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Regulation of Skeletal Muscle Insulin Action by SIRT1 and STAT3

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Amanda T. White

Committee in charge:

       Professor Simon Schenk, Chair
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2013

The Dissertation of Amanda T. White is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2013

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ACKNOWLEDGEMENTS

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ABSTRACT OF THE DISSERTATION

Regulation of Skeletal Muscle Insulin Action by SIRT1 and STAT3

by

Amanda T. White

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2013

Professor Simon Schenk, Chair

Professor Richard Lieber, Co-Chair

Muscle insulin resistance is a primary metabolic defect underlying the etiology of type 2 diabetes. Consumption of a high-fat/high-calorie diet (HFD) is associated with the development of insulin resistance and type 2 diabetes, while calorie restriction (CR) (60% of ad libitum [AL] food intake) robustly reverses muscle insulin resistance. Remarkably, the signaling mechanisms linking nutrient status to muscle insulin sensitivity are incompletely defined. Considering that the prevalence of diabetes

continues to grow at an alarming rate, addressing this fundamental gap in knowledge has the potential to significantly impact human health. Thus, the objective of this dissertation was to define the mechanisms underlying muscle insulin action in the context of nutrient availability. Sirtuin 1 (SIRT1), a NAD^+-dependent protein deacetylase, has been proposed to link perturbations in nutrient availability to muscle insulin action. In line with this, SIRT1 activity in skeletal muscle is increased by CR and decreased by HFD. Furthermore, mice with transgenic overexpression of SIRT1 exhibit a metabolic profile resembling CR, and small molecule activators of SIRT1 enhance insulin sensitivity in rodent models of insulin resistance. Nevertheless, little is known regarding the regulatory role of SIRT1 in muscle insulin action, particularly in vivo. We recently identified the SIRT1 target, signal transducer and activator of transcription 3 (STAT3), as an important regulator of muscle insulin sensitivity in response to CR. Furthermore, STAT3 is activated in insulin resistant states and inhibition of STAT3 in liver restores hepatic insulin sensitivity. Thus, we hypothesized that SIRT1 and STAT3 regulate muscle insulin sensitivity in response to nutrient availability. To address this hypothesis, we investigated muscle insulin signaling and sensitivity in response to HFD and CR using novel mouse models in which we modulated SIRT1 and STAT3 activity in skeletal muscle. Our findings demonstrate that muscle-specific overexpression of SIRT1 does not enhance muscle insulin action under AL or CR conditions or prevent HFD-induced obesity or insulin resistance. Finally, we showed that knockout of STAT3 in skeletal muscle does not enhance muscle insulin sensitivity on a control diet or HFD. Taken together, the studies from this dissertation broaden our understanding of the roles of SIRT1 and STAT3 in muscle insulin action in response to nutrient availability.

Introduction

Skeletal muscle is a key metabolic tissue responsible for the uptake of macronutrients, particularly glucose, after meal ingestion (1,2). This process is insulin-dependent, and in nutrient excess and obesity the ability of skeletal muscle to take up glucose in response to insulin is impaired, resulting in a state of insulin resistance (1,2). If left unchecked, this increases an individual's risk for developing type 2 diabetes (1,2). Accompanying a significant monetary burden, the personal cost of obesity and type 2 diabetes is considerable, including increased risk of heart disease and stroke, kidney disease, amputation, and even death (3). In humans and in rodent models, a high-fat/high calorie diet (HFD) can induce insulin resistance in skeletal muscle (4–6) while calorie restriction (CR) robustly reverses muscle insulin resistance (7–16). Taken together, this suggests that skeletal muscle is intrinsically sensitive to perturbations in nutrient flux, although how muscle detects, integrates, and translates such perturbations into a metabolic adaptation is unknown.

The mammalian ortholog of *Sir2*, sirtuin 1 (SIRT1), a NAD^{+}-dependent protein deacetylase, has been proposed to link perturbations in energy flux to subsequent cellular adaptations, including insulin action (17–22). Polymorphisms of the SIRT1 gene are associated with human health, including healthy aging, energy expenditure, adaptive response to lifestyle interventions, and risk for developing obesity, diabetic nephropathy, and type 2 diabetes (23–29). Mice with whole-body overexpression of SIRT1 demonstrate a metabolic profile resembling CR (30,31), and pharmacological activation of SIRT1 improves skeletal muscle insulin sensitivity in rodent models of insulin

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resistance (32,33). Additionally, SIRT1 overexpression in skeletal muscle cell models enhances insulin sensitivity under insulin resistant conditions (34,35). Nevertheless, while the therapeutic potential of SIRT1 activation to treat muscle insulin resistance is well appreciated (17–22), studies supporting this notion have primarily been conducted in vitro or have used activators of SIRT1 (32–42). This is important as SIRT1 is highly expressed in other key metabolic tissues such as brain, adipose tissue, and liver, and therefore it is possible that improvements in muscle insulin sensitivity following pharmacological treatment occur due to activation of SIRT1 in these other tissues. Thus, the role of skeletal muscle SIRT1 in insulin action in response to altered nutrient availability remains to be fully defined.

We recently identified the SIRT1 target, signal transducer and activator of transcription 3 (STAT3), as a potential mediator of skeletal muscle insulin sensitivity in response to reduced nutrient availability (7). Supporting this notion, circulating cytokines such as IL-6 that are elevated in insulin-resistant states enhance STAT3 activation (43). In response to cytokine binding, STAT3 is recruited to phosphotyrosine residues on activated receptors and is then itself phosphorylated at a key tyrosine residue (Y705) by Janus kinase (JAK). This leads to STAT3 dimerization and translocation to the nucleus where it affects transcription of its target genes, including suppressor of cytokine signaling 3 (SOCS3) (44). It has been proposed that STAT3 activation causes insulin resistance via increased SOCS3 activity and inhibition of insulin signaling at the level of the insulin receptor and the insulin receptor substrate proteins (45,46). STAT3 has been implicated as a mediator of IL-6-induced insulin resistance (47–56), and indeed, inhibition of STAT3 activity in liver cells and adipocytes protects against IL-6 induced

impairments in insulin signaling (54), and amino acid-induced insulin resistance in hepatocarcinoma cells is prevented by STAT3 knockdown (52). However, many of these studies focused on STAT3 in tissues other than muscle and less is known about the role of skeletal muscle STAT3 in insulin sensitivity. What has been demonstrated is that STAT3 phosphorylation is increased in muscle from patients with impaired glucose tolerance and constitutive activation of STAT3 in skeletal muscle, in vitro, leads to insulin resistance that is attenuated by STAT3 inhibition (57,58). However, the role of STAT3, in vivo, in the development of skeletal muscle insulin resistance has not been determined and is somewhat controversial. Indeed, while IL-6 increases activation of STAT3, this does not consistently lead to skeletal muscle insulin resistance in vivo (53,59), and in some cases even improves insulin sensitivity (60,61). Thus, the mechanistic role of STAT3 in skeletal muscle insulin sensitivity under control and insulin-resistant conditions, in vivo, remains to be fully elucidated.

The three studies of this Dissertation were designed to investigate whether modulation of SIRT1 and STAT3 activity in muscle under different nutrient conditions would enhance muscle insulin sensitivity. We hypothesized that increased SIRT1 activity or decreased STAT3 activity in skeletal muscle would improve muscle insulin action. Specifically, since CR activates SIRT1 and decreases STAT3, we investigated whether overexpression of SIRT1 or knockout of STAT3 in skeletal muscle recapitulates the beneficial effects of CR on skeletal muscle insulin action. Furthermore, since SIRT1 activity is decreased and STAT3 activity is increased in insulin resistant states, we investigated whether augmentation of SIRT1 or knockout of STAT3 in skeletal muscle would prevent insulin resistance and glucose intolerance in a HFD mouse model.

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CHAPTER 1:

Skeletal muscle-specific overexpression of SIRT1 does not enhance whole-body energy expenditure or insulin sensitivity in young mice

Abstract

The NAD^+-dependent protein deacetylase sirtuin (SIRT)1 is thought to be a key regulator of skeletal muscle metabolism. However, its precise role in the regulation of insulin sensitivity is unclear. Accordingly, in Study 1 we sought to determine the effect of skeletal muscle-specific overexpression of SIRT1 on skeletal muscle insulin sensitivity and whole-body energy metabolism. At 10 weeks of age, mice with muscle-specific overexpression of SIRT1 and their wild-type littermates were fed a standard diet with free access to chow or a calorie-restricted (60% of standard) diet for 20 days. Energy expenditure and body composition were measured by indirect calorimetry and magnetic resonance imaging, respectively. Skeletal muscle insulin-stimulated glucose uptake was measured ex vivo in soleus and extensor digitorum longus muscles using a 2-deoxyglucose uptake technique with a physiological insulin concentration of 360 pmol/l (60 μU/ml). *Sirt1* mRNA and SIRT1 protein levels were increased by approximately 100- and 150-fold, respectively, in skeletal muscle of mice with SIRT1 overexpression compared with wild-type mice. Despite this large-scale overexpression of SIRT1, body composition, whole-body energy expenditure, substrate oxidation and voluntary activity were comparable between genotypes. Similarly, skeletal muscle basal and insulin-stimulated glucose uptake were unaltered with SIRT1 overexpression. Finally, while

20 days of energy restriction enhanced insulin-stimulated glucose uptake in skeletal muscles of wild-type mice, no additional effect of SIRT1 overexpression was observed. These results demonstrate that upregulation of SIRT1 activity in skeletal muscle does not affect whole-body energy expenditure or enhance skeletal muscle insulin sensitivity in young mice on a standard diet with free access to chow or in young mice on energy-restricted diets.

Introduction

Skeletal muscle insulin resistance is a primary metabolic defect underlying the development of type 2 diabetes (1). Recently, the mammalian ortholog of *Sir2*, sirtuin (SIRT)1, an NAD^+-dependent protein deacetylase, was proposed to be a key signaling node linking alterations in energy flux to insulin action (2,3). Mice with whole-body overexpression of SIRT1 demonstrate a metabolic phenotype similar to that seen with energy intake restriction (CR) (4,5), while pharmacological activation of SIRT1 improves skeletal muscle insulin sensitivity in rodent models of insulin resistance (6,7). Although the therapeutic potential of SIRT1 activation for the treatment of skeletal muscle insulin resistance is well appreciated, studies supporting this notion have primarily been conducted in vitro or have used pharmacological activators of SIRT1 (6-9). Given that SIRT1 is highly abundant in other key metabolic tissues (e.g. brain, adipose tissue, liver), it is possible that improvements in muscle insulin sensitivity following pharmacological treatment occur secondarily to activation of SIRT1 in these other tissues.

In L6 myotubes and primary muscle myotubes, SIRT1 overexpression has been shown to enhance insulin-stimulated activation of Akt, while SIRT1 knockdown has the opposite effect (8). However, the functional effect of these changes in Akt signaling on glucose uptake was not determined (8). In contrast, in C2C12 myotubes, SIRT1 overexpression did not enhance insulin-stimulated activation of Akt or glucose uptake (9). Similarly, in a gain of function mouse model that included modest overexpression of SIRT1 in skeletal muscle, glucose disposal during a hyperinsulinaemic–euglycaemic clamp was not enhanced (4). We have also recently demonstrated in skeletal muscle that

knockout of SIRT1 deacetylase activity did not impair in vivo or ex vivo insulin-stimulated glucose uptake (10). It is clear from these studies that the precise role of SIRT1 in the regulation of skeletal muscle insulin sensitivity remains controversial. To address this, we generated mice with muscle-specific overexpression of SIRT1 (herein referred to as mOX mice) to determine whether increased SIRT1 activity in skeletal muscle enhances muscle insulin sensitivity. In addition, since CR robustly enhances skeletal muscle insulin action (10-12) and increases SIRT1 activity (10), we sought to determine whether SIRT1 overexpression in conjunction with CR would result in an additional enhancement of muscle insulin sensitivity.

Methods

Animals. All studies were conducted in male mice on a C57BL/6 background. To generate mOX mice, mice harboring loxP sites flanking a transcriptional stop element (FLX^STOP) upstream of the Sirt1 gene (13) (kindly provided by D. A. Sinclair, Harvard Medical School, Boston, MA, USA) were crossed with mice expressing Cre recombinase (Cre) under the control of the muscle creatine kinase (MCK) promoter (Cre-MCK). After Cre-mediated recombination, the STOP element is removed and Sirt1 gene expression is driven by a constitutive promoter (CAGGS) that lies immediately upstream of Sirt1 (13). The FLX^STOP mice that lack Cre-MCK are referred to herein as wild-type (WT) and were used as controls for all studies. Ex vivo measurements of basal and insulin-stimulated glucose uptake and in vivo supra-physiological insulin stimulation were performed from 13:00 to 15:00 hours in anaesthetized (150 mg/kg Beuthanasia-D Special; Schering-Plough Animal Health, Union, NJ, USA) mice. For these experiments, mice with free access to a standard diet were fasted for 4 to 6 h, while CR mice received their food at 17:00 hours on the previous day. Mice were housed on a 12 h light/12 h dark cycle. All experiments were approved by and conducted in accordance with the Animal Care Program at the University of California, San Diego.

CR diet. The CR studies were performed as previously described (10). Briefly, at 9 weeks of age, food intake of mice with free access to a standard chow diet (CON) (catalogue number 5001; LabDiet, Brentwood, MO, USA) was measured daily at 17:00 hours for 7 days. At 10 weeks of age, mice either continued CON intake or were

switched to a CR (60% of CON) diet for 20 days. Food was provided daily to CR mice between 16:00 and 17:00 hours.

Isolated muscle 2-deoxyglucose uptake. Ex vivo muscle insulin sensitivity was measured by the 2-deoxyglucose uptake (2DOGU) technique in isolated soleus (SOL) and extensor digitorum longus (EDL) muscles as described previously (10). The insulin concentration for insulin-treated muscles was 360 pmol/l (60 μU/ml) (Humulin R; Eli Lilly, Indianapolis, IN, USA). Frozen SOL and EDL muscles were weighed to 0.1 mg, homogenized and 2DOGU was calculated as previously described (10).

In vivo supra-physiological insulin stimulation. In anaesthetized CON-fed mice, one gastrocnemius (GA) was dissected and mice were intravenously injected with 6,000 pmol/kg (1 U/kg) insulin (Humulin R; Eli Lilly) diluted in a sterile 0.9% wt/vol. saline solution containing 1% wt/vol. BSA. At 5 min after the insulin injection, the contralateral GA was dissected. Immediately after dissection, the GA was rinsed in sterile saline, blotted dry and frozen in liquid nitrogen. Samples were homogenized for SDS-PAGE as previously described (10).

Nuclear isolation. Nuclear fractions were isolated from 50 mg GA muscle using a commercially available kit (NE-PER, catalogue number 78835; Thermo Fisher Scientific, Rockford, IL, USA) as per the manufacturer's instructions. The total protein concentration was determined by the bicinchoninic acid assay (Thermo Fisher Scientific).

SDS-PAGE. SDS-PAGE was performed using standard methods, as previously described (10). Briefly, 20 μg protein was boiled in 1X SDS buffer and loaded onto 3 to 8% TRIS-acetate gels. The following primary antibodies from Cell Signaling Technology (Danvers, MA, USA) were used for immunoblotting: phosphorylated Akt (p-Akt)^{Ser473}, catalogue number 9271; p-Akt^{Thr308}, catalogue number 9275; Akt, catalogue number 9272; p53, catalogue number 2524; glycogen synthase kinase (GSK)-3α/β, catalogue number 5676; phosphorylated GSK (p-GSK)-3α/β$^{Ser21/9}$, catalogue number 9331; acetylated p53 (Ac-p53)^{Lys379}, catalogue number 2570; insulin receptor (IR)β, catalogue number 3025; phosphorylated IR (p-IR)β$^{Tyr1150/1151}$, catalogue no. 3024. Other primary antibodies used were: histone H2B, catalogue number ab9408 (AbCam, Cambridge, MA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalog number 10R-G109a (Fitzgerald Industries, Acton, MA, USA).

Real-time RT-PCR. RNA was extracted from EDL, GA, plantaris and SOL muscles, and from liver and adipose tissue using the phenol/chloroform method. cDNA was synthesized from 1 μg of RNA using a kit (i-Script cDNA Synthesis, catalogue number 170-8891; Bio-Rad Laboratories, Hercules, CA, USA). Real-time RT-PCR was performed using a Mastercycler ep realplex (Eppendorf, Hamburg, Germany), SYBR Green Taq Polymerase (catalogue number 17-8880; Bio-Rad) and custom-designed primers. Target gene expression for each sample was calculated relative to Gapdh. Primer sequences were as follows: Gapdh 5′-ATTCAACGGCACAGTCAAG-3′ and 3′-TCCACGACATACTCAGCAC-5′; Sirt1 5′-GGCTACCGAGACAACCTCCTG-3′ and 3′-AGTCCAGTCACTAGAGCTGGC-5′.

Blood glucose and plasma insulin. Fasting blood glucose was measured in conscious mice (Ascensia Contour; Bayer Healthcare, Mishawaka, IN, USA) before anesthesia for 2DOGU. Plasma insulin concentrations were measured using a commercially available kit (Mouse Ultrasensitive Insulin ELISA, catalogue number 80-INSMSU-E01; Alpco Diagnostics, Salem, NH, USA) according to the manufacturer's instructions. For this, blood was collected in EDTA from the inferior vena cava of anaesthetized mice and centrifuged (5,000 g, 5 min, 4°C), and the supernatant fraction was frozen (−80°C) for subsequent measurement.

Measurement of AMP-activated protein kinase activity. GA muscle powdered under liquid nitrogen was lysed by shaking on an orbital shaker (1,500 g, 4°C) in AMP-activated protein kinase (AMPK) lysis buffer (50 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l dithiothreitol, 0.1 mmol/l benzamidine) with 0.1 mmol/l phenylmethanesulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor and 1% vol./vol. Triton X-100. Lysates were cleared by centrifugation for 15 min at 16,200 g and 4°C. Protein content was determined (bicinchoninic acid assay; Fisher Scientific, Loughborough, UK). AMPK activity was determined as previously described (14). Briefly, AMPK α1 and α2 antibodies (a kind gift from G. Hardie, University of Dundee, Dundee, Scotland, UK) were used to independently immunoprecipitate α1 and α2 complexes. AMPK activity was calculated as the difference in counts between AMARA (AMPK substrate: AMARAASAAALARRR)-containing and AMARA-negative samples as pmol ATP incorporated per min per mg protein (or mU/mg).

Energy expenditure and body composition. The volume of oxygen consumption, the volume of carbon dioxide expired per unit time, the respiratory exchange ratio (RER), physical activity (total movement [x-total], ambulatory activity [x-amb], rearing activity [z-total]) and food intake were assessed using the Comprehensive Lab Animals Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA). Measurements were made for three consecutive days and values were averaged from the light and dark phases recorded on days 2 and 3. Fat oxidation (kJ/h) was calculated from values for RER and heat (kJ/h), as previously described (15). Body composition was measured by magnetic resonance imaging (MRI) (EchoMRI, Houston, TX, USA).

Statistics. Statistical analyses were performed using SigmaPlot 11.2 (Systat Software, Chicago, IL, USA). Data were analyzed by two-way ANOVA (with repeated measures when necessary) for main effects of diet and genotype, followed by Tukey's post-hoc analysis, with significant differences at $p < 0.05$. Since for 2DOGU and insulin signaling data, there was an effect of insulin vs. basal for all groups, a two-way ANOVA was performed within basal and within insulin samples. All data are expressed as mean $\pm$ SEM.

Results

Muscle-specific SIRT1 overexpression occurs in mOX mice. mRNA levels were approximately 100-fold higher in skeletal muscle from mOX vs. WT mice, but were comparable in adipose tissue and liver (Figure 1.1a). SIRT1 protein levels were approximately 150 times higher than WT in soleus and EDL muscles from mOX mice (Figure 1.1b). To determine whether this large-scale overexpression of SIRT1 resulted in a functional increase in SIRT1 activity, we measured Ac-p53^{Lys379}, a known target of SIRT1 (16), in nuclear lysates from GA muscle. In line with the increased gene and protein expression of SIRT1 in mOX mice, nuclear Ac-p53^{Lys379}:total p53 was significantly reduced by approximately 35% in mOX vs. WT mice (Figure 1.1c). Total p53 abundance in the nucleus was approximately 30% higher in mOX vs. WT mice (WT 1.00 ± 0.01, mOX 1.31 ± 0.11, $p < 0.05$, n $= 4$) (Figure 1.1c). However, there was no difference in histone H2B abundance, indicating equal loading of the nuclear fraction (WT 1.00 ± 0.03, mOX 1.02 ± 0.01, $p > 0.05$, n $= 4$) (Figure 1.1c).

Energy expenditure, spontaneous activity, food intake and supra-physiological insulin signaling are not altered in mOX mice. While expected diurnal variations were observed, large-scale overexpression of SIRT1 in skeletal muscle did not result in alterations in, RER or fat oxidation in mOX mice (Figure 1.2a-c). Moreover, the number of recorded beam breaks as a measure of total spontaneous activity (Figure 1.2d), ambulatory activity (Light: WT 45 ± 2, mOX 70 ± 16 beam breaks; Dark: WT 254 ± 66,

mOX 271 ± 25 beam breaks, $p > 0.05$) and rearing activity (Light: WT 9 ± 21, mOX 13 ± 5 beam breaks; Dark: WT 97 ± 45, mOX 132 ± 32 beam breaks, $p > 0.05$) did not differ between genotypes. Food intake was similar in WT and mOX mice (Light: WT 1.34 ± 0.16 g, mOX 1.27 ± 0.12 g; Dark: WT 3.01 ± 0.24 g, mOX 3.37 ± 0.11 g, $p > 0.05$). In addition, supra-physiological, in vivo insulin stimulation for 5 min resulted in robust increases in phosphorylation of IRβ (p-IRβ$^{Tyr1150/1151}$), Akt (p-Akt^{Ser473} and p-Akt^{Thr308}) and GSK3β (p-GSK3β^{Ser9}) above basal in GA muscle, but there were no differences in these variables between genotypes (Figure 1.2e, f).

Body composition, fasting blood variables and skeletal muscle AMPK activity are not altered in mOX mice. In CON-fed mice, body weight, lean mass, fat mass and percent body fat did not differ between WT and mOX mice. With CR, these variables decreased similarly in both genotypes (Figure 1.3a,b). No effect of genotype on fasting glucose concentrations was observed in CON-fed mice, while CR decreased fasting glucose levels to similar degrees in WT and mOX mice (Figure 1.3c). No significant effects of diet or genotype on plasma insulin concentrations were observed (WT-CON 69 ± 7, WT-CR 42 ± 6, mOX-CON 42 ± 4, mOX-CR 46 ± 11 pmol/l, $p > 0.05$, n = 6–10). We also found no effect of diet or genotype on AMPKα1 or α2 activity (Figure 1.3d).

Skeletal muscle insulin sensitivity and signaling are not enhanced in mOX mice. To determine the effects of muscle-specific SIRT1 overexpression on insulin-stimulated glucose uptake, we measured basal and insulin-stimulated glucose uptake ex

vivo in isolated SOL and EDL muscles. We observed no effects of genotype or diet on basal glucose uptake (WT-CON 0.45 ± 0.03, WT-CR 0.39 ± 0.04, mOX-CON 0.36 ± 0.06, mOX-CR 0.34 ± 0.06 µmol $[20 \text{ min}]^{-1}$ $[\text{g muscle}]^{-1}$, $p > 0.05$). Insulin-stimulated 2DOGU (insulin 2DOGU − basal 2DOGU) in SOL and EDL muscles did not differ between WT and mOX mice on an CON diet (Figure 1.4a). Moreover, while CR enhanced insulin-stimulated 2DOGU in SOL and EDL muscles as compared with CON in both genotypes, no additional effect on insulin sensitivity was seen in mOX mice (Figure 1.4a). Complementing the 2DOGU findings, insulin-stimulated p-Akt$^{\text{Ser473}}$ and p-Akt$^{\text{Thr308}}$ in SOL muscle were enhanced by CR above CON in WT and mOX mice (Figure 1.4b,c). Downstream of Akt signaling, insulin-stimulated p-GSK-3β$^{\text{Ser9}}$ was enhanced during CR vs. CON in WT mice and showed a trend ($p = 0.054$) to increase in mOX-CR mice (Figure 1.4b,c). Notably, in insulin-treated muscles from CR mice, p-Akt$^{\text{Thr308}}$ and p-GSK-3β$^{\text{Ser9}}$ were significantly lower, and p-Akt$^{\text{Ser473}}$ showed a trend to be lower ($p = 0.065$) in mOX vs. WT mice (Figure 1.4b,c). In CON-fed mice, there were no differences in p-Akt$^{\text{Ser473}}$, p-Akt$^{\text{Thr308}}$ or p-GSK-3β$^{\text{Ser9}}$ between genotypes (Figure 1.4b,c). Moreover, no effects of CR or genotype were observed on total Akt or GSK3β abundance (Figure 1.4b), or on basal p-Akt$^{\text{Ser473}}$ (WT-CON 0.13 ± 0.03, WT-CR 0.07 ± 0.01, mOX-CON 0.07 ± 0.02, mOX-CR 0.08 ± 0.03, relative to WT-CON insulin), p-Akt$^{\text{Thr308}}$ (WT-CON 0.08 ± 0.02, WT-CR 0.05 ± 0.01, mOX-CON 0.08 ± 0.02, mOX-CR 0.10 ± 0.03, relative to WT-CON insulin), or p-GSK-3β$^{\text{Ser9}}$ (WT-CON 0.37 ± 0.03, WT-CR 0.33 ± 0.04, mOX-CON 0.37 ± 0.05, mOX-CR 0.31 ± 0.05, relative to WT-CON insulin).

Discussion

SIRT1 has been proposed to be an important regulator of insulin sensitivity in key insulin target tissues, including skeletal muscle (2,3). To date, however, the precise role of SIRT1 in the regulation of skeletal muscle insulin sensitivity has not been fully elucidated. To address this, in Study 1 we generated mice with muscle-specific overexpression of SIRT1. Our results demonstrate that large-scale overexpression of SIRT1 in skeletal muscle does not enhance muscle insulin sensitivity or alter energy expenditure in young, lean mice. In addition, SIRT1 overexpression in CR mice did not further enhance skeletal muscle insulin action. Taken together, these results suggest that augmenting SIRT1 activity in skeletal muscle does not increase skeletal muscle insulin sensitivity or whole-body energy expenditure in young, lean mice.

The therapeutic potential of SIRT1 activation for the treatment of muscle insulin resistance has been proposed for some years (2,3). Accordingly, treatment of obese rodents with SIRT1-specific activators has been demonstrated to improve in vivo skeletal muscle insulin action in response to a supra-physiological insulin infusion (6,7). An important limitation of pharmacological studies is that activation of SIRT1 occurs in other metabolic tissues, including adipose tissue, liver and brain. Thus, it is not possible to determine whether improved muscle insulin sensitivity is due to direct effects in muscle or whether it manifests secondarily to SIRT1 activation in these other tissues. Indeed, SIRT1 activation inhibits inflammatory pathways in macrophages and reduces adipose tissue inflammation in obese rodents (17,18), which can have beneficial effects on peripheral insulin sensitivity, as reviewed by others (19). Few studies have directly investigated the role of SIRT1 in skeletal muscle insulin action. Frojdo and colleagues

demonstrated in primary muscle and L6 myotubes that SIRT1 overexpression enhances, while SIRT1 knockdown impairs insulin-stimulated activation of Akt (8). Insulin-stimulated glucose uptake was not determined in this study, so it is not known whether these changes in Akt signaling resulted in functional alterations in insulin sensitivity. Additionally, the authors used supra-physiological (10,000–100,000 pmol/l) insulin concentrations, which may not accurately represent alterations in insulin sensitivity under physiological conditions. Our results clearly demonstrate that SIRT1 overexpression does not enhance skeletal muscle insulin action in response to a physiological insulin concentration. Supporting our findings, SIRT1 overexpression in C2C12 myotubes did not enhance insulin-stimulated glucose uptake or Akt signaling at a supra-physiological (100 nmol/l) insulin concentration (9). Similarly, we found no difference in in vivo insulin signaling in response to a supra-physiological insulin dose. In addition, knockout of SIRT1 deacetylase activity in skeletal muscle did not impair in vivo or ex vivo insulin-stimulated glucose uptake, although SIRT1 is required for CR to enhance muscle insulin action (10). Taken together, while cell-based studies potentially support a role for SIRT1 in the regulation of muscle insulin action, studies in bona fide skeletal muscle question a possible role for SIRT1 in the regulation of insulin action in this tissue under physiological conditions.

In this investigation, we studied lean, young mice; however, it is possible that any beneficial effects of muscle SIRT1 overexpression may only manifest in an insulin-resistant model. Indeed, when C2C12 myotubes were stimulated with 100,000 pmol/l insulin, SIRT1 overexpression prevented fatty-acid-induced insulin resistance (9). In contrast to these studies in C2C12 myotubes, Banks et al (4) found in a whole-body

SIRT1 gain of function model that skeletal muscle insulin sensitivity (as measured by a hyperinsulinaemic–euglycaemic clamp) was not improved in 11-month-old mice or in a high-fat diet model of insulin resistance. These findings provide additional support for the contention that SIRT1 does not directly regulate skeletal muscle insulin action, even in models of insulin resistance, though studies in a muscle-specific model, such as the mOX mouse, will help to add weight to this line of argument. It is important to acknowledge that germline overexpression of SIRT1 occurs in our model, as well as large-scale (i.e. approximately 150-fold) SIRT1 overexpression. Thus, it is possible that chronic overexpression of SIRT1 or such large-scale overexpression may preempt changes that are beneficial to insulin action. Notably, however, in the aforementioned study by Banks et al (4), muscle insulin action was not affected by two- to threefold SIRT1 overexpression from germline.

CR enhances insulin-stimulated glucose uptake due in part to greater activation of insulin signaling at the level of phosphatidylinositol 3 kinase (10,12) or Akt (10,12,20). As expected, in WT mice we observed beneficial effects of CR on insulin-stimulated glucose uptake and on activation of insulin signaling at Akt and its downstream target GSK3β. Since we have previously demonstrated that skeletal muscle SIRT1 deacetylase activity is required for the effects of CR on muscle insulin sensitivity to occur (10), and mice with whole-body overexpression of SIRT1 demonstrate a metabolic phenotype similar to that seen with CR (4,5), we hypothesized that an increase in SIRT1 activity in combination with CR would have an additional effect on muscle insulin action. However, we observed no additional effect on muscle insulin-stimulated glucose uptake during CR in mOX mice in response to a physiological insulin concentration. We interpret these data

to suggest that endogenous levels of SIRT1 are sufficient to maximize the ability of CR to enhance muscle insulin action. Interestingly, in muscle from CR mice, but not in that from CON mice, insulin-stimulated activation of p-Akt^{Thr308} and p-GSK3α/β was reduced in mOX compared with WT mice. While the reasons for this are not readily apparent, measurements of insulin signaling were made in muscles that were stimulated with insulin for 50 min, and if mOX mice display faster insulin signaling kinetics, these signals may be turned off faster in mOX mice with CR. Indeed, we found no differences in the activation of insulin signaling at the level of IR, Akt or GSK3β between WT and mOX mice at 5 min after a supra-physiological insulin dose.

Since SIRT1 is thought to be involved in the regulation of mitochondrial biogenesis in muscle (21,22), it is possible that SIRT1 overexpression in skeletal muscle may affect whole-body energy expenditure and/or substrate utilization. In line with this, energy expenditure is slightly elevated in mice with moderate whole-body (including skeletal muscle) overexpression of SIRT1 (23). In contrast, mice with SIRT1 gain of function that resulted in modest SIRT1 overexpression in skeletal muscle demonstrate decreased oxygen consumption (4). Intriguingly, whole-body SIRT1-null mice display increased metabolic rates and a decrease in RER (24). The reasons for such contrasting results are not readily apparent, but may be due to the different strains of mice used or to the way in which SIRT1 was manipulated. In any case, the above-mentioned studies certainly suggest that SIRT1 can play a role in the regulation of whole-body energy expenditure, although they do not reveal which tissue or tissues contribute to this effect. In a mouse model of SIRT1 overexpression in which SIRT1 was not overexpressed in muscle or liver, increased oxygen consumption was observed (5), suggesting that tissues

other than muscle or liver may mediate the aforementioned effects of SIRT1 on energy expenditure. In further support of this notion, decreased energy expenditure was observed in a recent study using brain-specific knockdown of SIRT1 (25). Our present results demonstrate that skeletal muscle SIRT1 overexpression does not affect whole-body energy expenditure, RER or fat oxidation. Additionally, we have found that loss of SIRT1 deacetylase activity in mouse skeletal muscle has no effect on energy expenditure (White A.T., Schenk S., unpublished observations). Thus, skeletal muscle SIRT1 does not affect energy expenditure and reported alterations in energy expenditure in models with manipulation of SIRT1 are likely to be due to adaptations in tissues other than muscle.

SIRT1 has been proposed to be an important regulator of AMPK activity through its ability to regulate liver kinase B1 (26-28), while other studies have demonstrated AMPK to be an important regulator of SIRT1 (29,30). AMPK has also been observed to be activated by CR and consequently is thought to mediate the beneficial effects of CR on insulin sensitivity (31,32). Here, we measured skeletal muscle AMPK (α1 and α2) activity to determine whether it was altered in response to SIRT1 overexpression and/or CR. We observed no effect of SIRT1 overexpression or CR on skeletal muscle AMPKα1 or α2 activity, which is in line with previous observations that AMPK activation is not altered in skeletal muscle during CR (10,12,20,33). Combined with our recent findings demonstrating that knockout of SIRT1 deacetylase activity had no effect on AMPK activation (10,34), the present results suggest that SIRT1 is not a major regulator of AMPK activity in skeletal muscle.

In summary, in Study 1 we have demonstrated that SIRT1 overexpression in skeletal muscle does not enhance skeletal muscle insulin signaling or insulin sensitivity, affect AMPK activity, or alter whole-body energy expenditure. Furthermore, while CR robustly improves muscle insulin action, SIRT1 overexpression has no additional effect above and beyond that achieved by CR alone. Together with other recent studies (4), these data demonstrate that overexpression of SIRT1 does not improve muscle insulin action in young mice.

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Chapter 1, in full, is a reprint of the material as it appears in Diabetologia 2013 (with edits for style). A. T. White, C. E. McCurdy, A. Philp, D. L. Hamilton, C. D. Johnson, S. Schenk. The dissertation author was the primary investigator and author of this paper.

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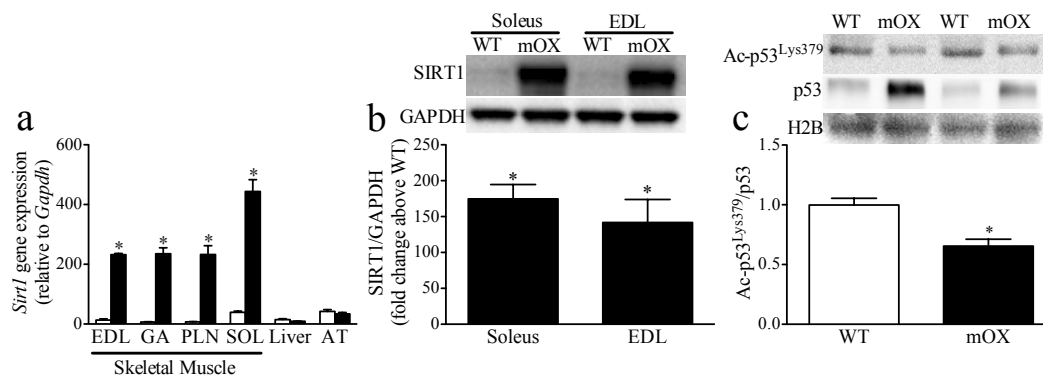
Figure 1.1. mOX mice display increased SIRT1 activity in skeletal muscle. (**a**) mRNA levels of *Sirt1* in soleus (SOL), extensor digitorum longus (EDL), gastrocnemius (GA), liver, and adipose tissue (AT) were measured relative to GAPDH by real-time RT-PCR in WT (white bars) and mOX (black bars) mice (n=3). (**b**) SIRT1 protein levels were measured in soleus and EDL muscles by immunoblot and quantified relative to GAPDH in WT and mOX mice. (**c**) Acetylated (Ac)-p53 (top image), total p53 (middle image) and histone H2B (bottom image) were analyzed by SDS-PAGE in nuclear fractions from GA muscle (n=4). The figure presents the quantification of Ac-p53 corrected for total p53 abundance. Values are mean ± SEM. *, effect of genotype, p<0.05.
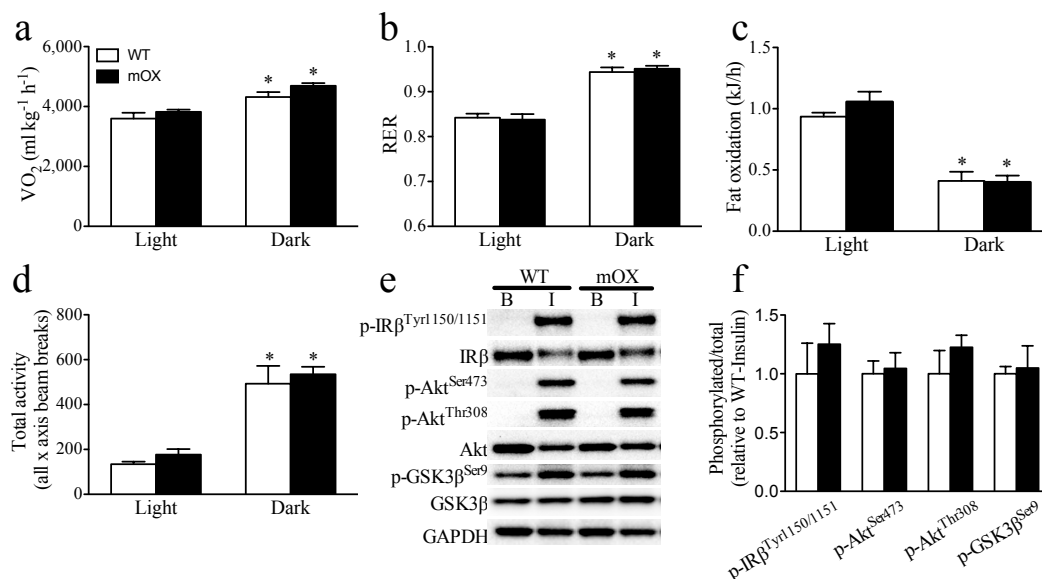
Figure 1.2. Overexpression of SIRT1 in skeletal muscle does not alter energy expenditure, spontaneous activity, or supra-physiological insulin signaling. Measurements were made using the CLAMS system (Columbus Instruments) over 3 consecutive days and averages for the light and dark cycles on days 2 and 3 are displayed for WT (white bars) and mOX (black bars) mice (n=6-8). (**a**) $\dot{V}O_2$ and (**b**) respiratory exchange ratio (RER) were measured by indirect calorimetry. (**c**) Fat oxidation was calculated from indirect calorimetry data as described by others [15]. (**d**) Total (x-total) activity was measured as all beam breaks on the horizontal axis. (**e,f**) Phosphorylation of IR, Akt, and GSK3β was measured by immunoblot in basal GA muscle and GA muscle subjected to 5 min of *in vivo* supraphysiological (6000 pmol/kg) insulin stimulation and was quantified relative to total protein levels (n=4). Values are mean ± SEM. *, effect of time, p<0.05.
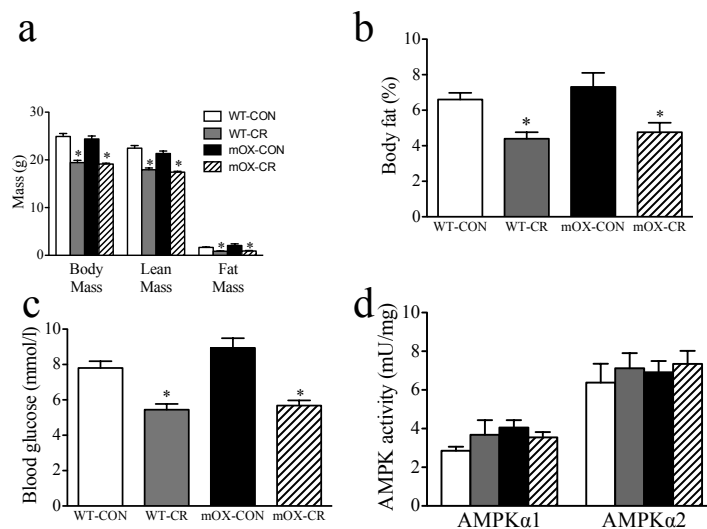
Figure 1.3. Metabolic profiles and skeletal muscle AMPK activity in WT and mOX mice. Beginning at 10 weeks old, WT and mOX were fed an ad-libitum (AL) or calorie restricted (CR) diet for 20 d. WT-AL (white bars), WT-CR (dark gray bars), mOX-AL (black bars), and mOX-CR (light gray bars) mice. (**a**) Body weight was measured to 0.1g and lean and fat mass were determined by MRI (n=10-19). (**b**) Percent (%) body fat was calculated from fat and body mass measurements (n=9-19). (**c**) Basal glucose levels (n=10-19) in fasted mice. (**d**) AMPK α1 and α2 complexes were immunoprecipitated and their activities were quantified (n=6) in GA muscle. Values are mean ± SEM. *, effect of diet, p<0.05.
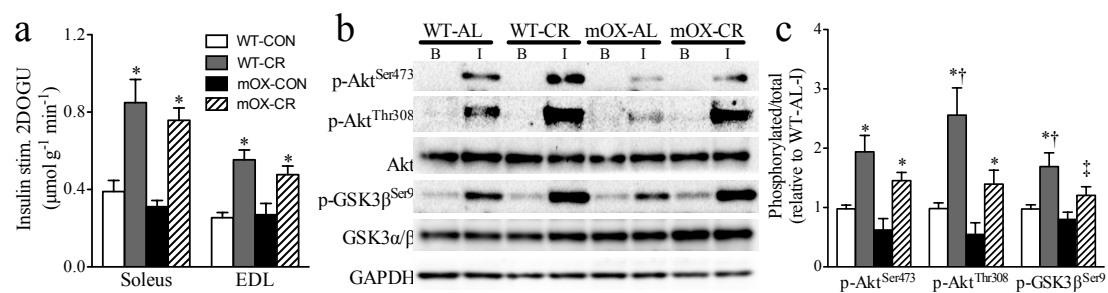
Figure 1.4. Insulin sensitivity and signaling are not enhanced in mOX mice. Beginning at 10 weeks old, WT and mOX were fed an ad-libitum (AL) or calorie restricted (CR) diet for 20 d. WT-AL (white bars), WT-CR (dark gray bars), mOX-AL (black bars), and mOX-CR (light gray bars) mice **(a)** Insulin-stimulated 2DOGU (insulin 2DOGU [360 pmol/l] − basal 2DOGU) in soleus and EDL muscles (n=7-11) were assessed after the 20 d diet period. **(b)** Insulin-stimulated Akt and GSK3β activation were measured in soleus muscle lysate by immunoblotting for p-Akt^{Ser473} (n=5-9), p-Akt^{Thr308} (n=7-11), and p-GSK-3β^{Ser9} (n=6-9). **(c)** Data are displayed as p-Akt^{Ser473}/total Akt, p-Akt^{Thr308}/total Akt, and p-GSK-3β^{Ser9}/total GSK3β, respectively. All samples were normalized to WT-AL-Insulin. Values are mean ± SEM. *, effect of diet; †, effect of genotype, $p<0.05$. ‡, p=0.054.

CHAPTER 2

High-fat diet-induced impairment of skeletal muscle insulin action is not prevented by SIRT1 overexpression

Abstract

Skeletal muscle SIRT1 expression is reduced under insulin-resistant conditions, such as those resulting from high-fat diet (HFD) feeding and obesity. While in Study 1 we investigated the effects of SIRT1 overexpression per se on muscle insulin action, in Study 2 we investigated whether constitutive activation of SIRT1 in skeletal muscle prevents HFD-induced muscle insulin resistance. To address this, mice with muscle-specific overexpression of SIRT1 (mOX) and wildtype (WT) littermates were fed a control diet (10% calories from fat) or HFD (60% of calories from fat) for 12 weeks. Magnetic resonance imaging and indirect calorimetry were used to measure body composition and energy expenditure, respectively. Whole-body glucose metabolism was assessed by an oral glucose tolerance test and insulin-stimulated glucose uptake was measured at a physiological insulin concentration in isolated soleus and extensor digitorum longus muscles. Though SIRT1 was significantly overexpressed in various muscles of mOX vs. WT mice, body weight and percent body fat were similarly increased by HFD, and $\dot{V}O_2$ was unaffected by diet or genotype. Importantly, impairments in glucose tolerance and insulin-mediated activation of glucose uptake in skeletal muscle that occur with HFD feeding were not prevented in mOX mice. These results demonstrate that activation of SIRT1 in skeletal muscle does not prevent HFD-induced glucose intolerance, weight gain, or insulin resistance.

Introduction

Impaired glucose uptake in response to insulin is a common metabolic derangement that can result from the consumption of a hypercaloric, high-fat diet (HFD), and is a key contributor to the etiology of type 2 diabetes (1,2). The NAD^+-dependent protein deacetylase sirtuin 1 (SIRT1) has been advocated as a key convergence point that links fluctuations in nutrient status to the regulation of insulin sensitivity (3). Mechanistically, this occurs via deacetylation of acetylated targets by SIRT1, which in turn regulates their cellular localization and function (4). Supporting its possible role as an energy sensor in skeletal muscle, SIRT1 activity has been shown to increase in low nutrient conditions (5) and to decrease in insulin resistant states, such as those present in patients with type 2 diabetes and with HFD feeding (6,7). Thus, interventions that activate SIRT1 hold promise for the treatment of insulin resistance and type 2 diabetes (3,8). Indeed, SIRT1 activation with small molecule activators (9–12), or via increasing NAD^+ availability (13–16) has been demonstrated to improve glucose homeostasis in insulin-resistant models.

To investigate the role of SIRT1 activation in vivo, several genetic mouse models have been created and have revealed metabolic benefits of SIRT1 overexpression. For example, mice with moderate SIRT1 overexpression in several important metabolic tissues including white adipose tissue, brown adipose tissue, and brain, are leaner, have elevated energy expenditure, and display improved glucose tolerance (17). However, SIRT1 overexpression in these mice was not present in liver or skeletal muscle (17), tissues that are generally considered to be key mediators of post-prandial glucose metabolism. In another mouse model, SIRT1 overexpression in multiple tissues

including liver, adipose tissue, and skeletal muscle, had no overt effects on glucose homeostasis on a low-fat diet, but prevented HFD-induced glucose intolerance (18). Similarly, in a SIRT1 gain-of-function mouse model, HFD-fed mice were protected from glucose intolerance, though these improvements were noted to be due to effects in the liver, and not skeletal muscle (19). Thus, while it is apparent from these transgenic mouse models that SIRT1 can provide protection from the deleterious effects of HFD on glucose metabolism, the contribution of skeletal muscle SIRT1 remains to be fully defined.

Regarding specific effects in skeletal muscle, SIRT1 overexpression in C2C12 myotubes has been demonstrated to protect against fatty acid-induced insulin resistance through transcriptional repression of protein-tyrosine phosphatase 1B (PTP1B) (6). Additionally, SIRT1 overexpression in L6 myotubes and primary human myotubes increases insulin-stimulated Akt activation, although whether this translates into functional improvements in insulin-stimulated glucose uptake, or maintains insulin action under insulin-resistant conditions, was not determined (7). In contrast to these cell-based studies, we showed in Study 1 that skeletal muscle-specific overexpression of SIRT1 does not enhance muscle insulin sensitivity in young, lean mice in response to a physiological insulin concentration (20). Since it is possible that SIRT1 is protective only under insulin-resistant conditions (6), and given that SIRT1 activation in the presence of insulin resistance is more therapeutically relevant, the goal of Study 2 was to determine whether SIRT1 overexpression in skeletal muscle prevents HFD-induced insulin resistance in mice.

Methods

     Animals. Generation of the muscle-specific SIRT1 overexpressing (mOX) mouse has been described in Study 1. The control/wildtype (WT) mice for all studies were floxed, Cre-negative littermates. All studies were conducted in male mice. At 10 weeks of age, mice were randomized to either continue control (10% calories from fat) diet (CON) or were switched to a high-fat (60% calories from fat) diet (HFD) for 12 weeks. Major endpoint measurements, including ex vivo insulin-stimulated glucose uptake and assessment of oral glucose tolerance, were performed in 21-22 week old, 4-6 h fasted mice between 1300-1500h. All experiments were approved by and conducted in accordance with the Animal Care Program at the University of California, San Diego.

     Isolated muscle 2-deoxyglucose uptake. Ex vivo muscle insulin sensitivity was measured by the 2-deoxyglucose uptake (2DOGU) technique in isolated soleus and extensor digitorum longus (EDL) muscles as described in Study 1. The insulin concentration for insulin-treated muscles was 0.36 nmol/L (60 μU/mL).

     Immunoblotting. Measurement of basal and insulin-stimulated signaling by SDS-PAGE and the antibodies used were as described in Study 1.

     Blood glucose, plasma insulin and leptin concentrations. Blood glucose concentration was determined from tail vein blood after a 4-hour fast prior to the OGTT using a standard glucose meter. Whole blood was collected with EDTA from the inferior vena cava of anesthetized mice, centrifuged at 5,000 g at 4°C for 5 min and the plasma

was frozen at -80°C for subsequent determination of plasma insulin and leptin concentrations with a Milliplex mouse adipokine kit (Millipore, Billerica, MA, USA).

OGTT. After 11 weeks on HFD, 4 h-fasted mice were orally gavaged with 5g/kg dextrose. Blood glucose concentration was measured using a standard glucose meter by tail vein at 0, 20, 40, 60, 80, and 120 min. The OGTT area under the curve (AUC) was calculated using the trapezoidal rule.

Energy expenditure and body composition. Energy expenditure, respiratory exchange ratio (RER), spontaneous activity, fat oxidation, and body composition were assessed in 21-22 week old mice as described in Study 1.

Real-time RT-PCR. RNA was extracted from gastrocnemius muscle using the phenol/chloroform method and real-time RT-PCR was performed as described for Study 1. Primer sequences for SIRT1 and GAPDH were described in Study 1. PTP1B primers were: 5′-   TTTTCA   AAGTCCGAGAGTCAG   -3′   and   3′-AGTAAGAGGCAGGTGTCAG -5′.

Statistics. Statistical analyses were performed using SPSS. Data were analyzed by three-way ANOVA with repeated measures when necessary, followed by Tukey's post-hoc analysis with significant differences at $p < 0.05$. For the 2DOGU and insulin signaling data, main effects were diet, genotype, and treatment (basal vs. insulin). Once a significant effect for treatment was found, data were separated and two-way ANOVAs

within basal and within insulin were performed. For EE data, a three-way ANOVA was performed for the main effects diet, genotype, and time (light vs. dark). Where there was a significant effect of time, data were analyzed by two-way ANOVA within light and within dark. OGTT AUC was analyzed with a two-way ANOVA for diet and genotype and two-way ANOVAs were used to compare blood glucose levels at each timepoint of the OGTT. All data are expressed as mean $\pm$ SEM.

Results

 Tissue SIRT1 expression. SIRT1 gene expression was approximately 60-fold higher in gastrocnemius muscle from mOX as compared with WT mice, but did not differ in liver or epididymal fat (Figure 2.1a). SIRT1 protein levels were significantly higher in soleus and EDL muscles of mOX vs. WT mice (Figure 2.1b).

 Body mass and plasma hormone concentrations. Body mass, fat mass, and percent body fat were significantly higher after 12 weeks of HFD feeding in both WT and mOX mice (Figure 2.2a,b). In line with this data, epididymal fat pad mass was significantly higher in HFD-fed mice, but there was no effect of diet or genotype on gastrocnemius muscle mass, liver mass, or heart mass (Table 2.1). Fasting glucose levels were increased in WT and mOX animals fed a HFD as compared with CON-fed mice (Table 2.2). Plasma insulin and leptin concentrations were increased similarly in WT and mOX mice on HFD as compared with CON (Table 2.2).

 Whole-body energy expenditure and spontaneous activity. $\dot{V}O_2$ was higher during the dark vs. the light phase, but was unaffected by diet or genotype (Figure 2.2c). RER was decreased equally by HFD as compared with CON in both WT and mOX mice (Figure 2.2d). Consistent with the RER data, fat oxidation was increased similarly by HFD for both genotypes (Figure 2.2e). Spontaneous activity, as measured by all beam breaks on the x axis (x-total), was increased during the dark phase as compared with the light phase, and was not significantly affected by genotype or diet, though HFD trended ($p=0.058$) to decrease activity in both WT and mOX mice as compared to CON (Figure

2.2f).

Oral glucose tolerance and skeletal muscle insulin sensitivity. Blood glucose concentrations during an OGTT were significantly higher in HFD- vs. CON-fed mice (Fig. 3*A*), resulting in increased AUC (Fig. 3*B*), but there was no effect of genotype on oral glucose tolerance (Fig. 3*A* and *B*). In line with our previous study (20), insulin-stimulated 2DOGU (insulin 2DOGU − basal 2DOGU) did not differ between WT and mOX mice on CON (Fig. 3*C*). As expected, insulin-stimulated 2DOGU was significantly decreased by HFD as compared to CON in soleus and EDL muscles of WT mice and, importantly, there was no effect of SIRT1 overexpression on the magnitude of this impairment (Fig. 3*C*). Complementing the 2DOGU data, insulin-stimulated activation of Akt and GSK3β in soleus (Fig. 3*D* and *F*) and EDL muscles (Fig. 3*E* and *G*) was impaired by HFD, but was unaffected by SIRT1 overexpression. There was no effect of diet or genotype on basal p-Akt^{Ser473}, p-Akt^{Thr308} or p-GSK3β^{Ser9}. There was no effect of diet or genotype on PTP1B gene expression (WT-CON: 1.0 ± 0.2; WT-HFD: 0.9 ± 0.1; mOX-CON: 1.2 ± 0.1; mOX-HFD: 1.0 ± 0.1, fold change vs. WT-CON).

Discussion

Skeletal muscle is responsible for a large proportion of glucose uptake from the blood in response to insulin, and insulin resistance in this important metabolic tissue, such as that induced by the consumption of a hypercaloric, high-fat diet (HFD) (1,2), is a key factor that contributes to the development of type 2 diabetes (21). Mechanistically, the NAD^+-dependent deacetylase SIRT1 has been proposed to be a central regulator of skeletal muscle insulin action in the face of changing nutrient availability (3). Given the potential benefit of SIRT1 activation for the treatment of insulin resistance (9–16), and the importance of skeletal muscle in post-prandial glycemia, we investigated whether overexpression of SIRT1 in skeletal muscle is sufficient to prevent obesity-related impairments in glucose tolerance and muscle insulin action. In line with Study 1, we observed no beneficial effect of skeletal muscle-specific overexpression of SIRT1 on insulin-stimulated glucose uptake in mice fed CON. Significantly, we now extend these findings to reveal that SIRT1 overexpression does not prevent HFD-induced impairments in insulin-stimulated glucose uptake in skeletal muscle.

The role of SIRT1 in the regulation of skeletal muscle insulin action is controversial. For example, our current data are in agreement with reports that show no effect of SIRT1 overexpression on glucose disposal in a hyperinsulinemic-euglycemic clamp (19), insulin-stimulated glucose uptake in isolated soleus and EDL muscles (Study 1), or in C2C12 myotubes under normal conditions (6). In contrast, under insulin resistant conditions induced by treatment with free fatty acids, insulin-stimulated glucose uptake is slightly enhanced in C2C12 myotubes (6). Additionally, in models of insulin resistance, glucose uptake during a hyperinsulinemic-euglycemic clamp and glucose

tolerance are improved by treatment with SIRT1 activators (9,11), SIRT1 overexpression (18), or by increasing NAD^+ availability (13–16). However, in these studies it is difficult to discern whether the observed beneficial effects on glucose metabolism are due to direct activation of SIRT1 in skeletal muscle, or if they are due to effects of SIRT1 activation in other important metabolic tissues, such as adipose tissue, pancreas, or liver. Indeed, activation of SIRT1 specifically in adipose tissue (22), β cells (23,24) or liver (25,26) of HFD-fed rodents has been shown to improve glucose homeostasis. Furthermore, improvements in glucose tolerance have been reported with moderate overexpression of SIRT1 in brain and adipose tissue, but not skeletal muscle, suggesting that effects in these other tissues play a role in the metabolic benefits of SIRT1 activation (17). Together, these studies highlight the importance of investigating the tissue-specific effects of SIRT1 activation.

With regard to skeletal muscle specifically, recent studies have shown that overexpression of SIRT1 in L6 myoblasts and primary human myotubes enhances insulin-stimulated Akt activation under normal conditions (7). Conversely, SIRT1 overexpression in C2C12 myotubes does not enhance insulin-stimulated Akt activation under normal conditions, but prevents impairments in Akt activation induced by treatment with palmitate (6). In this study, protection from palmitate-induced impairments in insulin signaling were found to be due to increased suppression of PTP1B by SIRT1 (6). Interestingly, we observed no effects of muscle-specific SIRT1 overexpression on PTP1B gene expression in CON- or HFD-fed mice. The discrepancy between our results and those reported previously (6,7) could be explained by the difference in model systems that were used since immortalized muscle cell lines and

primary muscle cells may not recapitulate the behavior of bone a fide skeletal muscle. In addition, we used a physiological insulin concentration (0.36 nmol/L) to study insulin action, while these other studies used insulin concentrations (100-1000 nmol/L) that are well above the normal physiological range (6,7). However, while the insulin concentration used is an important consideration, we reported in Study 1 that SIRT1 overexpression in skeletal muscle does not alter insulin signaling even in response to a supra-physiological insulin dose in young, lean mice (20), further highlighting differences between in vitro muscle models and studies in skeletal muscle.

In addition to its purported role in insulin sensitivity and signaling, SIRT1 has been advocated as a key regulator of energy expenditure and metabolism (27,28). For example, moderately higher energy expenditure has been observed in SIRT1-overexpressing mice on a HFD (18). Furthermore, treatment with small molecule activators of SIRT1 has been demonstrated to protect against HFD-induced obesity (10,11) and to increase $\dot{V}O_2$ in animals fed a HFD (11). In contrast, $\dot{V}O_2$ and body weight were reported to be unaltered with moderate whole-body overexpression of SIRT1 in young mice fed normal chow (18). Interestingly, SIRT1 gain-of-function in mice fed a HFD does not affect body weight gain, spontaneous activity, or metabolic rate (19), but decreases metabolic rate in mice fed normal chow (19). Clearly, the role of increased SIRT1 activity in the modulation of energy expenditure and body weight/composition is controversial and discrepancies in reported data may reflect tissue-specific effects of SIRT1 activation. Until now, the muscle-specific effects of SIRT1 activation on whole-body energy metabolism in HFD-fed mice have not been reported. In the present study, we observed increased body weight and fat mass, decreased RER, and increased fat

oxidation with HFD feeding, but did not observe any effects of muscle-specific SIRT1 overexpression on whole-body energy expenditure or body composition. Thus, it is evident that the observed benefits of whole-body SIRT1 overexpression on energy expenditure and protection from HFD-induced obesity likely occur due to contributions from tissues other than skeletal muscle. For example, enhanced energy expenditure has been observed in SIRT1-overexpressing mice in which SIRT1 activity is not increased in skeletal muscle (17). Additionally, SIRT1 overexpression in the brain significantly increases $\dot{V}O_2$ (29) and SIRT1 activation induces the "browning" of white adipose tissue, which is associated with increased metabolic rate (30).

In summary, skeletal muscle insulin resistance often precedes the development of type 2 diabetes, and SIRT1 activation has been put forth as a promising therapeutic approach for the treatment of this increasingly prevalent disease. However, since systemic SIRT1 upregulation by genetic and pharmacological means leads to SIRT1 activation in multiple tissues, it is important to understand the contribution of specific tissues to the insulin sensitizing effects of SIRT1 activation. In Study 2 we report that skeletal muscle-specific overexpression of SIRT1 in mice does not attenuate the deleterious effects of HFD-induced obesity on glucose tolerance or skeletal muscle insulin sensitivity. Furthermore, SIRT1 overexpression in muscle does not alter whole-body energy expenditure or prevent weight gain during HFD feeding. In conclusion, it is apparent that improvements in skeletal muscle insulin sensitivity, glucose tolerance, and body composition that manifest due to whole-body SIRT1 activation in HFD models result from SIRT1 activation in tissues other than skeletal muscle. This should be an important consideration in future studies aimed at understanding the potential of SIRT1

activators as therapeutics for the treatment of insulin resistance and type 2 diabetes.

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Table 2.1. Tissue mass (mg)

	WT-CON	WT-HF	mOX-CON	mOX-HF
Gastrocnemius	127 ± 2	130 ± 3	120 ± 3	124 ± 4
Epididymal fat	350 ± 28	1702 ± 165*	331 ± 31	1436 ± 151*
Liver	1319 ± 53	1278 ± 68	1255 ± 42	1635 ± 162
Heart	134 ± 2	142 ± 4	139 ± 5	150 ± 6

Tissues were weighed to the nearest mg. Data reported as mean ± SEM, n=7-24/group, * $p < 0.05$ vs. CON

Table 2.2. Blood parameters

	WT-CON	WT-HF	mOX-CON	mOX-HF
Fasting glucose (mg/dL)	123 ± 5	144 ± 10*	111 ± 7	150 ± 15*
Plasma insulin (pg/mL)	623 ± 88	1135 ± 184*	518 ± 25	971 ± 177*
Plasma leptin (pg/mL)	806 ± 133	30386 ± 4486*	1269 ± 257	19677 ± 5448*

Data reported as mean ± SEM, n=7-24/group, * $p < 0.05$ vs. CON
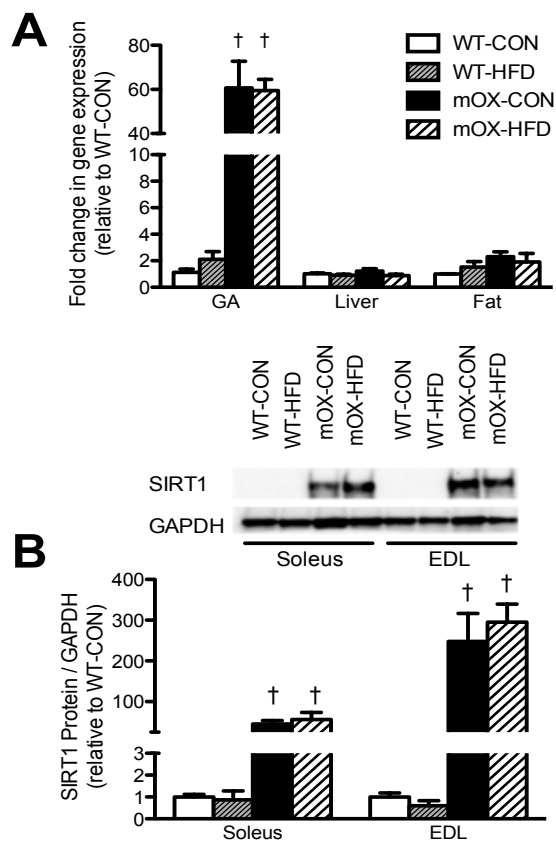
Figure 2.1. mOX mice have increased SIRT1 gene and protein expression in skeletal muscle. WT and mOX mice were fed a CON of HFD for 12 weeks. **(A)** Real-time RT-PCR measurement of SIRT1 gene expression in gastrocnemius (GA) muscle, liver, and epididymal fat of mOX and WT mice fed CON or HFD. **(B)** SIRT1 protein content in soleus (SOL) and extensor digitorum longus (EDL) muscles. Data reported as mean ± SEM, n=4-6/group, † p<0.05 vs. WT
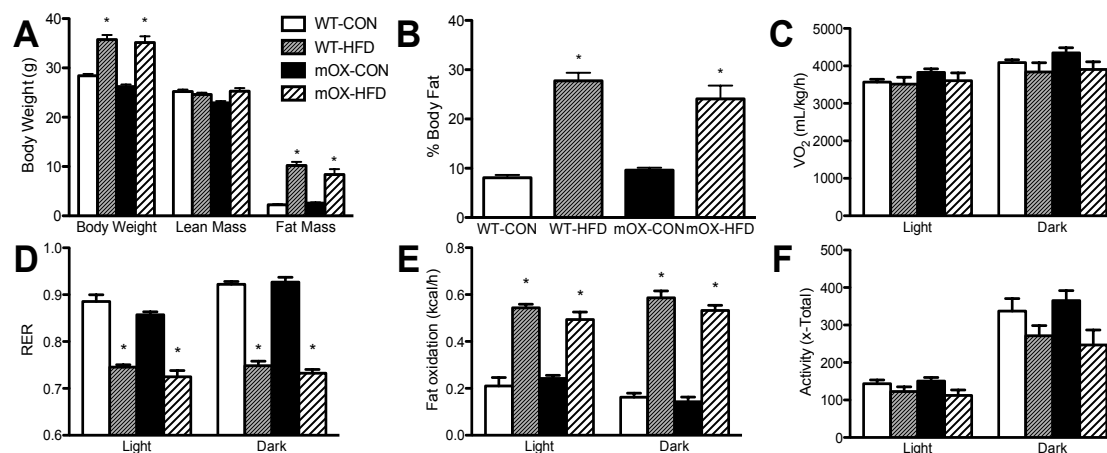
Figure 2.2. HFD feeding increases body fat and fat oxidation in WT and mOX mice.
WT and mOX mice were fed a CON of HFD for 12 weeks. **(A)** Body mass, lean mass, fat mass, and **(B)** percent body fat. **(C-F)** Energy expenditure and spontaneous activity measurements were made using the CLAMS system over 3 consecutive days and averages for the light and dark cycles on days 2 and 3 are presented. (**C**) V̇O$_2$ and (**D**) respiratory exchange ratio (RER) were measured by indirect calorimetry. **(E)** Fat oxidation was calculated from indirect calorimetry data. (**F**) Total (x-total) activity was measured as all beam breaks on the horizontal axis**.** Data reported as mean ± SEM, n=6/group, * p<0.05 vs. CON
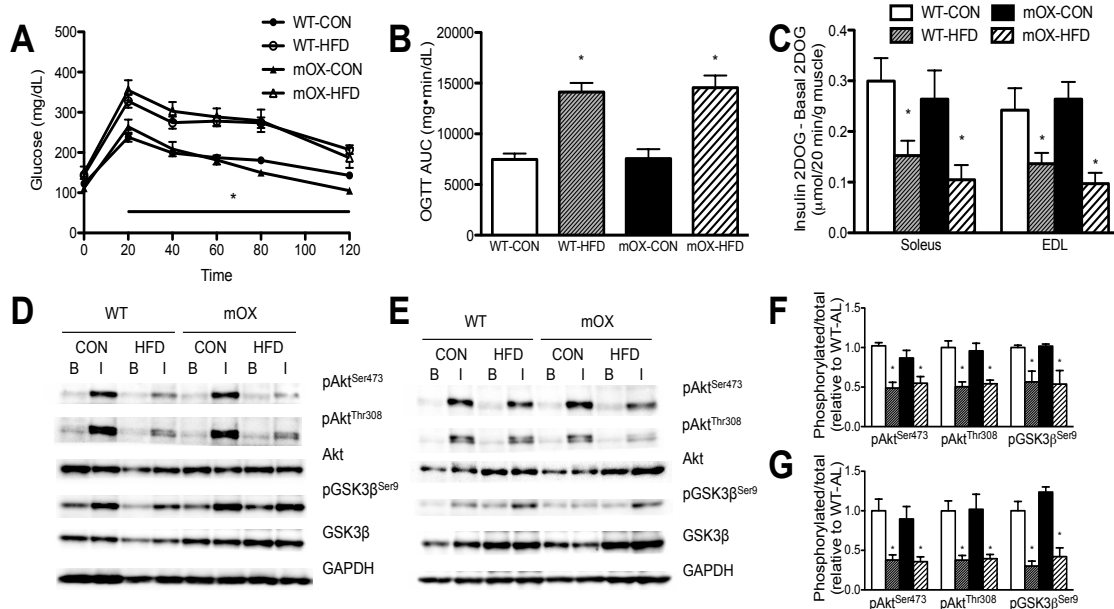
Figure 2.3. SIRT1 overexpression in muscle does not protect against HFD-induced impairments in glucose tolerance or insulin-stimulated glucose uptake. WT and mOX mice were fed a CON of HFD for 12 weeks. **(A)** Blood glucose concentrations and **(B)** area under the curve (AUC) quantification during a 120 minute oral glucose tolerance test (5 g/kg). **(C)** Insulin-stimulated 2-deoxyglucose uptake (2DOGU), calculated as insulin (60 µU/mL) 2DOGU − basal 2DOGU in isolated soleus and EDL muscles. **(D and E)** Phospo-Akt^{Ser473} (pAkt^{Ser473}), phospho-Akt^{Thr308} (pAkt^{Thr308}), total Akt, phospho-GSK3β^{Ser9} (pGSK3β^{Ser9}), and total GSK3β in basal and insulin-stimulated (B and I, respectively) **(D)** soleus and **(E)** EDL muscles. **(F and G)** Quantification of pAkt^{Ser473}, pAkt^{Thr308}, and pGSK3β^{Ser9} compared to total protein abundance of Akt and GSK3β in **(F)** soleus and **(G)** EDL muscles. Data reported as mean±SEM, n=6-15/group, * $p < 0.05$ vs. CON

CHAPTER 3

Knockout of STAT3 in skeletal muscle does not enhance muscle insulin action or prevent HFD-induced insulin resistance

Abstract

Signal transducer and activator of transcription 3 (STAT3) is elevated in skeletal muscle from patients with impaired glucose tolerance. Accordingly, aberrant STAT3 signaling has been implicated in the development of skeletal muscle insulin resistance, though a causal role for STAT3 in the pathophysiology of skeletal muscle insulin resistance in vivo remains to be elucidated. The purpose of Study 3 was to use a novel mouse model to determine whether knockout of STAT3 in skeletal muscle enhances insulin-stimulated glucose uptake and whether it prevents insulin resistance in a high-fat diet (HFD) mouse model. Mice with skeletal muscle-specific knockout of STAT3 (mKO) were generated by crossing floxed mice containing loxP sites flanking exon 22 of STAT3 (a region required for activation and nuclear translocation) with mice harboring Cre recombinase under the control of the muscle creatine kinase promoter. Beginning at 10 weeks of age, mKO mice and their floxed/wildtype (WT) littermates either continued consuming a low fat, control diet (CON) (10% of calories from fat) or were switched to a HFD (60% of calories from fat) for 20 days. As expected, STAT3 gene and protein expression were reduced ~50-80% in muscle from mKO vs. WT mice. Fat mass and body fat percentage did not differ between WT and mKO mice on CON, and were increased equally by HFD. Moreover, energy expenditure did not differ between WT and

mKO mice on either diet, and HFD decreased RER similarly for both genotypes. Interestingly, insulin-stimulated 2-deoxyglucose uptake (2DOGU) in CON-fed mice did not differ between genotypes in soleus or extensor digitorum longus muscles. In addition, HFD significantly decreased insulin-stimulated 2DOGU to the same extent in mKO and WT mice. Taken together, the results from Study 3 demonstrate that knockout of STAT3 does not enhance skeletal muscle insulin action, nor does it protect against HFD-induced insulin resistance in skeletal muscle.

Introduction

Consumption of a hypercaloric, high-fat diet (HFD) and the ensuing obesity has been implicated in the pathogenesis of insulin resistance and type 2 diabetes (1). Skeletal muscle is a key mediator of insulin-stimulated glucose uptake in vivo (2,3) and interventions that prevent skeletal muscle insulin resistance in the face of nutrient overload hold promise for the treatment of type 2 diabetes. Nevertheless, the molecular mechanisms through which nutrient overload and obesity lead to skeletal muscle insulin resistance are incompletely defined. The transcription factor signal transducer and activator of transcription 3 (STAT3) is activated in response to circulating cytokines such as IL-6 that are elevated in obese, insulin-resistant states (4). Typically, activation of STAT3 occurs upon interaction of cytokines with a membrane receptor that leads to activation of Janus kinase (JAK), which then phosphorylates STAT3 at a key tyrosine residue (Y705) that is required for STAT3 dimerization and translocation to the nucleus (5). Once activated and translocated to the nucleus, STAT3 affects the transcription of various target genes, including suppressor of cytokine signaling 3 (SOCS3). In insulin resistant states, STAT3 activation is increased (6,7), as is SOCS3 activity, which negatively regulates insulin signaling at the level of the insulin receptor substrate proteins (8–14). Given the signaling crosstalk between STAT3 and SOCS3, and subsequent effects on insulin signaling, we hypothesized that knockout of STAT3 in skeletal muscle would enhance insulin sensitivity and prevent HFD-induced insulin resistance.

STAT3 activation in the liver is elevated with HFD feeding and in genetically obese mice (15,16), and inhibition of STAT3 activity in liver cells and adipocytes protects against IL-6- and amino acid-induced impairments in insulin signaling (17,18).

Thus, inhibition of STAT3 is a plausible approach for the abrogation of HFD-induced insulin resistance in the liver, although whether STAT3 plays a causal role in the development of insulin resistance in skeletal muscle is unknown. STAT3 phosphorylation is increased in muscle from patients with impaired glucose tolerance (6,7) and palmitate-induced constitutive activation of STAT3 in L6 myotubes increases SOCS3 protein abundance in conjunction with impaired insulin signaling at the level of Akt (6). Importantly, STAT3 siRNA attenuates the impairments in insulin signaling caused by palmitate in L6 myotubes (6) and by IL-6 in human skeletal muscle myoblasts (7). In addition, mice with knockout of SOCS3 in skeletal muscle are refractory to HFD-induced insulin resistance (12). Taken together, these studies identify increased STAT3 activation as a possible underlying mechanism of insulin resistance in skeletal muscle and highlight the potential of STAT3 inhibition for the treatment of insulin resistance and type 2 diabetes. However, mechanistic studies have only been performed in skeletal muscle cell models, and not skeletal muscle per se, so the role of STAT3 in vivo in the pathogenesis of skeletal muscle insulin resistance has not been fully elucidated. Indeed, increased activation of STAT3 in response to IL-6 has been observed in the absence of impairments in insulin sensitivity (19) and IL-6 treatment has been shown to improve muscle and whole-body insulin sensitivity, even in the face of increased STAT3 activation (20,21). Thus, to address the controversial role of STAT3 in skeletal muscle insulin action, for Study 3 of this Dissertation we generated a novel mouse model with muscle-specific knockout of STAT3 (mKO). Our goal was to determine whether mKO mice have enhanced skeletal muscle insulin sensitivity, and whether they are protected from HFD-induced insulin resistance.

Methods

Animals. The mKO mice were generated by crossing mice with loxP sites flanking exon 22 of the STAT3 gene (22,23) with mice expressing Cre recombinase under the control of the muscle creatine kinase promoter. In this model, the deleted region of the STAT3 gene eliminates an essential acetylation site (K685) and an essential phosphorylation site, (Y705), which are necessary for STAT3 activation (22–25). The floxed mouse has been used extensively to study STAT3 biology (22–24,26). The control/wildtype (WT) mice for all studies were floxed, Cre-negative littermates, and all studies were conducted in male mice. At 10 weeks of age, mice were randomized to either continue control (10% calories from fat) diet (CON) or were switched to a high-fat (60% calories from fat) diet (HFD) for 20 days. Major endpoint measurements, including ex vivo insulin-stimulated glucose uptake and assessment of oral glucose tolerance, were performed in 13-week-old, 4-6 h fasted mice between 1300-1500 h. All experiments were approved by and conducted in accordance with the Animal Care Program at the University of California, San Diego.

Isolated muscle 2-deoxyglucose uptake. Ex vivo muscle insulin sensitivity was measured by the 2-deoxyglucose uptake (2DOGU) technique in isolated soleus and extensor digitorum longus (EDL) muscles as described in Study 1. The insulin concentration for insulin-treated muscles was 0.36 nmol/L (60 μU/mL).

Immunoblotting. Measurement of basal and insulin-stimulated signaling by SDS-PAGE and the antibodies used were as described in Study 1. Additional antibodies

used were: p-STAT3 (Catalog no. 9138) and STAT3 (Catalog no. 9132) from Cell Signaling Technology (Danvers, MA, USA).

Blood glucose, plasma insulin and leptin concentrations. Blood glucose concentration was measured from tail vein blood after a 4 h fast and immediately prior to the start of the OGTT with a standard glucose meter. Plasma insulin and leptin concentrations were measured as described in Study 2.

Oral glucose tolerance test (OGTT). For the OGTT, after 15-17 days on HFD, 4 h-fasted mice were orally gavaged with 5g/kg dextrose. Blood glucose concentration was measured at 0, 20, 40, 60, 80, and 120 min.

Energy expenditure and body composition. Energy expenditure, respiratory exchange ratio (RER), and spontaneous activity were measured by the CLAMS system as described in Study 1. Body composition was assessed by magnetic resonance imaging. All measurements were made ~18 days after the start of dietary intervention.

Real-time RT-PCR. RNA was extracted from gastrocnemius muscle using the phenol/chloroform method and real-time RT-PCR was performed as described for Study 1. Primer sequences for GAPDH were described in Study 1 and primer sequences for STAT3 were 5' - CCT GAA GAC CAA GTT CAT CTG TGT GAC - 3' and 5' - CAC ACA AGC CAT CAA ACT CTG GTC TCC - 3'.

Statistics. Statistical analyses were performed using SPSS as described in Study 2. Briefly, 3-way ANOVAs were run for the main effects genotype, diet, and time (for energy expenditure measurements) or genotype, diet, and treatment (for basal and insulin 2DOGU). Upon finding a significant effect of time or treatment, data were analyzed by 2-way ANOVA within light/dark or basal/insulin. All data are expressed as mean ± SEM.

Results

STAT3 expression. STAT3 protein abundance was significantly decreased in soleus and EDL muscle from mKO mice as compared with wildtype, but did not differ in liver (Figure 3.1a). STAT3 gene expression was approximately 50% lower in gastrocnemius muscle from mKO vs. WT mice (Figure 3.1b). STAT3 gene expression was significantly increased by HFD in both genotypes, but STAT3 gene expression in mKO-HFD mice was similar to that of WT-CON animals (Figure 3.1b). Phosphorylated and total STAT3 protein levels were significantly reduced by ~50% in the cytosolic fraction of gastrocnemius muscle from mKO vs. WT mice (Figure 3.1c). In addition, nuclear STAT3 was reduced by ~75% in gastrocnemius muscle from mKO vs. WT mice (Figure 3.1c). Taken together, these data validate that STAT3 signaling was significantly reduced in mKO vs. WT muscle, and thus supports the use of the mKO mouse to study the role of STAT3 in the regulation of muscle insulin action.

Body mass and energy expenditure. Fat mass and percent body fat were significantly increased after 20 days of HFD feeding in both WT and mKO mice, while there was no effect on total body weight (Figure 3.2a-b). In line with this data, epididymal fat pad mass was significantly higher in HFD-fed mice, but there was no effect of diet or genotype on gastrocnemius or tibialis anterior muscle mass, liver mass, or heart mass (Table 3.1). Caloric intake was increased for both WT and mKO mice fed HFD, largely resulting from increased feeding during the light phase (Figure 3.2c), indicating that HFD-fed mice were hypercaloric at the time of metabolic measurements. Spontaneous activity, as measured by all beam breaks on the x axis (x-total), was

increased during the dark phase as compared with the light phase, and was not significantly affected by genotype or diet (Figure 3.2d). $\dot{V}O_2$ was higher during the dark vs. the light phase, but was unaffected by diet or genotype (Figure 3.2e). RER was decreased equally by HFD as compared with CON in both WT and mKO mice (Figure 3.2f).

Plasma hormone concentrations. Fasting glucose and plasma insulin levels were similar in WT and mKO mice on CON and HFD (Table 3.2). Plasma leptin concentrations were increased similarly in WT and mKO mice on HFD as compared with CON (Table 3.2).

Oral glucose tolerance. Blood glucose concentrations during an OGTT were significantly higher in HFD- vs. CON-fed mice, but there was no effect of genotype (Figure 3.3a). Accordingly, the area under the curve for the OGTT was ~30% higher in HFD-fed vs. CON-fed mice (WT-CON 23032 ± 789, WT-HFD 28187 ± 786, mKO-CON 21528 ± 814, mKO-HFD 27442 ± 1144 g/dL, $p < 0.05$, n $= 6$–10).

Skeletal muscle insulin sensitivity. Basal and insulin 2DOGU were decreased similarly by HFD as compared to CON in both WT and mKO mice in soleus (Figure 3.3b) and EDL muscle (Figure 3.3c). Insulin-stimulated 2DOGU (insulin 2DOGU – basal 2DOGU) did not differ between WT and mKO mice on CON in soleus or EDL muscle (Figure 3.3d). As expected, insulin-stimulated 2DOGU was significantly decreased by HFD as compared to CON in soleus and EDL muscles of WT mice and, importantly,

there was no effect of STAT3 knockout on the magnitude of this impairment (Figure 3.3d). Complementing the 2DOGU data, insulin-stimulated activation of Akt in soleus muscle was impaired by HFD, but was unaffected by STAT3 knockout (Figure 3.3e,f).

Discussion

STAT3 has been proposed to be a regulator of insulin resistance in obese and insulin-resistant states largely due to the fact that it is activated by substrates such as IL-6 and leptin that are increased by nutrient overload and inflammation (4,27–31). Indeed, STAT3 activation is increased in IL-6-induced hepatic insulin resistance (15,30,32–35), and while a limited number of studies have observed increased STAT3 activation in skeletal muscle cell models of insulin resistance (6,7), to our knowledge no one has investigated the mechanistic role of STAT3 in the pathophysiology of skeletal muscle insulin resistance in vivo. To address this gap in knowledge, in Study 3 we investigated whether STAT3 knockout in skeletal muscle enhances insulin sensitivity in CON-fed mice or prevents HFD-induced insulin resistance. In this model, STAT3 gene expression was increased by HFD feeding in WT and mKO mice but was markedly reduced in mKO vs. WT mice on both diets, concomitant with dramatically reduced protein levels of cytosolic and nuclear STAT3. Interestingly, despite attenuation of STAT3 signaling in mKO mice, our results from Study 3 demonstrate that knockout of STAT3 does not enhance skeletal muscle insulin sensitivity or prevent HFD-induced impairments in insulin sensitivity, insulin signaling, or glucose tolerance.

STAT3 activation is enhanced in skeletal muscle from patients with impaired glucose tolerance, and as such, STAT3 has been implicated in the etiology of skeletal muscle insulin resistance (6,7). However, IL-6-stimulated activation of STAT3 does not consistently lead to skeletal muscle insulin resistance (19,32), and in some instances even results in enhancement of muscle insulin action (20,21). In fact, treatment of C2C12 myotubes with a selective alpha-7 nicotinic receptor agonist increases glucose uptake in a

STAT3-dependent manner (36). Conversely, in L6 myotubes, palmitate-induced impairments in Akt activation are associated with increased STAT3 activation and STAT3 siRNA attenuates this impairment (6). Additionally, impairments in Akt signaling and insulin-stimulated glucose uptake resulting from IL-6 treatment in human skeletal muscle myoblasts is reversed by STAT3 siRNA treatment (7). These studies reveal that there is inconsistency as to whether skeletal muscle STAT3 activation impairs or enhances insulin action, and to our knowledge, until now the contribution of skeletal muscle STAT3, in vivo, in the regulation of glucose homeostasis has not been investigated. In the present study, we hypothesized that knockout of STAT3 in skeletal muscle would enhance muscle insulin action and prevent HFD-induced insulin resistance. However, we observed no beneficial effects of skeletal muscle STAT3 knockout on insulin-stimulated glucose uptake in mice fed CON or HFD. Additionally, unlike previous studies in muscle cell models that observed a reversal of impairments in Akt signaling with knockdown of STAT3 (6,7), we found that Akt signaling was unaffected by STAT3 knockout in skeletal muscle in mice on CON and that reduced Akt signaling with HFD feeding was not ameliorated in mKO mice. These results contrast with previously published studies in muscle, possibly because previous studies used immortalized muscle cell lines that more closely model embryonic muscle, rather than bone-fide skeletal muscle (6,7,20,21). In addition, a supra-physiological dose of insulin (100-120 nmol/L) was used to measure insulin-mediated increases in glucose uptake in cell culture studies (6,7,20,21), whereas we studied insulin action using a physiological dose of insulin (0.36 nmol/L). Regardless, our results demonstrate that knockout of STAT3 in skeletal muscle in vivo does not enhance muscle insulin action under control

or insulin-resistant conditions.

Increased body weight, fat mass, and percent body fat have been observed in mice with constitutive activation of STAT3 in POMC neurons along with increased food intake (37), suggesting that modulation of STAT3 activity may alter whole-body energy metabolism. However, the effects of STAT3 modulation in neurons may reflect the central role of STAT3, for example in leptin signaling (29), and peripheral STAT3 may play a separate role in energy homeostasis. For example, adipose-specific knockout of STAT3 does not alter food intake or energy expenditure, and increased body weight and adiposity in this model were noted to be due to impairments in leptin signaling (38). In the present study, we observed no differences in body weight, fat mass, percent body fat, or food intake in mKO vs. WT mice on CON, and HFD increased these parameters equally for both genotypes with no overall increase in body weight. Considering the emerging role of STAT3 in mitochondrial bioenergetics (39–46), we thought it possible that modulation of STAT3 in skeletal muscle could impact whole-body energy metabolism. However, we did not observe any effects of muscle-specific STAT3 knockout on $\dot{V}O_2$ or RER, suggesting that skeletal muscle STAT3 is not a major regulator of whole-body energy homeostasis.

Despite the potential of STAT3 inhibition to ameliorate insulin resistance in cell models (6,7,17), STAT3 has been implicated as a positive mediator of glucose homeostasis through inhibition of gluconeogenic genes in the liver, and STAT3 knockout in liver increases blood glucose and plasma insulin concentrations, and impairs glucose tolerance (47–49). Additionally, knockout of hypothalamic STAT3 increases body weight, plasma glucose and insulin concentrations, and impairs glucose tolerance (50),

suggesting that ablation of STAT3 in this tissue interferes with normal glucose homeostasis. Clearly, the potential benefits of STAT3 inhibition are clouded by these conflicting results, and require further resolution. Herein, we demonstrated that muscle-specific knockout of STAT3 does not alter blood glucose concentration, plasma insulin levels, or glucose tolerance on CON, or protect against HFD-induced glucose intolerance. Taken together, these results suggest that the role of STAT3 in the modulation of whole-body glucose homeostasis is tissue-specific.

In conclusion, STAT3 signaling is elevated in insulin-resistant states in multiple tissues, including skeletal muscle. However, limited studies have investigated the mechanistic role of STAT3 activation in skeletal muscle insulin action per se. Thus, in Study 3 we investigated whether muscle-specific knockout of STAT3 enhances insulin sensitivity, whole-body energy homeostasis, or glucose tolerance. Furthermore, since inhibition of STAT3 has been observed to reverse the detrimental effects of palmitate and cytokines on insulin signaling, we employed a HFD mouse model to determine whether muscle-specific STAT3 knockout prevents HFD-induced insulin resistance. Overall, we found that knockout of STAT3 in skeletal muscle does not improve insulin signaling or sensitivity in CON-fed mice or ameliorate the detrimental effects of HFD feeding on insulin sensitivity, glucose tolerance, or body composition. Thus, we conclude that inhibition of skeletal muscle STAT3 is not a promising approach for enhancing insulin sensitivity or preventing HFD-induced insulin resistance.

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S.S. and A.T.W. were responsible for the conception and design of the study, and the analysis and interpretation of data. A.T.W was responsible for the design and drafting of the manuscript and S.S. revised the manuscript critically. All authors gave final approval.

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Chapter 3, in full, is currently being prepared for publication. A. T. White, S. Schenk. The dissertation author was the primary investigator and author of this paper.

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Table 3.1. Tissue mass (mg)

	WT-CON	WT-HFD	mKO-CON	mKO-HFD
Gastrocnemius	120 ± 3	123 ± 3	123 ± 3	122 ± 4
Tibialis anterior	43 ± 1	45 ± 1	45 ± 1	45 ± 1
Epididymal fat	327 ± 25	527 ± 41*	288 ± 17	621 ± 51*
Liver	1192 ± 37	1011 ± 32	1170 ± 57	1066 ± 30
Heart	122 ± 3	132 ± 4	126 ± 3	136 ± 4

Tissues were weighed to the nearest mg. Data reported as mean ± SEM, n=19-24/group, * $p<0.05$ vs. CON

Table 3.2. Blood parameters

	WT-CON	WT-HFD	mKO-CON	mKO-HFD
Fasting glucose (mg/dL)	123 ± 6	136 ± 3	133 ± 6	136 ± 4
Plasma insulin (pg/mL)	962 ± 147	1096 ± 109	1102 ± 279	1099 ± 203
Plasma leptin (pg/mL)	1351 ± 436	$4182 \pm 1003*$	938 ± 164	$3974 \pm 699*$

Data reported as mean $\pm$ SEM, n=7-24/group, * $p < 0.05$ vs. CON
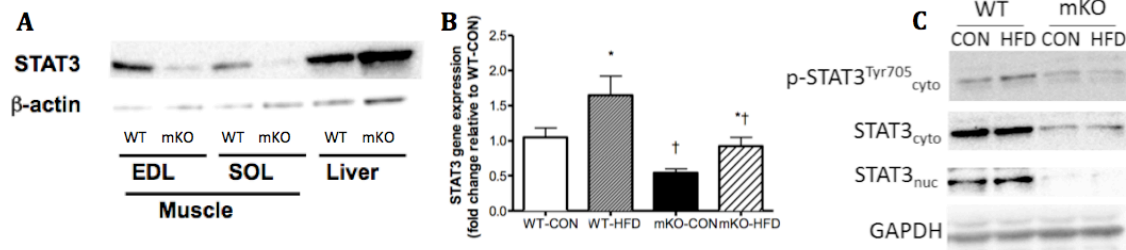
Figure 3.1. mKO mice have decreased STAT3 gene and protein expression in skeletal muscle. WT and mKO mice were fed a CON or HFD for 20 days. (**A**) Total STAT3 protein content in soleus (SOL), extensor digitorum longus (EDL), and liver from WT and mKO mice (**B**) Real-time RT-PCR measurement of STAT3 gene expression in gastrocnemius (GA) muscle of mKO and WT mice fed CON or HFD. (**C**) Phosphorylated (p-STAT3^{Tyr705}) and total STAT3 protein content in nuclear (nuc) and cytosolic (cyto) fractions of GA muscle. Data reported as mean ± SEM, n=4-6/group, † p<0.05 vs. WT
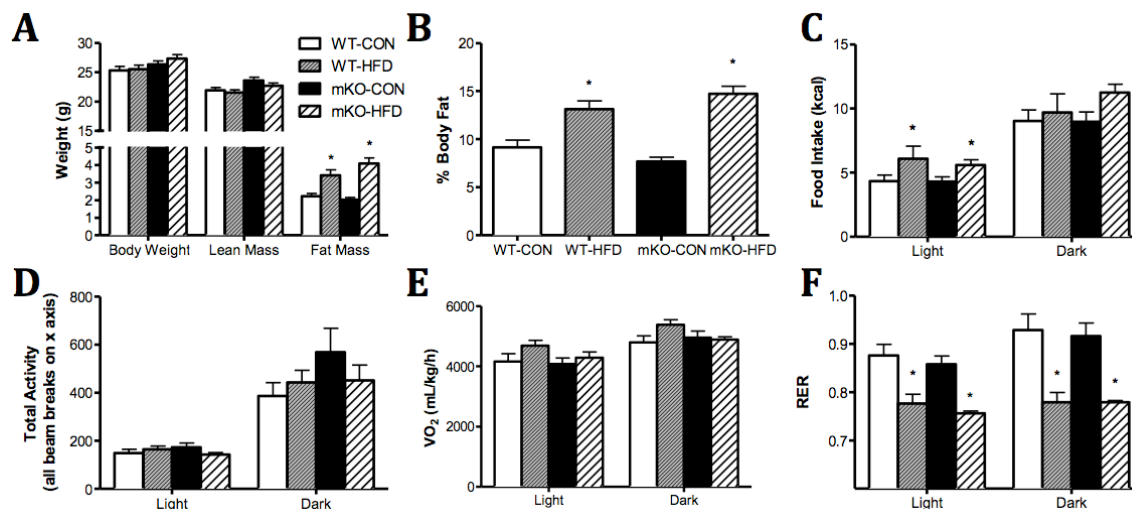
Figure 3.2. HFD feeding increases body fat and decreases RER in WT and mKO mice. WT and mKO mice were fed a CON or HFD for 20 days. **(A)** Body mass, lean mass, fat mass, and **(B)** percent body fat, as measured by MRI. **(C-F)** Food intake, energy expenditure, and spontaneous activity measurements were made using the CLAMS system over 3 consecutive days and averages for the light and dark cycles on days 2 and 3 are presented. **(C)** Cumulative food intake. **(D)** Total (x-total) activity was measured as all beam breaks on the horizontal axis. **(E)** $\dot{V}O_2$ and **(F)** respiratory exchange ratio (RER) were measured by indirect calorimetry. Data reported as mean ± SEM, n=6/group, * p<0.05 vs. CON
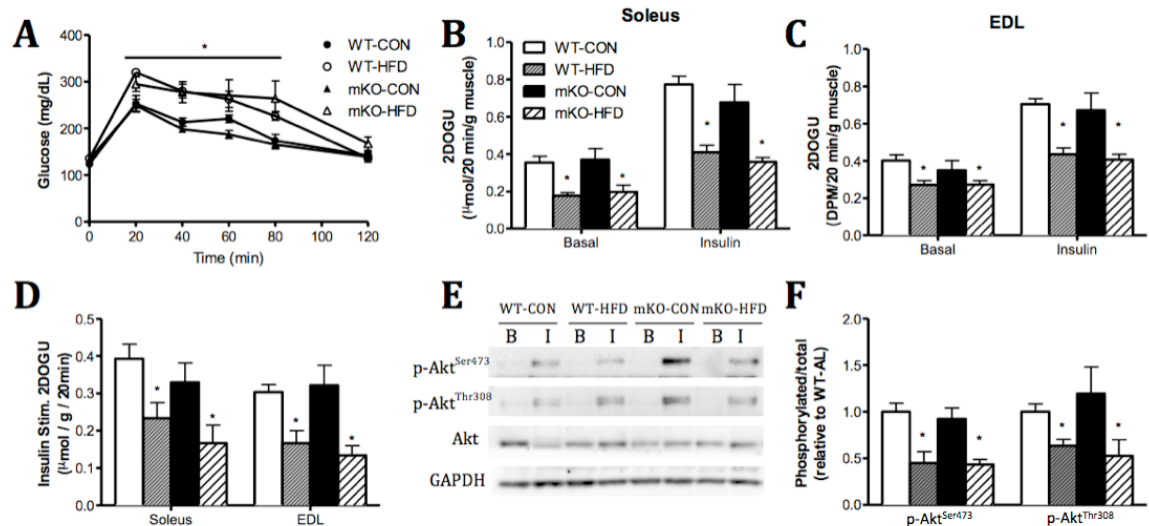
Figure 3.3. STAT3 knockout in muscle does not protect against HFD-induced impairments in glucose tolerance or insulin-stimulated glucose uptake. WT and mKO mice were fed a CON or HFD for 20 days. **(A)** Blood glucose concentrations during a 120 minute oral glucose tolerance test (5 g/kg). **(B and C)** Basal and insulin (60 μU/mL) 2-deoxyglucose uptake (2DOGU) in **(B)** soleus and **(C)** EDL muscles. **(D)** Insulin-stimulated 2DOGU, calculated as insulin 2DOGU – basal 2DOGU in isolated soleus and EDL muscles. **(E)** Phospo-Akt^{Ser473} (pAkt^{Ser473}), phospho-Akt^{Thr308} (pAkt^{Thr308}), and total Akt in basal and insulin-stimulated (B and I, respectively) soleus muscle. **(F)** Quantification of pAkt^{Ser473} and pAkt^{Thr308} compared to total protein abundance of Akt in soleus muscle. Data reported as mean±SEM, n=6-15/group, * $p<0.05$ vs. CON

CHAPTER 4

Conclusion

"O wretched and unhappy Italy, canst thou not see that intemperance kills every year amongst thy people as great a number as would perish during the time of a most dreadful pestilence, or by the sword or fire of many bloody wars!... For there is a remedy by which we may banish this fatal vice of intemperance – an easy remedy, and one of which every man may avail himself if he will; that is, to live in accordance with the simplicity of Nature, which teaches us to be satisfied with little, to follow the ways of holy self control and divine reason, and to accustom ourselves to eat nothing but that which is necessary to sustain life. We should bear in mind that anything more than this will surely be followed by infirmity and death." – Louis Cornaro, "The Temperate Life: A Sure and Certain Method of Attaining a Long and Healthy Life," 1558.

For centuries it has been known that overeating is associated with poor health, and that consuming fewer calories improves health and increases lifespan. Regardless of our long-standing knowledge of the deleterious effects of overeating and of the benefits of reducing calorie intake, eating a healthy diet is something that millions of people still struggle with every day. Clearly, Cornaro's words went unheeded as evidenced by the increasing prevalence of "lifestyle" diseases including obesity, heart disease, and type 2 diabetes. Accompanying the increased prevalence of these diseases is an immense economic burden, with type 2 diabetes alone incurring costs of $245 billion in 2012 in the United States. In addition, the personal consequences of type 2 diabetes are severe, and include complications such as foot amputation, blindness, and kidney disease. With this in mind, the aim of this dissertation was to elucidate some of the mechanisms by which diet impacts our health, with a specific focus on how changes in nutrient availability translate into alterations in skeletal muscle insulin sensitivity. The reason for focusing on

skeletal muscle insulin sensitivity is that skeletal muscle insulin resistance is an important clinical complication that when sustained for many years predicts an individual's risk for developing type 2 diabetes.

Together, the three studies from this dissertation have expanded our understanding of the contributions of SIRT1 and STAT3 to skeletal muscle insulin sensitivity. The major findings of this dissertation were: (1) skeletal-muscle specific overexpression of SIRT1 does not enhance muscle insulin sensitivity alone, or in combination with calorie restriction, (2) overexpression of SIRT1 in skeletal muscle does not prevent insulin resistance or glucose intolerance induced by high-fat diet feeding, and (3) STAT3 knockout in skeletal muscle does not improve muscle insulin sensitivity or protect against high-fat diet-induced impairments in insulin signaling, insulin sensitivity, or whole-body glucose homeostasis.

It is well appreciated that calorie restriction has many health benefits. However, calorie restriction as a lifestyle intervention requires discipline and dedication, and as such, is not feasible for most individuals. Thus, considerable effort has gone into exploring the cellular/molecular mechanisms by which calorie restriction improves health, increases lifespan, and enhances insulin sensitivity. With this in mind, over the past decade, the posttranslational modification of acetylation/deacetylation has gained attention for its capacity to alter the activity of target proteins and thus impact cellular metabolism. The deacetylase SIRT1 has emerged as a candidate that mediates the beneficial effects of calorie restriction, largely due to its dependence on NAD^+, which is increased under low nutrient conditions. Accordingly, small molecule activators of SIRT1 have been developed with hopes that they can be used to treat diseases of aging

and nutrient excess, including skeletal muscle insulin resistance and type 2 diabetes. Since these activators presumably increase SIRT1 activity in most or all tissues, and since skeletal muscle is the main site of insulin-stimulated glucose uptake, Studies 1 and 2 of this dissertation aimed to provide a deeper understanding of the possible benefits of SIRT1 activation specifically in skeletal muscle insulin action.

One of the major hypotheses tested in this dissertation was that SIRT1 is a key mediator of skeletal muscle insulin sensitivity in response to changing nutrient availability. As part of this hypothesis, we hypothesized that muscle-specific SIRT1 overexpression would mimic the effects of calorie restriction on muscle insulin sensitivity. We directly tested this hypothesis by measuring insulin-stimulated 2-deoxy-glucose uptake in isolated skeletal muscles from wildtype mice and transgenic mice with muscle-specific overexpression of SIRT1. In Study 1, we measured insulin-stimulated glucose uptake in mice that were fed a control (10% of calories from fat) diet ad libitum, or mice that were calorie restricted to 60% of their ad libitum intake. This degree of calorie restriction has been well documented to produce robust enhancements in muscle insulin sensitivity. While we did observe the expected increase in insulin-stimulated glucose uptake with calorie restriction in wildtype and SIRT1-overexpressing mice, there was no additional enhancement in insulin sensitivity with increased SIRT1 expression, suggesting that increased SIRT1 activity does not lead to an additive or synergistic improvement in muscle insulin action when combined with calorie restriction. Importantly, and somewhat surprisingly, we did not observe a beneficial effect of SIRT1 overexpression alone on insulin-stimulated glucose uptake in skeletal muscle. This suggests that while skeletal muscle SIRT1 may be necessary for the insulin-sensitizing

effects of calorie restriction, simply supplementing SIRT1 activity in muscle is not sufficient to mimic the effects of calorie restriction on muscle insulin action. Thus, treatments that increase skeletal muscle SIRT1 in insulin-sensitive patients are unlikely to provide benefits to skeletal muscle that parallel those of calorie restriction.

In insulin resistant states, such as in obesity and type 2 diabetes, SIRT1 activity is diminished, so replenishing SIRT1 in this context may provide the best opportunity to observe any beneficial effects on muscle insulin action. Supporting this, the majority of studies demonstrating a positive effect of SIRT1 activators on insulin sensitivity and health have been conducted in rodent models of obesity. Experimentally, feeding mice a high-fat/high-calorie diet is a well-established means to quickly and effectively create a model of insulin resistance. Because we did not see an effect of muscle SIRT1 overexpression on muscle insulin sensitivity in lean animals, we felt it reasonable to investigate whether muscle insulin action would be enhanced by SIRT1 overexpression under insulin-resistant conditions. Thus, as part of our hypothesis that SIRT1 modulates muscle insulin sensitivity in response to alterations in nutrient availability, and considering the connection between SIRT1 activity and insulin resistant states, in Study 2 we hypothesized that augmentation of SIRT1 would protect against muscle insulin resistance induced by high-fat diet feeding. To test this hypothesis, we placed wildtype and SIRT1-overexpressing mice on a control diet that contains 10% of calories from fat or a hypercaloric/high-fat diet containing 60% of calories from fat for 12 weeks. Twelve weeks of high-fat diet feeding is a sufficient amount of time to result in insulin resistance. Interestingly, we observed no protection from high-fat diet-induced insulin resistance with muscle SIRT1 overexpression, suggesting that SIRT1 activation in skeletal muscle,

even under insulin resistant conditions, is not a promising approach for improving muscle insulin action, and that further efforts should be made to understand the tissue-specific effects of SIRT1 activation.

Another main hypothesis of this dissertation was that STAT3 is a mediator of muscle insulin sensitivity in the face of changing nutrient levels. STAT3 is a transcription factor that is activated by leptin and circulating cytokines, such as IL-6, that are increased in obesity and insulin resistance. Activated STAT3 induces the transcription of SOCS3, which inhibits insulin signaling at the level of the insulin receptor substrate proteins. STAT3 and SOCS3 activation are increased in patients with impaired glucose tolerance and many of the detrimental effects of IL-6 treatment can be reversed via inhibition of STAT3 in liver and liver cells. Though STAT3 activity is also increased in skeletal muscle from insulin-resistant patients, its role in mediating the impairments in skeletal muscle insulin action that occur with nutrient excess is incompletely defined. Thus, the goal of Study 3 of this dissertation was to provide novel insights into the function of STAT3, and to determine if it is a signaling node that links perturbations in nutrient intake to alterations in skeletal muscle insulin sensitivity.

In Study 3 we chose to use a model where mice consume high-fat diet for only 20 days. This differs from that in Study 2 and instead focused on a time when calorie excess caused insulin resistance without significant weight gain or obesity. In this model, adiposity was increased by high-fat diet feeding, which indicates that the mice were in caloric excess, and thus allows us to test whether genetic knockout of STAT3 in muscle could prevent the deleterious effects of nutrient overload on muscle insulin sensitivity. Interestingly, muscle-specific STAT3 knockout did not enhance skeletal muscle insulin

sensitivity in mice fed a control diet, and furthermore, did not prevent insulin resistance or glucose intolerance due to high-fat diet feeding. Our findings are somewhat surprising since STAT3 has been implicated in the pathogenesis of insulin resistance in other tissues such as liver, where STAT3 inhibition has been shown to restore insulin sensitivity. However, our results suggest that muscle STAT3 is not a major regulator of whole-body glucose homeostasis and that a tissue-specific approach is necessary to fully understand the role of STAT3 in the pathogenesis of insulin resistance.

In conclusion, skeletal muscle insulin resistance is a major contributor to the development of lifestyle diseases, including type 2 diabetes. It is well appreciated that consumption of a high calorie diet can potentiate the development of muscle insulin resistance while a diet low in calories can reverse these deleterious effects. The mechanisms underlying the ability of muscle to detect and respond to these changes in nutrient availability have not been fully elucidated. The findings from the three studies of this dissertation provide unique and important insight into the roles of SIRT1 and STAT3 in the regulation of skeletal muscle insulin sensitivity in the context of altered nutrient intake. Specifically, Study 1 and Study 2 of this dissertation revealed that muscle-specific SIRT1 overexpression does not enhance muscle insulin action in control-fed animals, or under conditions of caloric restriction or high-fat diet feeding. Study 3 showed that knockout of STAT3 in skeletal muscle does not improve muscle insulin sensitivity or prevent insulin resistance caused by high-fat diet feeding. Taken together, the findings of this dissertation highlight the importance of investigating the tissue-specific effects of SIRT1 activation and STAT3 inhibition if they are to be targeted as therapies for the treatment of insulin resistance and type 2 diabetes. Though Cornaro's words ring true as

much today as they did centuries ago, hopefully continued efforts to understand the underlying mechanisms that regulate physiological adaptations to "temperance" and "intemperance" will result in the development of treatments to improve human health.

APPENDIX

NAD+/NADH and skeletal muscle mitochondrial adaptations to exercise

Abstract

The pyridine nucleotides, NAD^+ and NADH, are coenzymes that provide oxidoreductive power for the generation of ATP by mitochondria. In skeletal muscle, exercise perturbs the levels of NAD^+, NADH and consequently, the NAD^+/NADH ratio, and initial research in this area focused on the contribution of redox control to ATP production. More recently, numerous signaling pathways that are sensitive to perturbations in NAD^+(H) have come to the fore, as has an appreciation for the potential importance of compartmentation of NAD^+(H) metabolism and its subsequent affects on various signaling pathways. These pathways, which include the sirtuin (SIRT) proteins, SIRT1 and SIRT3, the poly(ADP-ribose) polymerase (PARP) proteins, PARP1 and PARP2, and C-terminal binding protein (CtBP), are of particular interest because they potentially link changes in cellular redox state to both immediate, metabolic-related changes and transcriptional adaptations to exercise. In this review we discuss what is known, and not known, about the contribution of NAD^+(H) metabolism and these aforementioned proteins to mitochondrial adaptions to acute and chronic endurance exercise.

Introduction

Nicotinamide (NAM) adenine dinucleotide (NAD$^+$; initially known as diphosphopyradine nucleotide [DPN$^+$]), is a ubiquitous cellular coenzyme that was first discovered by Arthur Harden and William Young, when they identified a heat-labile fraction of cell-free glucose fermentation containing ATP, Mg^{2+} and NAD$^+$, which they coined, "cozymase" (78). Our understanding of the role of NAD$^+$ and its reduced form, NADH, in cellular function and metabolism was subsequently expanded by a "who's who" of biochemistry, with researchers such as Hans von Euler-Chelpin, Otto Warburg, Conrad Elvehjem, Arthur Kornberg, Albert Lehninger and Britton Chance, all making substantial contributions. Four of the aforementioned researchers were awarded the Nobel Prize, with Harden and von Euler-Chelpin sharing the Nobel Prize in 1929 for their work on the fermentation of sugar and fermentative enzymes, which included the identification of the "nucleotide sugar phosphate", NAD$^+$. Subsequently, Warbug demonstrated that NAD$^+$ acted as a carrier of hydrogen and transferred it from one molecule to another, which was key to understanding the metabolic function of NAD$^+$ (128). Ultimately, it was work by Freidkin and Lehninger (55) that showed that NADH was an integral component of ATP production via oxidative phosphorylation. Thus, for many years the primary cellular function of NAD$^+$ was considered to be its ability to harness energy from glucose, fatty acids, and amino acids in pathways such as glycolysis, β-oxidation, and the citric acid cycle.

In recent years, however, the importance of NAD$^+$ as a central signaling molecule and substrate that can impact numerous fundamental biological processes has come to the fore. Indeed, a remarkable number of regulatory pathways that utilize NAD$^+$ in signaling

reactions have been identified, and these cover broad aspects of cellular homeostasis including functions in energy metabolism, lifespan regulation, DNA repair, apoptosis and telomere maintenance (11, 12, 84, 97, 190). Thus, while the tissue NAD^+/NADH ratio was once thought to be 'simply' a balance of the redox state, the complexity of NAD^+ metabolism has evolved considerably with the discovery of highly integrated networks of NAD^+ consuming pathways and NAD^+ biosynthetic and salvage pathways (11, 12, 84, 97, 128, 144, 190). Part of the reason for the renaissance of NAD^+ has been the discovery of NAD^+-consuming enzymes, particularly, sirtuins (SIRT). SIRT1 is the most well-described of the seven mammalian sirtuins, and based on its dependence for NAD^+ as a substrate (and therefore its sensitivity to perturbations in NAD^+), SIRT1 has been put forth as a key regulator of acute and chronic exercise-mediated mitochondrial adaptations in skeletal muscle (40, 70, 72, 76, 174, 185, 193). In addition, SIRT3 and poly-ADP-ribose (PAR) polymerases (PARPs), which also use NAD^+ as a substrate, have been proposed as important regulators of mitochondrial function and/or biogenesis (40, 76, 125, 174, 185, 193). In this review our aim is to provide an overview of NAD^+ metabolism in skeletal muscle and the changes that occur in NAD^+, NADH, and the NAD^+/NADH ratio in response to acute and chronic endurance exercise. Our intention is not to discuss the impact of the redox state and NAD^+/NADH ratio on cellular bioenergetics and substrate utilization, which is covered in highly informative reviews by others (9, 26, 106, 109, 110). Rather, our goal is to discuss the changes in pyridine nucleotide redox state that occur with exercise in the context of what we know and do not know about the effects of SIRT1, SIRT3, the PARPs and carboxyl-terminal binding protein (CtBP), on mitochondrial adaptations to exercise in skeletal muscle. It is of

course difficult to extrapolate the findings from one cell line or tissue type to another, and we acknowledge that we do not discuss many important studies that have contributed to our understanding of NAD$^+$ metabolism and SIRT1, SIRT3 and PARP biology in cell lines and tissue types other than skeletal muscle and muscle cell lines. For a more general and encompassing discussion on NAD$^+$ metabolism and its potential clinical implications, readers are encouraged to read some excellent and comprehensive reviews (see, (11, 12, 84, 97, 128, 144, 190)).

Where in the cell is NAD$^+$?

It is broadly accepted that NAD$^+$ is primarily found in three distinct cellular pools, 1) the cytosolic, 2) the mitochondrial, and 3) the nuclear pools. A general overview of the compartmentation of NAD$^+$ and NADH is provided in Figure 1, and provides a point of reference for the ensuing discussion on NAD$^+$(H) compartmentation and their movement into the mitochondria and nucleus. Initial studies used differential centrifugation methods, cell disruption methods, and compounds, to modulate mitochondrial NAD$^+$(H) metabolism in order to determine NAD$^+$(H) location. More recently, the 'compartmentation' of NAD$^+$, which was originally suggested by Ragland and Hackett (146), has been extrapolated from the localization of enzymes in the NAD$^+$ consuming, biosynthetic, and salvage pathways, and the use of innovative molecular biology techniques (11, 12, 84, 97, 144, 190). Thus, Dölle et al. (43) used a novel PAR Assisted Protein Localization AssaY (PARAPLAY) in HeLa S3 cells, in which they targeted the catalytic domain of PARP1 (which consumes NAD$^+$) to various cellular compartments. The idea behind this method is that if NAD$^+$ is present in the compartment to which

PARP1 is targeted, then PAR will accumulate and can be detected by immunocytochemistry (43). Using PARAPLAY, NAD^+ was found in the mitochondria (specifically the matrix but not intermembrane space) and peroxisomes, and surprisingly to the endoplasmic reticulum (ER) and Gogli complex (43, 112). Cytosolic NAD^+ was not detected in this study, most likely due to the fact that PAR glycohydrolase (PARG), which consumes PAR, is most abundant in the cytosol. Little is known about the role of NAD^+ and NADH in regulating Golgi complex and ER function, and certainly its function in skeletal muscle is unknown. Furthermore, surprisingly very little is known about nuclear NAD^+ levels in general, and to our knowledge nuclear $NAD^+(H)$ levels have not been measured in skeletal muscle. Overall, the free cytosolic and nuclear $NAD^+(H)$ compartments are traditionally thought to be in equilibrium, with $NAD^+(H)$ being able to freely pass through pore complexes in the nuclear membrane (46, 98-103, 187, 190). In Cos7 cells the free nuclear NAD^+ concentration is estimated to be ~10-100 μM (53, 188), which is comparable to the estimations for the cytosol (~150 μM) of muscle (42, 119). Thus, in response to exercise, it would be expected that the pyridine redox state in the nucleus reflect changes that occur in the cytosol. The relevance of nuclear $NAD^+(H)$ to adaptations to exercise will be covered when discussing SIRT1, PARPs, and C-terminal binding protein (CtBP).

NAD^+ and NADH concentrations in skeletal muscle at rest.

While PARAPLAY provides qualitative insight into the location of NAD^+, determining the precise concentration of NAD^+ in various compartments remains challenging. Typically, absolute concentrations of NAD^+ and NADH have been

calculated using biochemical and extraction methods, whilst the metabolite indicator method (MIM) has be used to extrapolate the 'free' cytoplasmic and mitochondrial NAD^+/NADH ratio by measuring the concentrations of specific cytoplasmic and mitochondrial redox couples. The MIM carries a number of assumptions, such as the selected dehydrogenase reaction being a near-equilibrium reaction and that the reaction occurs in one cellular compartment, at pH 7.0 (63, 107, 179). In skeletal muscle, the most common application of the MIM is calculation of the cytosolic free NAD^+/NADH ratio, via measurement of lactate and pyruvate levels, based on the lactate dehydrogenase (LDH) reaction (107, 179). The mitochondrial free NAD^+/NADH ratio, can be determined by measuring the concentrations of glutamate, α-ketoglutarate ,and NH_3, and is based on the glutamate dehydrogenase (GDH) reaction (107, 179), although GDH activity is low in skeletal muscle (10, 179).

In resting human muscle, total NAD^+ and NADH concentrations are estimated to be ~1.5-1.9 and ~0.08-0.20 mmol/kg dry weight (dw) muscle, respectively (62, 80, 93, 154, 155, 159, 160). Based on the approximate volumes of distributions of mitochondria, the extra-mitochondrial space (i.e., cytosol) and their mass fractions (i.e., % of cell volume: cytosol = 90% and mitochondria = 10% (50)), Cabrera and colleagues (42, 119) estimate the total, mitochondrial, and cytosolic compartment concentrations in skeletal muscle for NAD^+ and NADH, respectively, to be approximately- Total: 0.45 and 0.05 mmol/kg cell wet weight [ww]; Cytosol: 0.15 and 0.00028 mmol/kg cytosolic ww; Mitochondria: 3.15 and 0.5 mmol/kg mitochondrial ww (Note: to convert to dw muscle, multiply by ~4.2 (145)). Thus, the NAD^+/NADH ratio in resting skeletal muscle is estimated to be much higher in the cytosol (~540) as compared to mitochondria (~6.3),

and overall, greater than ~95% of cellular NADH is estimated to be in the mitochondrial compartment. The nucleus comprises ~1% of muscle cell volume (50), and considering that the nuclear-to-cytosolic $NAD^+(H)$ levels are considered to be in equilibrium, the nuclear NAD^+ and NADH concentrations would be estimated to be comparable to the aforementioned values for the cytosol. Although higher than estimates in other cells (NAD^+: ~10-100 μM; NADH: ~130 nM (53, 188), considering the high density of mitochondria and metabolic turnover of skeletal muscle, these approximations seem reasonable.

Relevant to the redox state and covalent activation of NAD^+- or NADH-dependent signaling proteins is the fact that most cellular NAD^+ and NADH is bound to proteins (13, 54, 171, 176, 179, 180). This makes it quantitatively difficult to determine the free NAD^+ and NADH levels (and the free NAD^+/NADH ratio), which ultimately represent the metabolically active forms of these coenzymes. Measurement of free $NAD^+(H)$ levels is further complicated by the fact that NADH binds proteins more firmly than NAD^+ (54, 171, 180). It should be noted, however, that studies in rat hippocampus using time-resolved fluorescence and anisotropy decay suggest the ratio of free-to-bound NADH to be ~0.78 (175). Whether this is the case in skeletal muscle is unknown. Based on the MIM for LDH, in resting skeletal muscle the free cytosolic NADH level is estimated to be ~0.5-1.5% of total cytosolic NADH (158).

In skeletal muscle, NAD^+ levels are highest in the mitochondria (42, 119), thus by extension one might infer that oxidative skeletal muscle (with a greater abundance of mitochondria) would have overall higher NAD^+ levels compared to glycolytic muscle. Supporting this notion, in human resting muscle, NAD^+ concentration is positively

correlated with the % of slow twitch fibers (62). However, in rat soleus and extensor digitorum longus (EDL) muscles, no differences in NAD^+ levels were noted, although differences in the degree of reduction of the NAD^+ couple were found (i.e., higher NAD^+ levels in soleus vs. EDL mitochondria), which may be indicative of the differing metabolic characteristics of these muscles (158).

Changes in NAD^+ and NADH concentrations and the NAD^+/NADH ratio in muscle during exercise.

Animal studies. Early studies by Britton Chance and colleagues (27, 28, 33) and others (61, 87, 88), typically in amphibian muscle, used fluorescence-based methods (128, 129) to demonstrate that NADH levels decrease (and thus NAD^+ levels increase) during muscle contraction. With respect to mammalian muscle, Jobsis and Stainsby (89) used the same technique to study NADH oxidation in the gastrocnemius-plantaris and gracilis muscle groups in dogs, and found that low-intensity (5 Hz) and tetanic contractions increased NAD^+ levels. By manipulating the ability of mitochondria to oxidize NADH, they concluded that the increase in tissue NAD^+ primarily occurs inside mitochondria (89). In contrast to studies that show that NAD^+ increase with contraction, Duboc et al. reported an increase in NADH during tetanic contractions in soleus and EDL muscles of the rat (44). A limitation of the fluorometric technique used in these studies is that it does not provide quantitative assessment of NAD^+, NADH, and the NAD^+/NADH ratio. Addressing this limitation, Edington and colleagues (48) measured NAD^+ biochemically, and estimated the NAD^+/NADH ratio using the MIM method (using the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios). Thus, in untrained and

trained rats, cytosolic and mitochondrial NAD^+ concentrations, as well as the NAD^+/NADH ratio, were increased by low-intensity muscle contraction of the gastrocnemius-plantaris muscles. As one would expect, the increase in the mitochondrial NAD^+/NADH ratio during the same absolute exercise was lower in trained rats (47, 48). In the soleus and EDL muscles of the rat, twitch or tetanic contractions increased NAD^+ levels (as measured by decreased NADH fluorescence) during contraction (178). Supporting this notion, studies in insect and canine muscle using the MIM method (based on the glutamate dehydrogenase [GDH] reaction) found that the mitochondrial NAD^+/NADH ratio is increased during exercise at a variety of exercise intensities (34, 135, 152, 153, 181). Chronic low-frequency (10 Hz) stimulation of the rat tibialis anterior muscle also increased NAD^+ levels after 15 min of contraction, and the NAD^+/NADH ratio was significantly increased for up to 24 h of stimulation (65). In mice, swimming exercise increased muscle NAD^+ levels (23), and in rats endurance exercise training resulted in a sustained (as samples were measured 2 days after the last exercise bout) increase in NAD^+ levels in gastrocnemius muscle of young and old rats (104). However, an increase in NAD^+ and the NAD^+/NADH ratio during exercise is not a universal finding. In one study NADH increased and the NAD^+/NADH ratio decreased during flight in insect muscle (77), whilst in mouse muscle no change in NAD^+ levels at the end of running exercise was found, though an increase 3 h after exercise was noted (22). In addition, in electrically-stimulated canine muscle (gastrocnemius-plantaris muscles), cytoplasmic NAD^+ levels were reduced during exercise (64), whilst in electrically-stimulated (5 Hz) soleus muscle, no change in NAD^+ levels was found (167).

Human studies. In human muscle, the effects of exercise on NAD^+ levels and the

NAD/NADH ratio are largely the opposite of those found in animal studies. Muscle NAD^+ levels were decreased when exercising at 65% and 100% of maximal oxygen uptake (VO_2max), and while increased muscle water accounted for ~73% of this decrease, NAD^+ levels were still reduced when assessed on a dry weight basis (62). The first studies to quantitatively measure both NAD^+ and NADH levels in human muscle at rest and during exercise were conducted by Dr. Kent Sahlin and colleagues (80, 93, 154, 155, 160). During maximal exercise and submaximal isometric contractions NADH increased ~140% above resting levels, whereas there was no significant change in NAD^+ levels (80, 155). In contrast, no change in total muscle NADH concentration was noted throughout exercise at 75% VO_2max (157), whilst NADH and the cytosolic NAD^+/NADH ratio were decreased during exercise at 50% VO_2max (93). Similar to this, a number of studies found that the cytosolic NAD^+/NADH ratio is reduced during exercise (66, 141), although the magnitude of reduction is lower after exercise training (141). Exercise intensity appears to be an important contributor to the differences in measured NAD^+(H) and NAD^+/NADH ratio during exercise in animal vs. human studies. For example, NADH decreased (and the cytosolic NAD^+/NADH ratio was unchanged) from resting values during exercise at 40% VO_2max, but both NAD^+ and the cytosolic NAD^+/NADH ratio were increased above resting values at 75% and 100% VO_2max (160). Moreover, a series of *in silico* studies (that distill the NAD^+ and NADH information from some of the aforementioned papers) predict that whole tissue, cytosolic, and mitochondrial NAD^+/NADH ratios are reduced during exercise at 60% VO_2max (119), but are increased during exercise at a lower intensity (65 watts) (21, 41). Interestingly, estimation of the mitochondrial redox state during exercise in human

muscle using the MIM method, estimated that the free NAD^+/NADH ratio is significantly increased at 75% and 100% VO_2max (63).

Summary. There are conflicting results in both animal and human studies as to whether or not exercise increases or decreases NAD^+, NADH and the NAD^+/NADH ratio. There are many reasons that may underlie these differences including training state, intensity of contraction, duration of exercise, time point of measurement during exercise, the analytical technique used to measure NAD^+(H) and the NAD^+/NADH ratio (e.g., fluorometric, biochemical, MIM method), and the compartment that was measured (whole tissue, mitochondrial or cytosolic). From a more 'big picture' perspective, because the majority of change in muscle NADH levels with exercise is presumed to occur within the mitochondrial compartment, a large increase in NADH during exercise would correspond to a decreased redox potential, which could be inhibitory on mitochondrial oxidative enzymes and limit TCA cycle flux (63). The simplest explanation for this would be a 'backing up' of the electron transport chain (ETC) due to limitations in the capacity to oxidize NADH. This is supported by the findings that elevated total muscle NADH concentrations decrease to resting levels during recovery from high intensity exercise (80, 155). Alternatively, an increase in the mitochondrial redox potential would be expected to facilitate generation of NADH by increasing the availability of NAD^+ for pyruvate dehydrogenase and the various dehydrogenase reactions of the TCA cycle and β-oxidation (63). In muscle, measurement and extrapolation of NAD^+(H) metabolism during exercise is further complicated by the fact that muscle comprises subsarcolemmal and intermyofibrillar mitochondria, which are known to have different capacities for substrate oxidation (32, 108, 184). Whether

NAD$^+$(H) kinetics during exercise is different within these mitochondrial populations is unknown, and it is likely that fluorometric studies of NAD$^+$(H) metabolism with contraction reflect changes in the subsarcolemmal compartment and not the 'whole' muscle. Considering these results and unresolved questions as a whole, it is clear that a major gap in our understanding of NAD$^+$(H) metabolism during exercise is that no study has directly measured the free NAD$^+$ and NADH levels or the subcellular localization and compartmentation of NAD$^+$(H) metabolism. Such analysis is clearly very technically challenging and will likely require the use of advanced techniques such as HPLC and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry in combination with tissue fractionation methods or two-photon excitation microscopy (139, 162, 182, 188). Ultimately, measuring the free NAD$^+$(H) levels is what is most important when it comes to regulation of proteins and pathways responsive to perturbations in NAD$^+$(H), such as SIRT1, SIRT3, and PARPs, and subsequent effects on cellular function and metabolism.

Shuttling of NADH into the mitochondria.

The inner mitochondrial membrane is impermeable to NAD$^+$ and NADH (115, 143), and shuttles are required to transport NADH from the cytosol to the mitochondria (138). This is accomplished via the exchange of metabolites that are reduced in the cytosol and oxidized in the mitochondria (138). In skeletal muscle these are the glycerol-3-phosphate (G3P; or α-glycerophosphate) shuttle and the malate-aspartate (M-A) shuttle (83, 138, 163-165). Considering that exercise training enhances the capacity of muscle to oxidize NADH, the activities of enzymes of the M-A shuttle are higher in trained vs.

untrained muscle (29, 83, 163, 165), as well as in oxidative vs. glycolytic muscle (29, 163). Moreover, muscle MDH activity decreases with detraining (29). In contrast, the activity of G3P dehydrogenase, a key enzyme in the G3P shuttle, is not increased by exercise training (163, 165), but is higher in glycolytic vs. oxidative muscle (83, 163). Reducing equivalents may also be transferred to the mitochondria via the lactate shuttle, which is explained in detail elsewhere (18, 60). Briefly, the lactate shuttle hypothesis posits that cytosolic pyruvate is primarily converted to lactate, which is then transported via facilitated diffusion into the mitochondria, where it is converted back to pyruvate by intramitochondrial LDH (18, 19, 60). Therefore, the lactate shuttle, via the LDH reaction, would allow for transfer of NADH from the cytosol to mitochondria in a manner similar to the G3P and M-A shuttles. It should be noted that as debated by others, there is significant controversy over the presence of LDH within pure mitochondria and the existence of a lactate shuttle in skeletal muscle mitochondria (16, 20, 59, 147, 156, 184). In recent years the NADH/cytochrome c (cyto c) electron transport shuttle has also been described, in which the direct transfer of electrons from cytosolic NADH to molecular oxygen inside the mitochondrial matrix is achieved at respiratory contact sites (i.e., where both mitochondrial membranes are in contact) (1, 123). The transfer capacity of the NADH/cyto c is reported to be equivalent to the malate-aspartate shuttle (1, 123). However, whether this system is active in skeletal muscle mitochondria, or is regulated by exercise training, is unknown.

Mitochondrial adaptations to endurance exercise: Role of SIRT1 and SIRT3

Sirtuins are a family of class III deacetylases that possess NAD^+-dependent

deacetylase and mono-ADP-ribosyltransferase activities (40, 76, 125, 174, 185, 193). Over the past decade there has been an explosion of research on the therapeutic potential of treating various diseases via activation of sirtuins, especially SIRT1, and more recently, SIRT3 (40, 76, 125, 174, 185, 193). In fact, a search on PubMed reveals that in just the past 12 years some 300 reviews have been published on sirtuins alone, with the majority of these focusing on SIRT1. The requirement of NAD^+ for the deacetylase function of SIRT1 and SIRT3 provides a fundamental link between the activity of these proteins and perturbations in $NAD^+(H)$ status during exercise. Accordingly, our focus here is to discuss the role of SIRT1 and SIRT3 in regulating the effects of acute and chronic exercise on mitochondrial function and biogenesis. A more general overview of sirtuin biology and function can be found elsewhere (40, 76, 125, 174, 185, 193).

SIRT1. SIRT1 is the most studied of the mammalian sirtuins and is mainly found in the nucleus, although it also has cytosolic targets (40, 76, 174, 185, 193). Of particular importance to the focus of this review was the discovery that SIRT1 deacetylates and positively regulates the activity of PGC1α, a master regulator of mitochondrial biogenesis (5, 57, 132, 150). Thus, SIRT1 has also been put forth as a principal regulator of mitochondrial biogenesis via its ability to regulate PGC1α function. Following this, a number of studies have noted that SIRT1 gene (31, 45, 127) or protein (68, 117, 118, 121, 122, 173) levels increase in skeletal muscle in response to acute or chronic exercise, in parallel with upregulation of mitochondrial content. However, other studies have found either no effect (25, 75) or a decrease (73-75, 104) in SIRT1 protein in skeletal muscle with chronic muscle contraction (via electrical stimulation) or endurance exercise. Complimenting these latter studies, skeletal muscle SIRT1 protein content does not scale

with muscle oxidative capacity or PGC1α abundance (73-75). Moreover, when SIRT1 was overexpressed in skeletal muscle, mitochondrial function and abundance (as measured by electron transport chain [ETC] and mitochondrial transcription factor A [mtTFA] protein abundance, citrate synthase activity), gene expression of mitochondrial proteins, and PGC1α gene and/or protein expression was not changed (56, 140) or even decreased (74). In C2C12 myotubes, overexpression of SIRT1 increased PGC1α gene expression and PGC1α promoter activity (5), although effects on mitochondrial biogenesis and function were not assessed. When SIRT1 protein (15, 22, 56, 57) or deacetylase activity (142) is knocked out in skeletal muscle of mice or C2C12 myotubes there is no reduction in mitochondrial function (e.g., O_2 consumption, proton conductance, activity of electron transport chain [ETC] enzymes or citrate synthase), number (as measured by mtDNA:nDNA ratio, ETC protein abundance), PGC1α gene and/or protein expression, or the gene expression of mitochondrial proteins. In contrast, PGC1α gene expression is lower in the TA, gastrocnemius, and soleus of SIRT1-null mice, although whether this reduction impacts PGC1α protein expression, mitochondrial biogenesis, or mitochondrial function was not assessed (5). Moreover, in studies in C2C12 and mouse primary myotubes, SIRT1 knockdown downregulates mitochondrial and fatty acid oxidation gene expression, fatty acid oxidation, and citrate synthase (CS) activity (22, 57), whilst SIRT1 overexpression increases PGC1α expression, transcriptional activity, and mitochondrial genes (5, 57). Despite reductions in PGC1α gene expression, SIRT1 knockdown in C2C12 myotubes does not reduce PGC1α protein expression (56, 57).

Possible reasons for discrepancies between these different studies have recently been reviewed (70, 72). An obvious reason for many of these differences relates to differences between studying SIRT1 biology *in vitro* using muscle cells (particularly C2C12 muscle myotubes), versus *in vivo* using rodent models and adenovirus techniques. Also, the precise definition of mitochondrial biogenesis and function is different across these studies, with measurement of the gene expression of PGC1α and mitochondrial genes being a common outcome measure. While measurement of gene expression provides important information, if positive or negative effects on mitochondrial biogenesis/function are to be concluded, it will be helpful in future studies to provide a more complete assessment of mitochondrial biogenesis/function, which may include measurement of mitochondrial protein synthesis and turnover, submaximal and maximal O_2 consumption, ETC enzyme activity and protein abundance, the mtDNA:nDNA ratio, or mitochondrial morphology by electron microscopy.

To resolve the incongruent findings regarding SIRT1 protein levels and mitochondrial adaptations to exercise, it has been proposed that SIRT1 activity might be the underlying mediator of these changes. Nuclear SIRT1 activity is positively correlated with oxidative capacity (i.e., CS activity, complex IV abundance) across different muscle types and is also associated with the onset of mitochondrial adaptations to acute exercise, as well as chronic changes in oxidative capacity that occur with exercise training (75). Other studies have also reported an increase in SIRT1 activity (as measured by the SIRT1 activity assay or deacetylation of PGC1α) with acute and chronic muscle contraction (22, 23, 25, 73, 75, 104, 117, 118), although no increase was found with voluntary wheel running (despite increased mitochondrial biogenesis) (25). Notably, the SIRT1 activity

assay uses a peptide substrate that contains Fluor de Lys, a non-physiological fluorescent moiety, and studies using this assay (25, 73-75, 104), may be complicated by the fact that measured SIRT1 activity is potentially an artifact of the fluorophore itself (17, 90). This assay also measures SIRT1 activity in the presence of maximal NAD^+, which does not reflect the NAD^+ levels in the muscle. With this in mind, measurement of the acetylation status of proposed SIRT1 targets (e.g., p53, FOXO, or PGC1α), SIRT1 binding to the promoters of known gene targets, or measurement of the gene expression of SIRT1 target genes would compliment measures of SIRT1 activity, and provide a more physiological readout of SIRT1 function.

It is important to note that SIRT1 activity can be regulated via phosphorylation (56, 69, 91, 161). Recently, Gerhart-Hines (56) demonstrated that SIRT1 was phosphorylated in its catalytic domain by protein kinase A (PKA), which is also activated by endurance exercise. In addition, activation of PKA (via forskolin) increased SIRT1 phosphorylation and activity, including induction of PGC1α expression in skeletal muscle (56). This occurred despite no increase in NAD^+ (56), perhaps suggesting that SIRT1 activity (and function) could be regulated independently of NAD^+ in skeletal muscle. However, the effects of exercise on SIRT1 phosphorylation in skeletal muscle are unknown.

A limitation of the aforementioned studies that investigate SIRT1 and exercise-induced mitochondrial biogenesis is that they are correlative, and do not address whether SIRT1 is required for exercise-induced mitochondrial biogenesis in skeletal muscle. To address this limitation, Philp et al. (142) studied the effects of acute and chronic exercise training on muscle function, PGC1α acetylation and mitochondrial biogenesis in mice

with muscle-specific knockout of SIRT1 deacetylase activity (mKO^{SIRT1}). In muscle from mKO^{SIRT1} mice there was no compensatory upregulation in the gene expression of SIRT2-7 or the protein abundance of SIRT3 and SIRT6 (unpublished observations; S. Schenk, A.T. White and A. Philp). Similar to previous studies in mice (14), no impairment in mitochondrial function or number (e.g., abundance and/or activity of complexes I-IV of the ETC, CS activity, mtDNA:nDNA ratio) in muscle from mKO^{SIRT1} vs. control mice was found, nor was muscle endurance capacity impaired (142). Interestingly, mKO^{SIRT1} and control mice also had comparable reductions in PGC1α acetylation and induction of exercise-response genes (e.g., mitofusin 2, PDH kinase 4, cytochrome c) after acute exercise, and normal mitochondrial adaptations (e.g., abundance and/or activity of complexes I-IV of the ETC, CS activity, mtDNA:nDNA ratio) to wheel running training (142). Thus, studies in mKO^{SIRT1} mice reveal that SIRT1 deacetylase activity is not required for normal function of mitochondria in skeletal muscle, nor is it required for exercise-induced adaptations. Regarding PGC1α acetylation, the authors found that the acetyltransferase that regulates PGC1α transcriptional activity, general control of amino acid synthesis 5 (GCN5) (57, 116, 132), is modulated by exercise, such that nuclear localization of GCN5 was reduced and less GCN5 co-immunoprecipitated with PGC1α after exercise (142). Similarly, whole-body deletion of SRC-3, an upstream activator of GCN5, results in decreased PGC-1α acetylation and increased mitochondrial biogenesis (36), whilst overexpression of GCN5 reduces mitochondrial gene expression and fatty acid oxidation (57). This study suggests, therefore, that the reduced acetylation of PGC1α with exercise is not due to increased deacetylation by SIRT1, but rather is a result of reduced acetylation by GCN5 (142). This

is an interesting finding, and demonstrates that PGC1α acetylation is a balance of the activities of the proteins that acetylate and deacetylate it. Currently, the mechanisms by which exercise regulates GCN5 activity, GCN5 translocation from the nucleus, and the GCN5-PGC1α interaction, are unknown.

How SIRT1 gene expression is regulated in response to exercise is also unknown. In liver cells, SIRT1 gene expression is regulated via opposing effects of cyclic AMP response-element-binding protein (CREB) and carbohydrate response-element-binding protein (ChREBP)(134), such that increased CREB binding to the SIRT1 promoter increases SIRT1 transcription, whereas ChREBP binding impairs it. CREB has also been shown to regulate PGC1α transcription (3, 4). Given that acute exercise activates CREB (49, 142), it is possible that this is responsible, at least in part, for increased SIRT1 gene transcription with exercise. The effects of exercise on ChREBP expression and activation in muscle have not been studied. It is also possible that SIRT1 gene expression is regulated by changes in NADH levels. To this end, SIRT1 gene expression is also regulated by C-terminal binding protein (CtBP) (189), a transcriptional corepressor that has a 100-fold greater affinity for NADH than NAD^+ (53, 188). While we discuss CtBP in more detail later in this review, of note here is that changes in NADH levels during or after exercise could reduce the repressive effects of CtBP on SIRT1 gene transcription in skeletal muscle.

SIRT3. SIRT3 is considered to be a mitochondrial-localized protein (8, 35, 71, 124, 136, 170, 172), although there have been some conflicting reports on its localization (166). Relevant to our discussion, in skeletal muscle SIRT3 appears to localize solely to mitochondria (71), and scales with markers of skeletal muscle oxidative capacity (71,

137). Additionally, SIRT3 is decreased in old vs. young sedentary individuals, but is higher in endurance-trained vs. sedentary individuals, regardless of age (111). In line with this, exercise training or chronic electrical stimulation (71, 82, 137), but not acute exercise (71, 82), increases skeletal muscle SIRT3 protein levels, and is specific to those muscles recruited during the exercise intervention. Complimenting these studies, knockdown of SIRT3 in C2C12 muscle cells decreases basal and maximal oxygen consumption rates and mitochondrial content, and prevents PGC1α-induced activation of mitochondrial genes (86, 105). Although knockdown of SIRT3 does not reduce the total mitochondria number as measured by the abundance of complexes I, III and V of the ETC (86), it does reduce skeletal muscle fatty acid oxidation by ~50%, due to hyperacetylation of long chain acyl CoA dehydrogenase (LCAD) (81). Alternatively, overexpression of SIRT3 in C2C12 myotubes increases mitochondrial DNA content (105). Taken together, these studies suggest that SIRT3 plays an important role in regulating skeletal muscle mitochondrial biogenesis, and potentially fatty acid oxidation, in response to long-term exercise training. However, a recent paper by Yang et al. (183) in C2C12 muscle cells and skeletal muscle from SIRT3 null mice counters this perspective. In their paper, the authors demonstrate that SIRT3 acts to reduce mitochondrial protein synthesis (and thus, mitochondrial biogenesis) via its ability to deacetylate mitochondrial ribosomal protein L10 (MRPL10) and negatively regulate the activity of mitochondrial ribosomes. Thus, rather than increase mitochondrial protein synthesis, SIRT3 appears to have the opposite effect in skeletal muscle. The teleological implications of this will be discussed shortly.

Increased ATP utilization during exercise is matched through increased mitochondrial ATP production, which occurs via oxidation of mitochondrial NADH produced in metabolic pathways such as glycolysis, the TCA cycle, β-oxidation, and the electron transport chain (ETC). Interestingly, up to one fifth of mitochondrial proteins are acetylated, as are many of the proteins in these metabolic pathways, which has important effects on their function (95, 177, 191). Indeed, SIRT3 appears to be responsible for much of the deacetylation of mitochondrial proteins (124, 136, 170, 172). Of potential interest to ATP generation in skeletal muscle during exercise, SIRT3 deacetylates and activates the TCA cycle and ETC enzymes, including succinate dehydrogenase (SDH) (30), ubiquinol-cytochrome c reductase hinge protein (a component of complex III) (114), malate dehydrogenase (137), NDUFA9 of complex I (2), GDH (124), ATP synthase (114), and isocitrate dehydrogenase 2 (ICDH2) (168). Also, SIRT3 deacetylates and activates the β-oxidation enzyme, LCAD (81). With this information in mind, we propose that a possible role of SIRT3 in skeletal muscle is the acute regulation of enzymes and pathways that generate ATP in response to ATP demand during exercise. This is supported by the fact ATP production in heart, kidney, and liver from SIRT3 null mice is reduced by more than 50% (2), although whether this is the case in skeletal muscle is unknown. In the context of the findings of Yang et al. (183) showing that SIRT3 reduces (rather than increases) mitochondrial protein synthesis, as measured by a [^{35}S]-methionine translation-based assay, this also would make teleological sense. Thus, during exercise it is necessary to generate ATP to maintain force production, so pathways that utilize energy, such as protein synthesis, would be momentarily halted. The actions of SIRT3, therefore, are akin to the effects of AMPK on enhancing energy

production and inhibiting pathways that use energy for processes other than to maintain

ATP production and muscle work (79, 85), albeit the effects of SIRT3 are specific to the

mitochondria. It will be of interest in future studies to determine if mitochondrial

biogenesis in response to exercise is impaired in SIRT3 null mice. Also, given that fatty

acid oxidation increases during endurance exercise (38), it will interesting to determine if

acute exercise alters substrate utilization in parallel with activation of SIRT3 activity and

deacetylation of its downstream targets. Studies using muscle-specific SIRT3 null mice

and exercise will no doubt be very informative regarding such questions.

PARPs and mitochondrial biogenesis in skeletal muscle

The PARPs are major consumers of nuclear NAD^+, and therefore compete with

SIRT1 for NAD^+ in the nucleus (40, 76, 174, 185, 193). Considering this, a series of

papers from the laboratory of Johan Auwerx recently investigated the effects of knocking

down PARP1 and PARP2 on skeletal muscle mitochondrial biogenesis in C2C12

myotubes and mice. PARP1 null mice had increased levels of NAD^+, reduced acetylation

of SIRT1 substrates such as PGC1α and FOXO1, and increased mitochondrial

biogenesis, as measured by mitochondrial gene expression, mitochondrial morphology,

SDH staining and mtDNA content, O_2 consumption (7). Increased muscle SIRT1 activity

may in part be due to increased protein content, although SIRT1 activity was increased in

HEK293 cells without an increase in SIRT1 protein content (7). Complimenting these

findings, treatment of mice with PARP-1 inhibitors increased NAD^+ levels and SIRT1

activity (7). The activity of other non-nuclear sirtuins including SIRT2 and SIRT3,

however, were unchanged in PARP1 null tissues (7), suggesting that the upregulation of

SIRT1 in the absence of PARP1 may be due to a local change in the NAD^+ pool in the nuclear compartment. Similar to PARP1, knockdown of PARP2 in C2C12 myocytes increased SIRT1 activity (6). In skeletal muscle this appeared to occur through both an increase in intracellular NAD^+ levels and modulation of the SIRT1 promoter by PARP2 (6). As expected, SIRT1 activity was increased in PARP1 and PARP2 null mice and these mice also had increases in skeletal muscle mitochondrial biogenesis (e.g., mtDNA, mitochondrial morphology and gene expression, SDH staining) and their muscle demonstrated a more oxidative phenotype (6, 7). Moreover, PARP2 null mice had increased endurance as measured by a treadmill endurance test (6). Whether this was due to improvements in skeletal muscle per se, or was a function of the changes in other tissues, such as the heart, was not determined. Collectively these studies are very interesting, and suggest that inhibition of PARPs could be used to enhance muscle mitochondrial biogenesis by increasing nuclear NAD^+ levels and increasing SIRT1 activation. If exercise leads to an increase in NAD^+ in the nuclear compartment, it will be interesting in the future to determine if acute exercise leads to inhibition of PARP1 and PARP2, so as to maximize NAD^+ levels and SIRT1 activation. Although, it is notable that *in vivo* SIRT1 deacetylase activity is not required for the ability of exercise to enhance mitochondrial biogenesis (142). Thus, studies that cross PARP1/2 null mice with mKO^{SIRT1} mice, or studies with PARP inhibitors in mKO^{SIRT1} mice, will help to definitively determine if PARP inhibition works through SIRT1, *in vivo*.

Contribution of NADH to mitochondrial adaptations to exercise: Possible role of CtBP

As discussed above, CtBP is a transcriptional corepressor that is greater than 100-fold more sensitive to perturbations in cellular NADH vs NAD^+ levels (53, 188). Considering that the cytosolic/nuclear content of NAD^+ in muscle is estimated to be ~540-fold higher than NADH (42, 119), conversion of NAD^+ to NADH, or vice versa, would therefore result in a greater change in the NADH levels. By extension, and as reasoned by others (53, 188, 189), changes in nuclear NADH, rather than NAD^+, could link perturbations in NAD^+/NADH ratio to gene transcription. To this end, CtBP regulates mitochondrial morphology and function in MEFs and liver-related cells, via its ability to regulate Bcl-2-associated X protein (Bax) (94). CtBP also represses the transcriptional activity of myocyte enhancer factor 2 (MEF2) (186), a key transcription factor in the regulation of mitochondrial biogenesis (39, 131) that shows increased DNA binding in response to exercise (130). The regulation of MEF2 transcriptional activity, however, is complex, as MEF2 is deacetylated by SIRT1, and deacetylation of MEF2 *in vitro* reduces (not increases) its transcriptional activity (126, 192). So clearly, the interplay of exercise on NAD^+(H), SIRT1, CtBP and MEF2, and the subsequent transcriptional response, may represents a balance of these activating and inhibitory signals, that likely involves additional levels of regulatory control, such as ubiquitination, sumoylation and phosphorylation (67, 92, 149). Taken together, these studies point to a potentially important role of CtBP, via its sensitivity to changes in NADH, in the modulation of mitochondrial biogenesis in skeletal muscle in response to exercise.

Replenishing NAD^+ levels in skeletal muscle: An important consideration

If an increase in NAD^+ during exercise leads to an increase in the activity (and thus consumption of NAD^+) by SIRT1, SIRT3, PARP1 or PARP2, then it would be important for skeletal muscle to replenish NAD^+ levels in the cytosolic, nuclear and mitochondrial compartments during or after exercise. In mammals, the NAD^+ biosynthetic and salvage pathways replenish NAD^+, and the specifics of these pathways are reviewed elsewhere (11, 12, 84, 97, 128, 144, 190); an overview of these pathways is presented in Figure 2. Except for research on NAM phosphoribosyltransferase (NAMPT; also known as pre-B-cell colony-enhancing factor 1 (PBEF1) or visfatin), the contribution of these pathways to replenishment of NAD^+ in skeletal muscle and in response to exercise is to date, essentially unstudied.

NAMPT is located in the nucleus, cytosol and mitochondria (96, 148, 182), and is part of the NAD^+ biosynthetic pathway that converts NAM to NAM mononucleotide (NMN) (40, 76, 174, 185, 193). This reaction is potentially important for maintaining the activity of SIRT1 and SIRT3, as nicotinamide (which is generated in the deacetylase reaction of sirtuins, including SIRT1 and SIRT3) is a negative regulator of SIRT1 and SIRT3 (11, 12, 84, 97, 128, 144, 169, 190). Indeed, in HEK293 cells, NAMPT plays an important role in protecting against cell death in response to genotoxic stress by maintaining mitochondrial NAD^+ levels and SIRT3 activation (182). However, in plasma from humans and mice NAM concentrations (which range from 0.3 to 5 μM) are lower than the reported IC_{50} for SIRT1 inhibition, but are in the range of the K_M for NAMPT (24, 148, 151). Thus, whether or not NAM levels in muscle reach a level sufficient to inhibit SIRT1/SIRT3 is unknown. This aside, in rodents, endurance exercise increases NAMPT gene and/or protein expression in parallel with increased tissue NAD^+ levels

(23, 104). Similarly, in humans, NAMPT protein abundance is higher in trained vs. untrained individuals, and is increased by exercise training, although whether this increased NAD^+ levels was not measured (37). Thus, in the context of increased SIRT1/SIRT3 activity during and after exercise, a coordinated increase in NAMPT activity may act to maintain SIRT1/SIRT3 activity by consuming NAM, and also replenishing NAD^+ (discussed below). The concentration of NAM in skeletal muscle is unknown. Therefore, it will be interesting in future studies to determine whether NAMPT activity is increasing specifically in the mitochondrial, nuclear and/or cytosolic compartments with exercise, and whether this coincides with changes in NAM levels. Altogether, such measurements will provide important information regarding the precise contribution of NAMPT to NAD^+ metabolism and the regulation of SIRT1 and SIRT3 activity in skeletal muscle in response to exercise.

To generate NAD^+, NMN generated by the NAMPT reaction is converted by NMN adenylyltransferas (NMNAT) to NAD^+. NMNAT can also convert nicotinic acid (NA) mononucleotide (NAMN) to NA adenine dinucleotide (NAAD), which is subsequently converted to NAD^+, by NAD^+ synthase. There are three isoforms of NMNAT: NMNAT1 and NMNAT2 are localized in the cytosol and nucleus, and NMNAT3 appears to be exclusively in mitochondria (113, 133). At the mRNA level, NMNAT1 is highly expressed in skeletal muscle (51, 52, 113), NMNAT2 is expressed at low levels, whilst NMNAT3 is very low or absent (113). The protein levels and activity of these proteins in skeletal muscle are unknown. The presence of NMNAT1 and to a lower extent, NMNAT2, in skeletal muscle suggests that they may play an important role in replenishing nuclear and cytosolic NAD^+ levels, and it will be interesting to see if

exercise coordinately increases NAMPT and NMNAT1/2 levels, in order to maintain the overall cytosolic/nuclear NAD^+ pools. Regarding replenishment of mitochondrial NAD^+, the inner mitochondrial membrane is impermeable to NAD^+ and NADH (115, 143), which poses a potential problem for maintaining the mitochondrial NAD^+ level, particularly if NAD^+ consumption by SIRT3 is increased during exercise. Only recently was it demonstrated in HeLa S3 cells that NMNAT3 is the only known enzyme of NAD^+ synthesis in mitochondria (133). Whilst NMNAT3 gene expression is very low in skeletal muscle, it will be of interest in future studies to determine if NMNAT3 activity in skeletal muscle correlates with mitochondrial density or if exercise increases the activity or abundance of NMNAT3, even independent of an increase in mitochondrial abundance. Alternatively, perhaps a different or an additional mitochondrial NAD^+ salvage or biosynthetic pathway is present in skeletal muscle mitochondria.

Concluding remarks: There are still many unanswered questions

It has been more than 100 years since the discovery of the pyridine nucleotides, NAD^+ and NADH. While for much of this time $NAD^+(H)$ was considered to primarily participate in metabolic reactions that led to generation of ATP through their ability to act as substrates for enzymes or as covalent modifiers of enzyme function, these coenzymes are potentially key mediators of the adaptive response to exercise. Indeed, changes in $NAD^+(H)$ levels in concert with known $NAD^+(H)$ sensing enzymes provides a logical link between exercise-induced metabolic stress and subsequent mitochondrial adaptations. Specifically, the effects of SIRT1, PARP1/2, and CtBP appear to manifest through their ability to directly or indirectly modulate the transcriptional response to

exercise; they likely do not contribute to an immediate increase in ATP production during acute exercise (Figure 3). Very little, however, is known about $NAD^+(H)$ dynamics in the nucleus of skeletal muscle, and how this affects the transcriptionally-based adaptations central to endurance exercise training. Regarding SIRT3, we propose that it acts as an acute regulator of mitochondrial ATP production via its ability to regulate the enzymatic activity of various TCA and ETC enzymes (and possible as yet to be discovered targets). An additional component of this acute regulation is proposed to include a reduction in mitochondrial protein synthesis during exercise (Figure 3). It is possible that during exercise, SIRT1 plays a similar role in regulating cellular protein synthesis in the cytosol via its ability to negatively regulate mammalian target of rapamycin (mTOR) and/or its interaction with tuberosclerosis complex 2 (TCS2) (58). Whether this regulation occurs in muscle or during exercise is not known. In addition, little is known regarding the coordination of NAD^+ consuming and regeneration pathways in skeletal muscle and whether these two opposing events are regulated by common mechanisms. Furthermore, our understanding of the compartmentation of $NAD^+(H)$ metabolism, and quantitative changes in NAD^+, NADH, and the $NAD^+/NADH$ ratio in subcellular compartments in skeletal muscle at rest and in response to exercise is poor. While technically challenging to measure, such investigation will be highly informative with respect to understanding the activation or inhibition of both $NAD^+(H)$-responsive proteins. For example, while NAD^+ can clearly activate sirtuins, NADH can act as a competitive inhibitor of SIRT1 (120). However, the relative binding affinity of NAD^+ for SIRT1, is ~1000-fold greater than NADH, and overall, the ability of NADH to inhibit SIRT1 activity is proposed to be minimal in an *in vivo* setting (169). Thus, determining the precise contribution of changes

in NAD^+, NADH, and the NAD^+/NADH ratio will be important. In the end it is likely that a combination of changes in free NAD^+ and NADH levels and the NAD^+/NADH ratio within specific subcellular compartments is important. Thus, as research on NAD^+(H) metabolism continues into its second century, there are still many important research questions to be resolved regarding their effect on the adaptive response to exercise in skeletal muscle. Ultimately, such research holds great promise for improving our fundamental understanding of skeletal muscle function in response to exercise, which has obvious and important implications for human health and treatment of skeletal muscle-related diseases.

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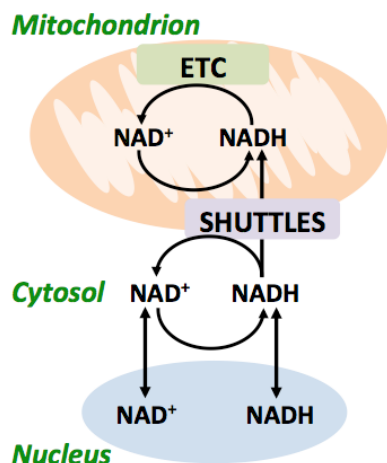
Figure A.1. **Compartmentation of NAD$^+$ and NADH in skeletal muscle.** NAD$^+$ and NADH move freely across pores in the nuclear membrane, and as such the cytosolic and nuclear compartment concentrations of NAD$^+$ and NADH are thought to be comparable. In the cytosol, NADH is generated by glycolysis. Because mitochondria are impermeable to NADH, the transfer of these reducing equivalents occurs via a variety of shuttles including the glycerol-3-phosphate shuttle, malate-aspartate shuttle, lactate shuttle, and the NADH/cytochrome c electron transport shuttle, as described in the text. Depending on the shuttle NADH is produced. The cytosolic/nuclear NAD$^+$ pool is replenished when NADH is converted back to NAD$^+$ in the reactions of the aforementioned shuttles, including the conversion of pyruvate to lactate. NAD$^+$ levels in the nuclear, cytosolic, and mitochondrial compartments are also replenished via specific *de novo* and salvage pathways that are discussed in the text and overviewed in Figure 2. Within the mitochondria, NADH is oxidized to NAD$^+$ in the electron transport chain (ETC).
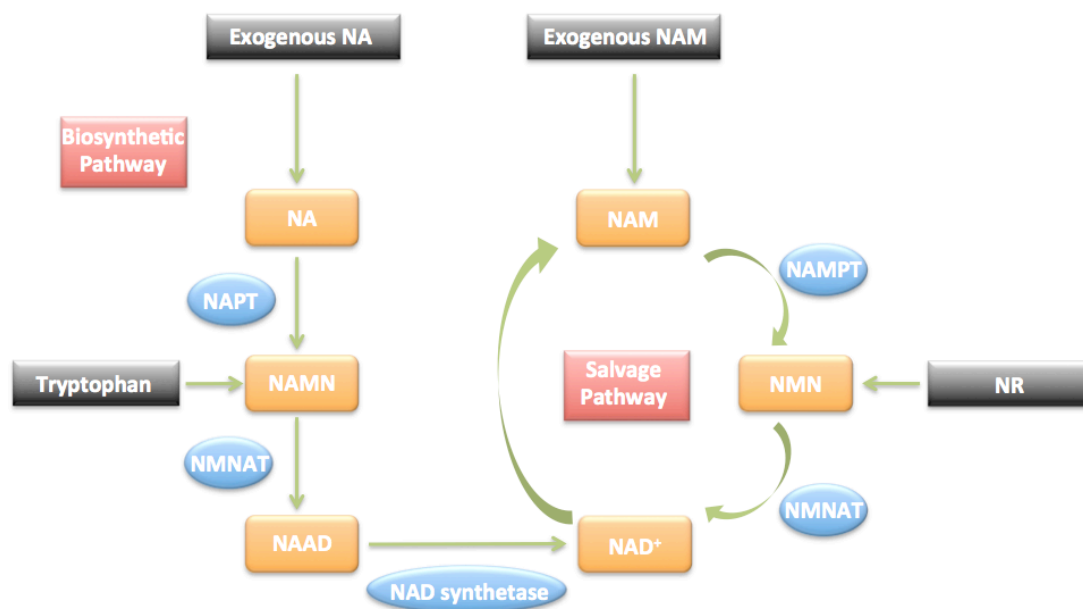
Figure A.2. Replenishment of NAD$^+$ through the biosynthesis (*de novo*) and salvage pathways. Given there are many NAD$^+$-consuming enzymes, it is essential that NAD$^+$ be replenished in order to maintain compartmental NAD$^+$ levels. This occurs through the salvage and biosynthetic pathways. Except for NAMPT, the role of these pathways in NAD$^+$ replenishment in skeletal muscle, and in response to exercise, are essentially unknown. Molecules generated in each pathway are in orange. Enzymes are in blue. NA, nicotinic acid; NAM, nicotinamide; NAMN, NA mononucleotide; NMN, NAM mononucleotide; NMNAT, NMN adenylyltransferase; NAAD, NA adenine dinucleotide; NAD$^+$, NAM adenine dinucleotide; NAPT, NA phosphoribosyltransferase; NAMPT, NAM phosphoribosyltransferase; NR, nicotinamide riboside.
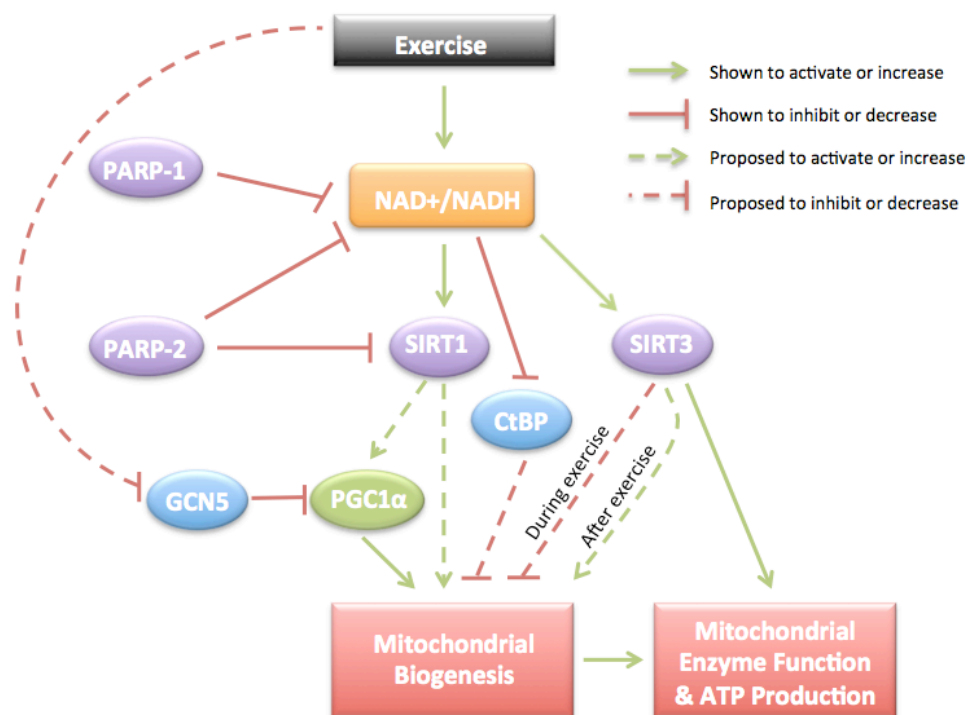
Figure A.3. Proposed mechanism for exercise-induced mitochondrial biogenesis via NAD$^+$/NADH metabolism. Increased ATP demand during exercise leads to an increase in the free cytosolic/nuclear and mitochondrial NAD$^+$ level and NAD$^+$/NADH ratio, which provides increased substrate for the NAD$^+$-consuming enzymes, SIRT1, SIRT3, PARP1 and PARP2. Exercise also reduces the availability of NADH, the predominant covalent activator of CtBP. In response to exercise SIRT1 is also activated by increased cytosolic/nuclear NAD$^+$ levels, and while it likely can contribute to mitochondrial biogenesis through PGC1α-dependent and -independent mechanisms, it is not required for exercise-mediated deacetylation of PGC1α. Rather, acute exercise appears to reduce the inhibitory effect of the acetyltransferase, GCN5, on PGC1α, via mechanism that is still to be determined. PARP1 and PARP2 are able to directly or indirectly modulate SIRT1 activity by competing for NAD$^+$, although the effects of exercise on the activity of these enzymes is unknown. Also, whether SIRT1 is required for the ability of PARP inhibition to induce mitochondrial biogenesis in skeletal muscle, *in vivo*, is not known. The transcriptional corepressor CtBP, is activated by NADH, and it is hypothesized that during or after exercise that reductions in the cytosolic/nuclear NADH level reduces the repressive effects of CtBP on transcriptional modulators of mitochondrial biogenesis. Legend: Dotted lines indicate that a hypothesized contribution of the pathway, or that the data to date provides an incomplete perspective. PARP, poly [ADP-ribose] polymerase; NAD$^+$, nicotinamide adenine nucleotide (oxidized); NADH, nicotinamide adenine nucleotide (reduced); SIRT1, sirtuin 1; SIRT3, sirtuin 3; GCN5, general control of amino acid synthesis; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; CtBP, C-terminal binding protein.