SIRT3: Molecular Signaling in Insulin Resistance

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Abstract

Post-translational modification of intracellular proteins through acetylation is recognized as an important regulatory mechanism of cellular energy homeostasis. Specific proteins called sirtuins deacetylate other mitochondrial proteins involved in glucose and lipid metabolism, activating them in metabolic processes. SIRT3 is a sirtuin of particular interest as it is found exclusively in mitochondria and has been shown to affect a variety of cellular metabolic processes. The activity of this enzyme is related to cellular insulin sensitivity. This study attempted to identify the relationship between insulin sensitivity and change in amount of SIRT3 following a bout of exercise in non-diabetic individuals. We find a moderate inverse correlation between insulin sensitivity and increase in SIRT3 abundance following exercise. This suggests that this protein may not be involved directly in cells' ability to regulate energy homeostasis or that it may act through another mechanism not investigated in this study.

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Introduction:

In normal individuals, mitochondria play a significant role in regulation of cellular metabolism and energy usage. These organelles are the site of ATP production as the final common pathway of glycolysis and lipolysis. Mitochondria also play a role in heat generation and regulation of reactive oxygen species production (Kim). In general, the activity of these processes is not determined by abundance of mitochondrial proteins, but by the activity level of the proteins. This can be regulated by posttranslational modifications including addition or removal of a phosphate or acyl group.

Sirtuins are a specific family of protein deacetylases found throughout the body. These proteins catalyze the cleavage of an acetyl group from lysine residue on the substrate protein, using NAD+ as a cofactor (Huang). Of the 7 orthologs of sirtuins in humans, three are located in the mitochondria and are proposed to have regulatory effects on proteins involved in oxidative phosphorylation and lipid metabolism (Palacios, Hirschey 2011). SIRT4 specifically interacts with glucose dehydrogenase, an enzyme that acts to convert glutamate to alpha-ketoglutarate, a reaction that generates ATP and stimulates insulin production in the pancreas. SIRT5 plays a critical role in the catabolism of amino acids.

SIRT3 is of particular interest relating to cellular metabolism and exercise particularly because of its location in the mitochondria and its known targets involved in energy production. It is highly expressed in the mitochondria of skeletal muscle, among other tissues. Interestingly, it is more highly expressed in slow twitch muscle than fast twitch (Palacios). SIRT3 is known to deacetylate long-chain acyl CoA dehydrogenase, a protein responsible for fatty acid oxidation (Hirschey 2010).It is also known to deacetylate proteins involved in the tricarboxylic acid cycle and electron transport chain, such as glutamate dehydrogenase, Acetylcoenzyme A synthase 2 (AceCS2), and various other proteins responsible for generation of ATP (Huang, Lombard, Shwer). Aside from its direct action, it increases other downstream molecules such as AMPK, CREB, and PGC-1α which have various functions, including increased mitochondrial biogenesis and oxidative capacity. (Palacios)

The activity level of SIRT3 is metabolic state of the cell as a result of feeding status and energy use. In mice, those fed a high fat diet had decreased SIRT3 activity compared to those on a normal diet (Jing, Hirschey 2011). Calorie restriction, prolonged fasting, and cold exposure cause increased levels of SIRT3 (Jing, Palacios).

SIRT3 has also been shown to increase in response to long term exercise training (Palacios, Lanza). However, the relationship between acute training and SIRT3 expression is not as well characterized. Acute exercise has been shown to increase the abundance of SIRT1, and it is reasonable to believe that other sirtuins might also increase in under the same stimulus (Dumke, Rodgers).

This relationship between exercise and increased SIRT3 production likely depends on insulin sensitivity of the cell. Hirschey showed that SIRT3 knockout mice developed obesity and diabetes mellitus. (Hirschey 2011). De Filippis showed a decreased expression of essential nuclear encoded mitochondrial genes in insulin resistant cells. This included possible upstream regulators of SIRT3 such as PGC1α, NF-KB, PI3-kinase, and Akt. (De Filippis, Jing). Since the action of SIRT3 is to deacetylate proteins involved in cellular respiration and fatty acid oxidation, it would be reasonable to suggest that if its action is attenuated in insulin resistance, it could be at least partly responsible for decreased metabolic adaptation in insulin resistant individuals.

The focus of our study was to investigate the change in SIRT3 in different individuals. Specifically, we measured the quantity of SIRT3 present in muscle biopsies of non-diabetic subjects with differing levels of insulin sensitivity and compare pre-exercise to post-exercise levels of SIRT3. We expected to see an increase in the amount of SIRT3 following exercise in all subjects, but to a higher degree in those subjects who are more sensitive to insulin.

Research Materials and Methods:

Subjects: The sample muscle tissue was taken from muscle biopsies from individuals who were participating in several studies that were approved by the Institutional Review Board at Arizona State University, and all subjects gave written, informed consent. Seven lean and nine non-diabetic subjects took part in those studies. These patients did not regularly exercise and had not had any significant changes in weight for the preceding 6 months. Prior to participation, all subjects were screened by medical history, physical exam, electrocardiogram, a complete chemistry panel, and a glucose tolerance test. For the present study, six de-identified muscle biopsy samples and associated clinical characteristics were selected at random to undergo SIRT3 quantification.

Exercise testing: In order to determine max heart rate and peak aerobic activity, patients used a cycle ergometer protocol. Resistance was begun at 40 W and gradually increased until exhaustion. Criteria for test completion were RER greater than 1.1 or no further elevation in heart rate. The RER and V_{O2} were plotted and maximum V_{O2} was estimated as the V_{O2} which corresponded to RER of 1.1 on the graph. During this bout of exercise, patients were monitored with 12 lead electrocardiography.

Determination of M-value: At least one week following V_{O2} testing, subjects came in following an overnight fast, having consumed nothing but water. Insulin sensitivity was determined using a euglycemic hyperinsulinemic clamp similar to that which has previously been described (Defronzo). The antecubital vein was cannulated to allow infusion of insulin at 80 $mU \bullet m^{-2} \bullet min^{-1}$ and radiolabeled [6,6-^{2}H] glucose. Enrichment of deuterated glucose was determined using GC/MS as described (DeFilippis), and steady state equations were used to calculate rates of glucose disposal. The M-value was determined as the infusion rate of glucose required to

Exercise protocol: The exercise bout took place on a different day from the peak aerobic activity and euglycemic hyperinsulinemic clamp, as described (De Filippis). Each subject came in fasting overnight. The subject then underwent an exercise protocol on a recumbent stationary bicycle. Each set consisted of eight minutes at 70% of max HR, two minutes of 90% HR and then two minutes with no resistance. A total of four of these sets was performed consecutively. Subjects rested in a recumbent position following exercise.

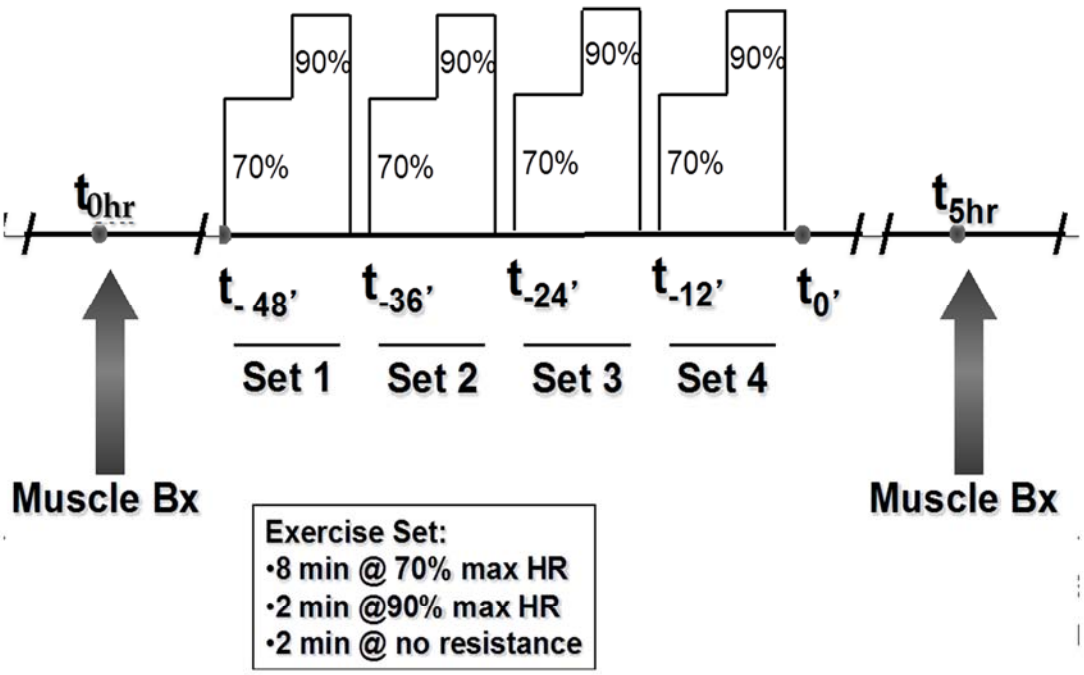

Figure 2: Representation of exercise protocol (not to scale). Before engaging in exercise, a biopsy was taken of the vastus lateralis under local anesthesia using a Bergstrom cannula. The subject then underwent a bout of cycling for eight minutes at 70% of heart rate, followed be 2 minutes at 90%, and finally 2 minutes of rest. This was repeated 3 times. A final biopsy was taken at 5 hours following completion of exercise.

Muscle biopsy: Fifty to 200g muscle biopsies were taken at various points during the protocol using a Bergstrom cannula. Each specimen was blotted dry of blood and immediately placed on dry ice. Mitochondria were isolated from fresh, non-frozen muscle biopsies as described (Lefort, Diabetes). Prior to analysis, Mitochondria were frozen and then homogenized using a Polytron in a detergent lysis buffer (Lefort et al, Diabetes). Lysates were stored at -80 C until use.

SDS-PAGE: Total SIRT3 protein was determined from the mitochondria of each subject in pre- and post-exercise biopsies. The muscle protein was denatured in SDS-polyacrylamide gel electrophoresis sample buffer. Equal amounts of protein were resolved on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Proteins were placed in a solution of TBST [20 mmol/l Tris · HCl (pH 7.5), 150 mmol/l NaCl, and 0.05% Tween 20] and nonfat dried milk for 1 hour at room temperature. The membranes were then incubated with SIRT3 antibodies (Aviva systems) at 1:1000 dilution at 4°C overnight. The next morning, the membranes was washed 5 minutes three times in TBST. Anti-rabbit IgG in TBST was then added in a 1:2000 dilution and the system allowed to incubate overnight at 4°C. Membranes were washed three consecutive times in TBST and then visualized on X-ray film using the enhanced chemiluminescence (ECL) detection system according to the manufacturer's protocol

Results:

Subjects:

Table 1 shows selected characteristics of the subjects. The range of the M-value as previously determined was from 3.7 to 9.7. BMI was calculated as mass (kg)/height $(m)^2$. This value ranged from 22.1 to 34.2. Raw values of SIRT3 for each subject at baseline and following exercise as well as quantity of complex II used to normalize the values. The normalized values were reported as well. Only one subject (GC48) showed an increase in amount of SIRT3 after normalization. The remaining four subjects manifested varying degrees of decreased SIRT3.

Subject Characteristics	
Sex(male/female)	4/1
Age, yr	31.6±4.7
BMI, kg/m^2	28.0±2.0
Body Weight, kg	85.3±7.7
Fat mass, kg	21.7±2.5
M, mg/kg/min	6.4±1.1
FPG, mmol/l	4.9±0.1
FPI, pmol/l	47.4±11.4
HbA_{1C}, %	5.2±0.1
Total Cholesterol, mmol/l	190.2±10.7
Triglycerides, mmol/l	147.8±34.2
HDL, mmol/l	52.0±9.8
LDL, mg/dl	113.6±9.2
SBP, mmHg	127.0±4.9
DBP, mmHg	80.2±5.5
Resting heart rate, beats/min	66.6±3.2
Maximum heart rate, beats/min	186.6±3.9

Table 1: Subject characteristics are baseline values expressed as means SE. BMI, body mass index; FM, fat mass; M, glucose disposal rate; FPG, fasting plasma glucose; FPI, fasting plasma insulin; Hb A1c, glycosylated hemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure.

SIRT3 protein determination by Western blot:

Quantification of Sirt3 protein showed mixed results. Only one (GC48) had an increase in Sirt3 protein after exercise. The rest of the samples had decreased levels of Sirt3 following exercise.

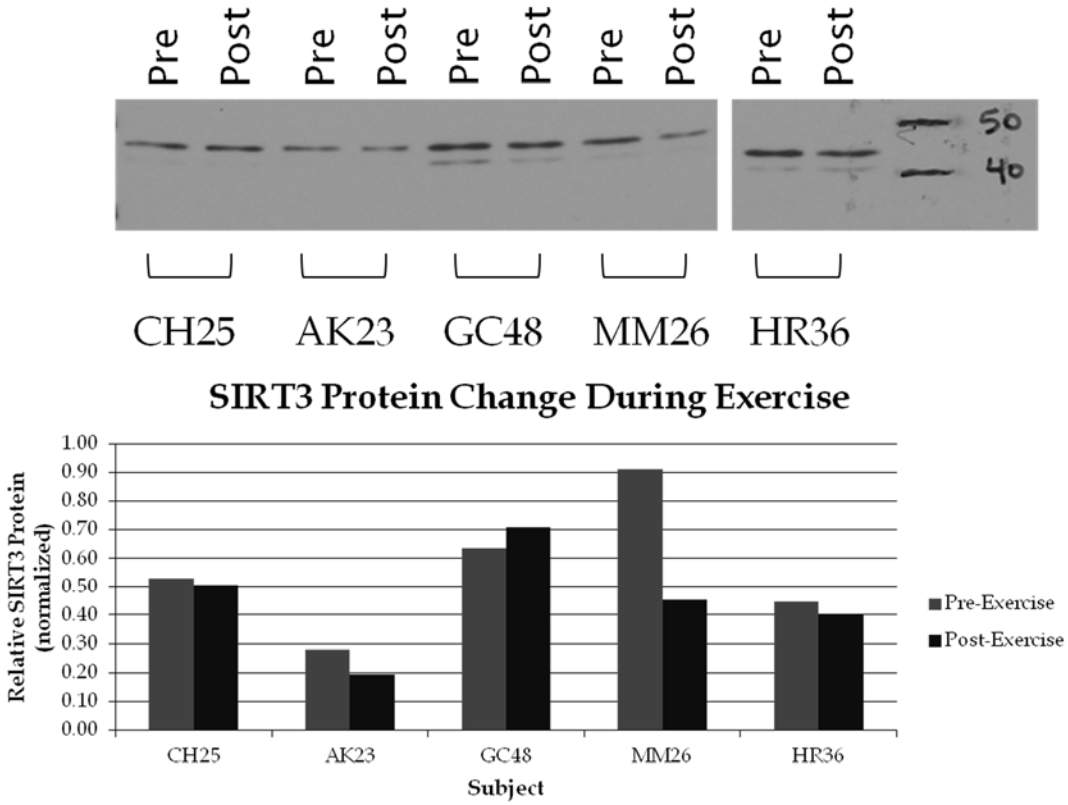

Figure 4: Effect of exercise on SIRT3 quantity before and after exercise in non-diabetic subjects. Muscle biopsies were taken pre- and post-exercise and quantity of SIRT3 measured using SDS-PAGE and band density quantification

SIRT3 quantification and M-Value:

The relative change in quantity of Sirt3 was compared to the previously determined M-Value. A moderate correlation in decreased Sirt3 and insulin sensitivity was demonstrated. There was a trend toward decreased change in SIRT3 protein with increasing M-value (r^2 = 0.7156).

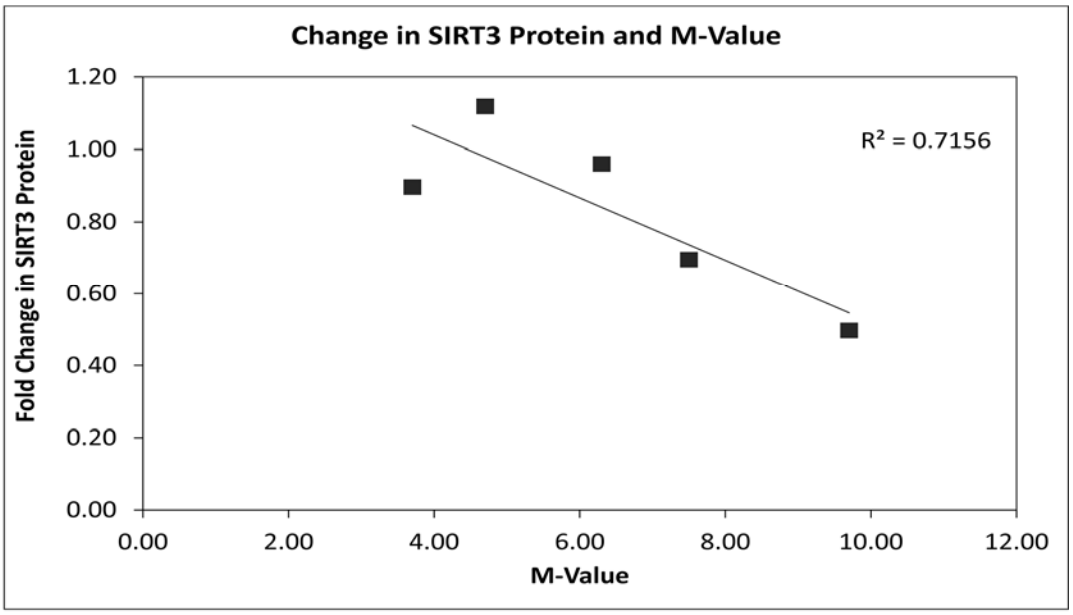

Figure 5: Graph showing relationship of change in Sirt3 value compared to M-value. The pre-determined M-value was plotted against the fold change of Sirt3 protein before and after exercise. This showed a moderate negative correlation with an r^2 value of 0.7156.

Discussion:

Cellular energy production involves a complex interplay of regulatory molecules and their substrates. The activity of these processes is based on a variety of different factors, including abundance of nutrition and physical activity of the cell. Inability to appropriately regulate the breakdown of energy results in a disruption of energy homeostasis and is likely a large contributor to disease states such as metabolic syndrome and diabetes.

The purpose of this study was to determine whether our previous findings that insulin resistant muscle had a reduced gene expression response to exercise extended to the main mitochondrial sirtuin, Sirt3. Sirt3 deacetylates numerous mitochondrial proteins and may contribute to metabolic flexibility in fuel choice. We found instead that Sirt3 protein abundance decreased 5 hours after exercise. Furthermore, the decrease was more pronounced in insulin sensitive muscle. Therefore, the concept of "exercise resistance", as we defined it (De Filippis), does not apply to Sirt3, the abundance of which changes more after exercise in insulin sensitive muscle.

The original hypothesis for this study were based on the following logic. As demand for energy in the form of ATP increases, insulin action stimulates fatty acid oxidation and oxidative phosphorylation to increase energy production. This relationship is consistent with the findings of DeFillipis who found insulin sensitive cells had a more pronounced increase in PGC-1α and AMPK, both of which are upstream upregulators of SIRT3 (DeFilippis).

During times of exercise, insulin resistant cells would have a dampened ability to influence downstream regulation of energy production via SIRT3 action. Dysfunction of SIRT3 in this condition would result in hyperacetylation of key proteins involved in fatty acid oxidation and oxidative phosphorylation, rendering them inactive. The resulting inability to increase production of ATP is consistent with reports of decreased oxidative capacity and reduced synthesis of ATP in metabolic syndrome and diabetes (Hirschey 2010, Szendroedi).

Nevertheless, our findings contradicted the original hypothesis. There could be several reasons for this finding. First, we do not know what transcription factors are responsible for

exercise-induced changes in Sirt3 expression, and they may differ from those responsible for expression of genes that are exercise resistant in insulin resistant muscle. Second, we only assayed protein abundance in this study. Changes in protein abundance can occur at the level of transcription, translation, or degradation. Therefore, it is possible that exercise in insulin sensitive muscle results in a decrease in gene transcription. It also is possible for there to have been a decrease in translation without a change in expression (unchanged mRNA levels). This could occur by exercise-induced increases in the expression of micro-RNAs that target the Sirt3 message. Very little is known regarding regulation of miRNAs after exercise in human muscle, however. Finally, it is possible that a greater amount of Sirt3 protein could have been targeted for degradation following exercise in insulin sensitive muscle. Unfortunately, although measurements of all of these factors are feasible, they are beyond the scope of the present study.

Conclusion:

Notwithstanding the results of our study, further elucidation of this pathway could offer greater understanding into the pathophysiology of the metabolic syndrome and identify potential pharmacological targets to maintain intra-mitochondrial levels of SIRT3. NAD+ is one of those targets as a metabolically active molecule used as a cofactor in the deacetylase reaction. A high ratio of NAD+/NADH has been observed in metabolic stress such as calorie restriction or prolonged fasting, which is consistent with increased quantity of SIRT3 (Ahn, Huang). Increasing the amount of NAD+ by supplementing key intermediates been suggested as a way of increasing SIRT3 (Yoshino). This could have implications in preventing progression of metabolic

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