CHAPTER 6.

DISCUSSION AND FINAL CONCLUSIONS

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6.1 Summary

It is becoming more readily obvious that alterations in lysine acetylation contribute significantly to changes in energy metabolism (6,152,176,192,201,204,206). In addition, major cardiovascular diseases such as heart failure, and myocardial infarction as well as metabolic syndrome are associated with changes in lysine acetylation and sirtuins activity (59,151,166,196,199,227). One of the major pathways of energy production namely fatty acid β-oxidation was shown to be markedly enhanced in the aforementioned diseases (58,89,94,121,243). Previous studies showed that increased lysine acetylation of fatty acid oxidation enzymes inhibit their activity (196,197,200,206,246). Furthermore, increases in lysine acetylation of insulin signaling proteins are suggested to contribute to the development of insulin resistance in various tissues (164-166,196,208,244). Finally, in HF, a selective reduction in glucose oxidation as a source of cardiac energy as well as increased reliance on fatty acid β-oxidation believed to impair cardiac efficiency and function (58,59,129,136-138,261,264).

The specific aim of this thesis was to understand whether alterations in lysine acetylation could contribute to changes in cardiac fatty acid β-oxidation seen during maturation and in obesity and HF. In the liver,

it is suggested that lysine acetylation is inhibitory signal for fatty acid oxidative enzymes (196,200,206,246). This concept is in direct contrast to other studies that suggested lysine acetylation as a stimulatory signal for fatty acid oxidative enzymes in cardiac myocytes, and skeletal muscles (159,201).

In the first part of this thesis we addressed the controversy whether lysine acetylation is a stimulatory or inhibitory signal for fatty acid β-oxidation in the heart, and demonstrated that increased lysine acetylation was associated with increased cardiac fatty acid β-oxidation during maturation. Fatty acid oxidation enzymes; β-HAD and LCAD both were hyperacetylated and activated during maturation. Our results are highly suggestive that lysine acetylation is a stimulatory signal for cardiac fatty acid β-oxidation, and argues against the notion that increasing lysine acetylation lead to inhibition of fatty acid β-oxidation. Following on these findings, we next investigated in this thesis contrary to popular theory, whether obesity increases lysine acetylation and accelerates fatty acid β-oxidation. We achieved this by utilizing HFD-induced obese mouse model. Previous studies from our lab and others showed that obesity induced by HFD feeding or genetic manipulation, such as leptin deletion leads to increases in cardiac fatty acid β-oxidation (61,88,98,243). Interestingly, we showed that HFD feeding increases cardiac fatty β-oxidation and led to increased lysine acetylation and activity of β-HAD and LCAD. Furthermore, using SIRT3 KO mice, we showed that increases in fatty

acid β-oxidation rates were accompanied with hyperacetylation and activation of both fatty acid oxidation enzymes β-HAD and LCAD, suggesting that lysine acetylation increases fatty acid β-oxidation in the heart.

The last part of this thesis focused on the role of lysine acetylation in regulating fatty acid β-oxidation and hypertrophy in pressure overload-induced HF mouse model. We showed that rates of fatty acid β-oxidation are increased in obese-HF and were associated with hyperacetylation and activation of LCAD. Furthermore, cardiac hypertrophy evident in obese-HF mice were associated with decreased lysine acetylation of FoxO1.

Overall, the novel findings of this thesis have clearly demonstrated that lysine acetylation enhances fatty acid β-oxidation in the heart. Furthermore, we have evidently demonstrated that inhibition of SIRT3 in the heart by HFD feeding or genetic deletion, leads to increases in fatty acid β-oxidation. The rest of this chapter will focus in more details about the implications of the findings, justification of the methods used in this thesis, and future directions.

6.2 Enzymatic versus Spontaneous Acetylation

Lysine acetylation is abundant both in mitochondria and cytosol. Acetyl CoA generated from the oxidation of fatty acids and glucose is the substrate for proteins acetylation. As the primary site for acetyl CoA

utilization, mitochondrial total CoA pool is about 2mM, which is 150 times higher than that of the cytosol in the heart (276). It was demonstrated previously that the high pH and acetyl CoA concentrations in the mitochondria are sufficient to drive enzyme-independent acetylation of mitochondrial proteins *in vitro* (277). Furthermore, incubating recombinant LCAD with acetic anhydride *in vitro* was able to acetylate LCAD on 13 lysine residues (206). However, the basis of this concept would be feeble if mitochondrial acetyltransferases are identified in mitochondria. Indeed, a mitochondrial acetyltransferase was recently identified in the heart, skeletal muscles, and liver (211). Interestingly, genetic knockdown of GCN5L1 without changing acetyl CoA concentrations results in a marked decrease in mitochondrial proteins acetylation (211). However, further characterizations of GCN5L1 kinetics and molecular components are needed. In addition, of the 13 chemically acetylated lysines (206), only 8 lysine residues in LCAD were identified as a target for lysine acetylation in the liver (200). These differences in the number of acetylated lysines further support that enzymatic acetylation is key for regulation of mitochondrial proteins acetylation. Furthermore, the abundance of lysine acetylation in the cytoplasm although concentrations of acetyl CoA are low and the non-alkaline pH argues against the notion of non-enzymatic acetylation. Moreover, in conditions that are accompanied by increased overall acetylation such as obesity, diabetes, and ischemia-reperfusion (142,191,201,210,246,278), the acetylation levels of specific proteins in

the mitochondria are not equal and could significantly differ from each other. For example, in this thesis I showed that mitochondrial enzymes LCAD and β-HAD were hyperacetylated in hearts isolated form obese mice. However, acetylation of another mitochondrial enzyme PDH was not changed, suggesting that lysine acetylation is a highly specific post-translational modification. In addition, Affinity of some acetyltransferases to acetyl CoA is similar to physiological acetyl CoA concentrations (279); this may suggest that fluctuations in acetyl CoA concentrations may regulate proteins acetylation. Finally, enzymatic site-specific acetylation may occur in parallel with spontaneous non-enzymatic acetylation, which adds more sophisticated level of how post-translational modifications might be controlled.

6.3 Differential Regulation of Sirtuins

An interesting aspect in sirtuins physiology is the differential regulation of sirtuins activity and expression in different tissues. Calorie restriction increases SIRT1 protein expressions in various tissues including brain, kidney, cardiomyocytes, skeletal muscles, and liver (148,158,280,281). Furthermore, protein expressions of SIRT3 were reported to be enhanced in liver, adipose tissue, cardiomyocytes, and skeletal muscles by calorie restriction (140,152,158,192,209,212,280) . However, it was reported by Schwer *et al* that mitochondrial protein lysine

acetylation is altered by calorie restriction in a tissue-specific manner (282). Their data show that calorie restriction markedly increases mitochondrial proteins acetylation in liver. However, mitochondrial proteins acetylation was significantly decreased by calorie restriction in adipose tissue. Interestingly, acetylation was unchanged in mitochondria isolated from heart, brain, and kidney (282). In fasting, SIRT3 protein levels were decreased in skeletal muscles but increased in liver (200,201,208). Furthermore, SIRT3 is active in the fed state in skeletal muscles, whereas in the liver SIRT3 is more active in the fasting state (201). Interestingly, metabolic effects of SIRT3 seem to be tissue-specific as well. For example, SIRT3 deletion results in hyperacetylation of LCAD and inhibition of liver lipid oxidation (200), whereas deletion of SIRT3 in skeletal muscles increases fatty acid β-oxidation (201). In addition, specific lysine residues could be targeted in different conditions. Four lysine residues in the enzyme superoxide dismutase are targets for SIRT3 deacetylation. Lysines 53 and 89 are deacetylated during calorie restriction (283), whereas lysines 122 and 68 are deacetylated by SIRT3 in response to ionizing radiation stress and increased reactive oxygen species levels, respectively (284,285). This dichotomous role of sirtuins in different tissues may explain the differences in metabolic effects we noticed in the heart compared to what others reported in the liver (196,200).

6.4 Mitochondrial Versus Total Heart Lysates

We used total heart lysates to determine the acetylation levels of nuclear, cytoplasmic, and mitochondrial enzymes. However, in previous studies mitochondria were isolated and then acetylation levels of mitochondrial proteins were evaluated (191,192,200,201,226). Our concern is that 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. It is important to emphasize, however, that our results from frozen heart tissue mimicked what was previously found in mitochondrial preparations of liver and skeletal muscles tissue (196,197,200,201). Furthermore, trichostatin A (TSA) which is a selective class I and II histone deacetylase inhibitor, and nicotinamide (NAM) which is a noncompetitive sirtuins inhibitor were added to mitochondrial isolation and immunoprecipitation experiments. The purpose of such intervention is to preserve acetylation status of proteins during the time of experiment. However, we did not use these inhibitors in our experiments. We believe that this intervention could be futile, since acetylation can be regulated by both acetyltransferases and deacetylases. Therefore, inhibiting only one arm of the reaction using deacetylases inhibitors such as TSA and NAM, although could inhibit deacetylases, acetylation catalyzed by

acetyltransferases could proceed uninterrupted. Nonetheless, we isolated mitochondria from non-perfused hearts in the presence and absence of TSA and NAM, and we found that overall proteins acetylation is not affected by inhibitors treatment.

6.5 Final Conclusions

Overall, the findings in this thesis have addressed very important questions regarding the contributions of lysine acetylation towards the regulation of cardiac energy metabolism. The novel findings in this thesis should open the doorway for a new avenue to explore in the treatment of obesity and heart failure. This avenue will have a central focus on the optimization of cardiac energy metabolism by targeting sirtuins based on the following conclusions:

1) Overall proteins acetylation is increased during maturation, accompanied by hyperacetylation and activation of fatty acid oxidative enzymes; β-HAD and LCAD.
2) Increased expression of the nuclear deacetylase SIRT1 and the decreases in SIRT6 cardiac protein levels during maturation were accompanied by decreased acetylation of PGC-1α and increased ATP production.

3) Hyperacetylation of the glycolytic enzymes; HK and PGM were associated with decreased glycolytic rates during maturation.
4) Decreased cardiac SIRT3 expression in obesity or by genetic deletion increases fatty acid β-oxidation, acetylation and activities of β-HAD and LCAD.
5) Last, cardiac hypertrophy evident in pressure overload-induced heart failure was associated with SIRT1 mediated inhibition of FoxO1. Such observations suggest that SIRT1 may induce cardiac hypertrophy.

6.6 Future Directions

Although exciting and novel data has been presented in this thesis, there remains a significant amount of experiments that need to be performed to validate the role of sirtuins and acetylation in regulation of cardiac energy metabolism. One of the most important aspects that need to be addressed is whether pharmacological activation of SIRT3 will improve cardiac metabolism and function in a disease states such as obesity, diabetes, and heart failure. Although cardiac-specific overexpression of SIRT3 protects the heart from hypertrophy and improves cardiac function, there are no specific activators or inhibitors for

SIRT3 and the data regarding its role in regulating cardiac energy metabolism is still scarce.

Another important area for future investigation lies on the fact that many lysine residues are acetylated in the same enzyme. Further investigation of the acetylated lysines and determination of SIRT3 target sites are key for improving our understanding about the mechanisms by which acetylation/deacetylation modulates enzymatic activities. Furthermore, acetylation, succinylation, and malonylation counterbalance each other because these post-translational modifications may occur in the same lysine residue. This suggests the existence of intricate interplay between acetylation, succinylation, and malonylation in the activation or inhibition of various metabolic enzymes. It will be fascinating to study their relationship in conditions such as fasting and cardiovascular diseases associated with obesity and diabetes.

The role of acetylases in controlling cardiac energy metabolism is never investigated. Compared to our understanding of deacetylation, the processes involved in acetylation of lysine residues of metabolic enzymes are less well characterized. A role for GCN5L1 as a mitochondrial acetylase and GCN5 as a nuclear acetylase has recently been described (178,211). Recently, MCD has also been proposed to be an acetyltransferase whereby it channels acetyl CoA that is produced via MCD into acetylation reactions (286). It will be very interesting to determine the role of MCD in cardiac acetylation. Furthermore, SIRT4

deacetylates and represses MCD activity. SIRT4 knockdown in liver and muscle results in a significant upregulation of MCD activity, as well as increased fatty acid β-oxidation (216). It will be just as interesting to investigate whether such mechanisms may also apply in the heart, as SIRT4 has received very little attention as a possible regulator of cardiac fatty acid β-oxidation. It is worth mention that MCD is also localized in the peroxisomes where it regulates the level of malonyl CoA and peroxisomal fatty acid β-oxidation (287,288). The finding that protein acetylation is abundant in human liver peroxisomes (210), could be a prelude to the existence of a similar mechanism in cardiac peroxisomes.

The last area that is going to require extensive investigation is to delineate the functions of each sirtuin in each subcellular compartment, and the impact of acetylation on TCA and ETC enzymes in the heart. In addition, several post-translational modifications include phosphorylation and ubiquitination have been identified to regulate SIRT1 activity (148,289). However, so far no post-translational modifications of SIRT3 have been identified (198). It is very important for future research to determine if post-translation modifications regulate SIRT3 activity, which will greatly help us to develop new therapeutic agents to target SIRT3 and treat obesity associated cardiovascular diseases, where SIRT3 seems to play a pivotal role in pathogenesis.