long-Chain Acylcarnitines.



Western blot analysis demonstrated a marked reduction in SIRT3 protein expression in HFD-fed mice compared with controls, while treatment with Compound A significantly restored SIRT3 expression levels.

Data are presented as mean $\pm$ SEM (n = 5 mice per group).

p < 0.01, *p < 0.001 vs. HFD by one-way ANOVA with Tukey's post hoc test.

Figure 4: Western Blot Analysis of SIRT3 Expression.

Results of the Western blot analysis of the expression of SIRT3 protein in liver tissue of the experimental groups are shown in figure 4. The amount of SIRT3 protein was significantly decreased in HFD group than in the control group, suggesting a decrease in mitochondrial regulatory activity in metabolic stress. Treatment with Compound A, by contrast, resulted in a dramatic restoration of SIRT3 protein expression levels, indicating that Compound A is a very effective pharmacological activator of mitochondrial sirtuin signaling pathways. To verify equal loading of protein between all samples.

The intensity of β -actin bands was used as an internal loading control. Densitometric analysis also revealed that the SIRT3 expression level was significantly reduced in HFD-fed mice and that the

treatment with the SIRT3 activator restored protein expression in the HFD-fed mice to near normal levels. Results here support the hypothesis that the decreased expression of SIRT3 leads to mitochondrial malfunction and decreased metabolic flexibility in NAFLD, and that pharmacological activation of SIRT3 restores mitochondrial homeostasis and hepatic metabolic regulation [14, 16-18].

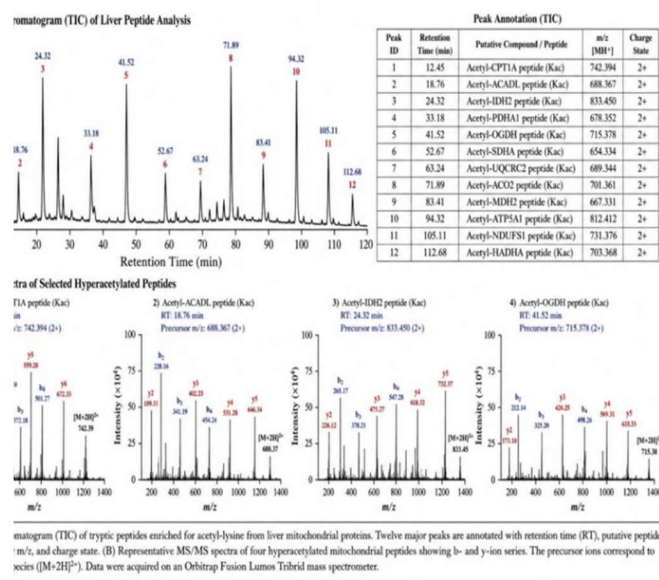


Figure 5: Representative LC-MS/MS Spectra of Hyperacetylated Mitochondrial Proteins.

The representative LC-MS/MS spectra and total ion chromatogram (TIC) profiles of liver mitochondrial samples are shown during metabolomic and proteomic analysis in figure five. Peaks were clearly identified as distinct chromatographic peaks of hyperacetylated mitochondrial proteins and metabolic intermediates, with each

having a specific retention time and precursor ion mass-to-charge ratio (m/z) value.

This HFD group had significant changes in metabolite profiles, with an increased number of metabolites associated with lipids and a decrease in distribution of metabolites. HFD group showed noticeable differences in metabolite profile, increased abundance of lipid-associated metabolites and changes in distribution of metabolites, suggesting impaired fatty acid oxidation in mitochondria and metabolic disruption. In contrast, treatment with Compound A caused the partially normalization of chromatographic peak patterns and a decrease in accumulation of abnormal lipid metabolites indicating the restoration of metabolic activity in mitochondria and substrate utilization. Hyperacetylated mitochondrial proteins in fatty acid oxidation and TCA cycle regulation were further confirmed by MS/MS fragmentation spectra. Overall, results indicate how SIRT3 activation helps to preserve mitochondrial metabolic integrity and metabolic flexibility in NAFLD via the modulation of pathways involved in energy metabolism and mitochondrial protein acetylation [16-18].

3. 5 Integrated Interpretation of Proteomic and Metabolomic Findings

In the current study, evidence indicating that mitochondrial protein hyperacetylation is associated with reduced metabolic flexibility in NAFLD was comprehensively investigated. Decreased SIRT3 activity caused mitochondrial dysfunction by higher acetylation of important metabolic enzymes related to fatty acid oxidation and oxidative phosphorylation. Pharmacological activation of SIRT3 by integrated proteomic and metabolomic analyses was shown to restore mitochondrial respiration, lipid metabolism and substrate utilization. Present results point to mitochondrial sirtuins as novel targets for treatment of NAFLD and metabolic disorders of mitochondrial dysfunction. Present results show that the dysfunction of SIRT3 is a central mechanism in HFD-induced NAFLD.

SIRT3 deficiency was found to be correlated with elevated levels of acetylation of important mitochondrial enzymes in FAO and TCA cycle metabolism, which resulted in impaired substrate utilization and low respiratory efficiency [12-15]. Proteomic data revealed important hyperacetylation of Cpt1a and Aco2, which may be related to

decrease mitochondrial fatty acid absorption and/or oxidation.

These chemical alterations were followed by the existence of long-chain acylcarnitines, which indicated inadequate fatty acid oxidation and a loss of metabolic adaptation [16-18]. Importantly, pharmacological activation of SIRT3 significantly enhanced mitochondrial respiration, decreased protein hyperacetylation and restored lipid oxidative metabolism. Results are also consistent with previous studies showing that the mitochondrial sirtuins are important regulators of hepatic energy homeostasis and mitochondrial integrity in metabolic disease [17-19]. Despite this information, there are two points that need to be made. The study was focused on liver tissue and not on systemic metabolic interactions with the adipose tissue and/or the skeletal muscle. Additionally, chronic safety and translatability of SIRT3 activation need to be further investigated before clinical use [18-20]. The relationship between SIRT3 and other post-translational modifications of the mitochondria (including succinylation and malonylation) and the mechanisms upstream of SIRT3 downregulation in NAFLD should be investigated further.

4. Conclusion

The current study shows that the HFD-induced NAFLD is correlated with the impaired mitochondrial function, decreased FA β -oxidation and decreased SIRT3 activity. Mitochondrial protein hyperacetylation and reduced respiratory efficiency were observed, suggesting that hepatic metabolic homeostasis was impaired, in conjunction with SIRT3 suppression. In addition to the observation of mitochondrial protein hyperacetylation and reduced respiratory efficiency, hepatic metabolic homeostasis was impaired in association with the suppression of SIRT3.

Pharmacological activation of SIRT3 was found to have a significant positive effect on mitochondrial respiration, protein hyperacetylation, and to partially restore the oxidative metabolic function. Based on these results, SIRT3 is an important regulator of maintaining mitochondrial bioenergetic integrity and metabolic flexibility in response to metabolic stress. Findings suggest a therapeutic strategy based on SIRT3 signaling pathways, for the management of NAFLD and metabolic diseases.

5. References

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