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Acute and chronic regulation of skeletal muscle mitochondrial function

Jonathan Lund Warren
University of Alabama at Birmingham

ACUTE AND CHRONIC REGULATION OF SKELETAL MUSCLE MITOCHONDRIAL FUNCTION

by

JONATHAN L. WARREN JR.

BARBARA A. GOWER, COMMITTEE CO-CHAIR
GORDON FISHER, COMMITTEE CO-CHAIR
W. TIMOTHY GARVEY
DOUGLAS R. MOELLERING
ROBERT A. OSTER

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ABSTRACT

Obesity and type 2 diabetes (T2D) represent a growing global burden on healthcare and financial resources. One hypothesis for the onset and exacerbation of these diseases is related to the role of mitochondria as the end users of products of the metabolism of the nutrients we consume and in mediating the oxidative state of the body through the production of reactive oxygen species (ROS). In order to meet the demands of a metabolic challenge and prevent excessive ROS production, there appear to be adaptations to mitochondrial physiology and morphology that can occur and we hypothesize that the ability of the mitochondria to display these adaptations adequately may be imperative to maintaining cardiometabolic health. The purpose of this dissertation was to characterize mitochondrial function in response to acute and chronic stimuli relevant to the understanding of the onset and prevention of obesity and cardiometabolic diseases like T2D. We describe mitochondrial function in the context of the chronic metabolic environment promoted by obesity, the acute effect of hyperinsulinemia, and the chronic changes induced by an aerobic exercise intervention. We found that total body fat was associated with greater fatty acid oxidative capacity and coupling efficiency. Additionally, we found that an acute hyperinsulinemic event promoted ROS production and mitochondrial inefficiency. Finally, a 16-week aerobic exercise training program improved mitochondrial oxidative capacity under a fatty acid substrate, likely as a function of mitochondrial biogenesis. These data contribute to the hypothesis that the

maintenance and/or restoration of mitochondrial oxidative capacity and adequate plasticity to meet both acute and chronic metabolic demands undoubtedly represents a potential target for the treatment and prevention of obesity, T2D, and other chronic diseases linked to mitochondria dysfunction and oxidative stress.

Keywords: mitochondria, metabolism, body composition, hyperinsulinemia, exercise

DEDICATION

This work is dedicated to my wife and family whose love and support gave me the strength to complete these works and this degree.

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ACUTE AND CHRONIC REGULATION OF SKELETAL MUSCLE MITOCHONDRIAL FUNCTION

INTRODUCTION

Obesity and type 2 diabetes (T2D) comprise a growing global burden on healthcare and financial resources, particularly as a function of the Westernization of diets and reduced activity levels across the world. The mitochondria within our cells are the end users of products of the metabolism of nutrients we consume in order to meet our energetic demands and thus may represent a viable target for the prevention and treatment of these and other cardiometabolic diseases. In order to develop effective prevention and treatment strategies, pursuing a further understanding of the role of skeletal muscle mitochondrial function in the risk for and treatment of obesity, T2D, and other cardiometabolic diseases is imperative.

Prevalence, physiology, and impact of T2D

T2D is a metabolic disease affecting over 30 million people in the US, nearly a quarter of whom are undiagnosed (1). The disease is often associated with obesity and is characterized by insulin resistance (IR) and beta-cell dysfunction. Insulin secretion by the beta-cells of the pancreas may increase initially to mitigate the increase in IR. However, beta-cell output may ultimately become insufficient to overcome the IR and/or the chronic stress of increased demand on the beta-cell may cause it to become completely dysfunctional.

The first treatment strategies for T2D, developed by renowned diabetes pioneer Dr. Elliot Joslin in the early 20th century, were carbohydrate restriction and vigorous exercise. Dietary and exercise interventions continue to represent the cornerstones for the modern management of T2D. In addition to these lifestyle strategies, there are currently a number of oral medications (e.g. metformin, thiazolidinediones) and insulin therapies available to improve glycemic control, though these therapies come with significant financial and social burdens. The prognosis for individuals with well-controlled T2D is quite good, however, if left undiagnosed or untreated, a number of complications can occur, including nephropathy, peripheral neuropathy, retinopathy, and amputations related to nerve damage and poor circulation. Patients with T2D are also at increased risk for cardiovascular events (2) and some cancers (3). Thus, effective strategies for prevention and non-pharmacological treatments deserve continued study in an effort to enhance quality of life for individuals living with and at risk for T2D. Investment in these interventions will provide significant savings to the patient and nation in healthcare dollars.

Mitochondrial energy production

Mitochondria are organelles within eukaryotic cells that are responsible for the production of ATP by oxidative phosphorylation, a process first proposed by Dr. Peter Mitchell in 1961 (4). The mitochondria exhibit a double membrane structure with two distinct compartments separated by the inner membrane: the matrix and intermembrane spaces. NADH and $FADH_2$ (reducing equivalents generated primarily by the metabolism of carbohydrates and fats consumed in the diet) within the matrix are oxidized at

complexes I and II of the electron transport system (ETS), respectively. Electrons are passed down the ETS until transferred to molecular oxygen at complex IV yielding two water molecules. The passing of electrons provides the energy necessary to translocate protons from the matrix space into the intermembrane space at complexes I, III, and IV, creating an electrochemical gradient. The free energy resulting from this gradient is then used to phosphorylate ADP to ATP as protons re-enter the matrix space via complex V, also known as ATP synthase. Both substrate availability and energetic demands can modify the function of the mitochondria in order to operate optimally. The quantity of mitochondria contained within a cell is dictated by the energetic requirements of the cell based on its type and function. Two subfractions of mitochondria exist within skeletal muscle, subsarcaolemmal (SS) and intermyofibbrillar (IMF), which differ in morphology, physiology, and their location within the cell (5).

Mitochondria as producers of reactive oxygen species

In addition to their role as energy producers, mitochondria are also a potent source of reactive oxygen species (ROS), an unavoidable byproduct of aerobic metabolism. Some level of ROS is required for a number of homeostatic processes, including insulin signaling (6). However, chronic exposure to excessive ROS sufficient to overwhelm the antioxidant capacity may result in the oxidation of nearby lipids, proteins, and nucleic acids, damaging these cellular components in the mitochondria and surrounding tissues (7). Mitochondrial ROS production is primarily a function of electrons leaking from the ETS to partially reduce molecular oxygen forming a superoxide anion at complexes I and III, albeit with some additional contribution of complex II (8). Superoxide is a highly

reactive and short-lived molecule acted upon by endogenous antioxidant enzymes (Mn superoxide dismutase if formed within the mitochondrial matrix and CuZn superoxide dismutase if generated within the intermembrane space at complex III) to form hydrogen peroxide. Hydrogen peroxide is relatively stable, can permeate the outer mitochondrial membrane, and is the most abundant of the ROS produced within the body. Hydrogen peroxide plays an important role as a signaling molecule within the cell and surrounding tissues by oxidizing cysteine and thiol residues of various ROS-sensitive proteins, modifying the confirmation and consequently the function of these proteins (9). However, excess hydrogen peroxide can also be scavenged by specific antioxidant enzymes such as catalase and glutathione peroxidase to produce water and oxygen. Hydrogen peroxide can also decompose in the presence of metal ions to become hydroxyl radical (10). Hydroxyl radical is the least abundant of the ROS, but exhibits a relatively rapid and indiscriminate oxidation capacity. As this molecule is so short-lived, humans have no endogenous enzymatic machinery to prevent its action.

The oxidative stress resulting from ROS produced in excess has been implicated in inflammation, atherosclerosis, epigenetic alterations, and apoptotic and mitophagic processes (11). Accumulation of damaged cellular components due to ROS has been linked to obesity and multiple chronic disease conditions, including T2D (11,12).

Acutely uncoupling oxidative phosphorylation from ATP synthesis by allowing protons to re-enter the matrix space through the inner mitochondrial membrane independent of ATP synthase is thought to be one mechanism that can limit ROS formation (13-16). This process ensures the continued utilization of substrate, promotes the continued movement of electrons through the ETS, and alleviates the elevated proton

motive force despite decreased ATP demand. While uncoupled respiration is thought to reduce ROS production, context should be considered. If mitochondrial respiration is chronically uncoupled, this will severely limit bioenergetic efficiency and restrict ATP availability when inevitably presented with a new energetic demand, highlighting the need for mitochondria to exhibit some level of flexibility to shift between a coupled and uncoupled state in order to adapt to the present metabolic environment. Mitochondrial plasticity is a term that has been used within the literature to describe this ability of the mitochondria to respond rapidly and adequately to metabolic demands characterized by the alterations in oxidative activity presented above, as well as adaptations to mitochondrial morphology (17).

Mitochondrial morphology

Early descriptions of the mitochondria depicted these organelles as discrete units. However more recent advances in our understanding of the mitochondria highlight an intricate and interconnected reticular organelle network capable of rapid changes in distribution (18). Even SS and IMF mitochondria, once thought to be independent mitochondrial compartments, are now considered to exhibit some level of connectivity and communication (19). This network is mediated by the balance of fusion, fission, and mitophaghic events, and aids in the overall preservation of mitochondrial health. Fusion processes create a larger, interconnected and filamentous mitochondrial network capable of sharing metabolites, protein, and mitochondrial DNA (mtDNA) (20). Fission dismantles this network and can package dysfunctional proteins and damaged mtDNA to be tagged for mitophagy. These processes also appear to be regulated by transient

metabolic demands and are capable of regulating oxidative capacity of the mitochondria. In particular, mitochondrial fission appears to be associated with elevated mitochondrial substrate and ROS production (21). Fission has also been shown to occur in response to increased ROS production due to hyperglycemia (22). Transgenic models of induced mitochondrial fission have displayed increased uncoupled respiration which was sufficient to relieve the proton motive force in a hyperglycemic model (23).

Assessment of mitochondrial function and ROS production

A number of methods and preparations exist with which to quantify mitochondrial function. Assessing mitochondrial oxygen consumption, or respirometry, allows for indirect measure of oxidative phosphorylation kinetics. The series of titrations used in this technique and the definitions of each classical steady-state measure were first described by Chance and Williams in 1956 (24) and later modified by Nicholls and Ferguson (25). Oxygen consumption by mitochondria is most often assessed using either phosphorescent probes (e.g. Seahorse Bioscience XF Extracellular Flux Analyzer) or amperometric oxygen sensors (e.g. OROBOROS Instruments Oxygraph O2K), each with unique strengths and limitations. The Seahorse apparatus provides a high-throughput, plate-based assay for measuring mitochondrial oxygen consumption in intact adherent cells. In contrast, the OROBOROS O2K offers limited capacity, but greater flexibility in sample type and experimental design. Various substrate combinations can be used to characterize mitochondrial capacity under different conditions. For example, pyruvate or succinate can be used to drive electron input to complexes I and II of the ETS,

respectively. Long-chain fatty acid esters, such as palmitoyl carnitine, can be used to examine mitochondrial fatty acid (FA) oxidation.

Isolation of mitochondria from tissue or cell homogenate by differential centrifugation can provide bioenergetic data independent of various other cellular components. Additionally, this technique can allow for further fractionation of SS and IMF skeletal muscle mitochondria. However, data obtained from isolated mitochondria are limited, as this technique often yields a small fraction of total mitochondria within a tissue sample, and may not accurately reflect mitochondria function *in vivo* (26).

In order to assess mitochondrial function in a more native state within tissues, methods for permeabilizing various tissues (e.g. skeletal muscle) have been developed (27). An appropriate titration with a cholesterol-binding detergent, such as saponin, can form pores within the cellular membrane while preserving the integrity of the mitochondria, which contain relatively low amounts of cholesterol in the outer membranes. These pores allow for the introduction of exogenous substrates, ADP, and inhibitors of various components of the ETS into the mitochondria in an experimental setting. This technique allows us to assess function of mitochondria within a more natural intracellular position, preserving interactions of the mitochondria with the cytoskeleton, nucleus, and other organelles (28). This technique may more closely mimic the *in vivo* environment compared to an isolated mitochondria preparation.

Finally, mitochondria within cultured cell lines also may be examined. While these methods provide a near-endless quantity of experimental tissue, they have unique limitations. These cells are often grown in non-physiological conditions and are impermeable to many relevant compounds including ADP.

Several non-invasive methods for the *in vivo* assessment of mitochondrial function and capacity have been developed using magnetic resonance approaches, such as ^{31}P magnetic resonance spectroscopy (MRS), which allow us to observe the concentrations of various high-energy phosphates (e.g. ATP, phosphocreatine) in tissues (29). Additionally, near-infrared spectroscopy (NIRS) methods can quantify changes in oxygenated versus deoxygenated hemoglobin in skeletal muscle, and in combination with arterial occlusions can provide an inexpensive and portable measure for assessing mitochondrial capacity (30). However, both MRS and NIRS are limited in that they provide no information related to the efficiency of oxidative phosphorylation (i.e. the amount of respiration occurring coupled to ATP production relative to uncoupled respiration).

A number of methods are used to examine ROS production, oxidative stress, and antioxidant capacity. Direct measure of ROS production is difficult, as ROS are relatively short-lived and unstable molecules, however direct measure of superoxide and hydrogen peroxide, among others, is possible using various fluorometric methods (31) that require "living" tissue. Oxidized DNA, lipids, and proteins within various tissue compartments (e.g. as blood and urine) are products of oxidative stress that are much more stable markers of ROS production compared to ROS themselves. These products are commonly measured using a wide array of commercially available assay kits. Finally, methods for the assessment of antioxidant capacity within the tissues can provide valuable data on the ability of various tissues to mitigate the damaging effects of ROS (32).

Role of mitochondria in obesity and disease

The mitochondria have been implicated in obesity, IR, and in a number of chronic disease states from T2D to cancer to Alzheimer's disease. However, a number of questions remain regarding the mitochondrial mechanisms contributing to these maladies.

Obesity has often been associated with mitochondrial dysfunction (33) and chronic oxidative stress (12). Many studies in obese persons report a lower mitochondrial oxidative capacity (34,35) and impairment in the flexibility to adapt to a fatty acid or carbohydrate substrate (36). In addition, a number of studies using *in vivo* methods for assessing mitochondrial function have indicated an enhanced capacity for fatty acid oxidation among obese individuals (37-39). It remains to be determined whether this translates to an inherent mitochondrial phenotype in obese persons and if this plays a significant role in obesity onset or progression.

Whether mitochondrial dysfunction is a cause or consequence of IR and T2D is debated. The most common hypothesis implicating mitochondrial dysfunction as a mediator in the onset of IR relates to decreased mitochondrial oxidative capacity leading to ROS production and the accumulation of lipid intermediates inhibiting the insulin signaling cascade (40). Elevated insulin, over-nutrition, and sedentary behavior create conditions of elevated substrate supply and low energetic demand, which together can promote the production of excessive ROS (6,41), as well as contribute to IR (42). Hyperglycemia (due to IR/glucose intolerance) also promotes excessive ROS production, exacerbating cardiometabolic complications (43). Developing treatments for limiting or preventing oxidative stress may be important for preventing the progression of T2D and associated comorbidities.

There are a number of notable differences in mitochondrial properties between patients with T2D and healthy counterparts. Numerous studies have demonstrated decreased mitochondrial size, content, biogenesis, and oxidative capacity in both patients with T2D and otherwise healthy individuals with a family history of T2D compared to healthy control subjects (20,44,45). Others have shown derangements in the ability of skeletal muscle mitochondria of patients with T2D to readily adapt to substrate availability (46). Corroborating these data are studies that have found lower whole-body aerobic capacity in patients with T2D (47,48). However, there are conflicting reports on whether mitochondrial function and plasticity are truly impaired in patients with T2D independent of mitochondrial content. Boushel et al. have demonstrated that while coupled and uncoupled respiration are lower in permeabilized fibers normalized to the wet weight of the fiber bundles, no such differences are apparent after normalizing to mtDNA or citrate synthase activity (49), suggesting that the differences observed are solely due to decreased mitochondrial content. The acute effects of insulin on mitochondrial function remain an active and incomplete area of study as well. Nair and colleagues purport that 8 hours of high dose insulin actually stimulates an increase in mitochondrial ATP production in healthy subjects compared to no change in participants with T2D, and that insulin deficiency (in participants with type 1 diabetes) decreases ATP production (50,51). These studies were limited to assessments performed in isolated mitochondria. Despite these controversies, improving both mitochondrial quality and quantity in patients with T2D to increase substrate oxidation, reduce ROS production, and reduce IR are considered viable methods for prevention and treatment.

If individuals with IR and T2D do not display the necessary mitochondrial plasticity to mitigate excessive ROS production promoted by a metabolic insult, it may promote continued oxidative damage and further complications. Impaired uncoupling capacity has been shown to be associated with T2D. For example, expression of uncoupling protein 3 (UCP3), a protein thought to facilitate uncoupling in skeletal muscle, is reduced by 50% in patients with T2D compared to healthy controls (52). Additionally, transgenic rodent models expressing high levels of UCP3 appear resistant to obesity and diabetes (53,54). Mitochondrial plasticity also encompasses the regulation of the dynamic mitochondrial reticular network by altering morphology through fission and fusion processes. Whether these mechanisms of plasticity are regulating acute ROS production and mitochondrial function (and subsequently, cardiometabolic risk) have yet to be determined. Assessment of mitochondrial responses to acute stimuli also warrants further investigation in order to better understand the role of mitochondria in mediating the acute redox environment.

Exercise-induced changes to mitochondrial function

The energetic demands placed on skeletal and cardiac muscle during aerobic/endurance exercise are met primarily by the oxidative phosphorylation capacity of the mitochondria and a number of beneficial mitochondrial adaptations are known to occur following exercise training. Exercise can promote the expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) (55), a key protein involved in mitochondrial biogenesis (56), function (57), and dynamics (56,58). Additionally, mRNA levels of key regulatory proteins in FA transport and metabolism, fatty acid translocase

(CD36) and carnitine palmitoyltransferase 1B (CPT1B), have been shown to be elevated after a short exercise program (59) and CD36 appears to be an important mediator in the increase in FA metabolism following exercise (60). In an aging population, 12 weeks of moderate intensity aerobic training was sufficient to enhance ETS activity and mitochondrial content (61). Increases in mitochondrial capacity do not appear to be limited to aerobic training paradigms, as resistance exercise training has also been shown to elicit qualitative and quantitative changes in mitochondrial respiration (62). Exercise interventions also seem to be able to improve insulin sensitivity (63) and lower oxidative stress (64) in at-risk populations, while AET can improve mitochondrial impairments such as low mitochondrial density and impaired fat oxidation (65) in participants with diagnosed T2D. In addition to these improvements, exercise appears to promote mitophagic processes (66), which allow the organelle to dispose of damaged mitochondrial machinery in order to maintain mitochondrial quality. However, whether these changes in mitochondrial function are the result of chronic or acute effects of exercise is not well understood. In addition, given the acute and chronic energetic deficit created by an aerobic training program, it remains unclear whether these effects are apparent following the restoration of energy balance.

Summary

The purpose of this dissertation is to 1) characterize the relationship between mitochondrial oxidative capacity and body composition, 2) assess changes in skeletal muscle ROS production and mitochondrial function in response to an acute hyperinsulinemic event, and 3) to assess changes in aerobic capacity and mitochondrial

capacity following 16 weeks of aerobic exercise training. Collectively, these studies will provide a comprehensive investigation into the regulation of mitochondrial health by both acute and chronic stimuli and further define the role of mitochondrial function in cardiometabolic health. We believe these data will assist in determining that the restoration of healthy mitochondrial function could represent a promising mechanism to target in order to prevent and treat of obesity, T2D, and other cardiometabolic diseases. The following describes the manuscripts contained herein.

Associations of mitochondrial fatty acid oxidation with body fat in premenopausal women

Higher *in vivo* fatty acid (FA) oxidation rates have been reported in obese individuals compared to lean counterparts, however whether this reflects a shift in substrate-specific oxidative capacity at the level of the skeletal muscle mitochondria has not been examined. This chapter provides evidence of a positive relationship between FA oxidative capacity at the level of the skeletal muscle mitochondria and body fat in a cohort of lean to obese women. We hypothesized that total fat mass would be associated with higher skeletal muscle mitochondrial FA oxidation. Additionally, given the inter-individual variability in body fat distribution of the cohort, we examined whether regional fat distribution patterns were independent predictors of mitochondrial FA oxidation. We found that total fat mass was positively associated with State 3 (ADP stimulated respiration) and the respiratory control ratio (RCR), an index of mitochondrial coupling efficiency. These data suggest that the increased FA oxidation observed in obese persons using *in vivo* methods originates at the mitochondrial level.

Effects of acute hyperinsulinemia on skeletal muscle mitochondrial function, reactive oxygen species production, and metabolism in premenopausal women

Skeletal muscle mitochondrial dysfunction and ROS production are thought to be important mediators in the onset and exacerbation of IR and T2D. Others have previously hypothesized that when mitochondria have the capacity to respond rapidly and adequately to meet a metabolic demand, ROS production may be limited. Characterizing the acute changes in skeletal muscle mitochondrial function and ROS production under a metabolic challenge will yield further understanding of normal physiological responses. In this chapter, we showed that following a hyperinsulinemic-euglycemic clamp, ROS production is elevated and mitochondrial coupling is decreased, suggesting a possible link between these processes.

Effects of aerobic exercise training on skeletal muscle mitochondrial function and lipid metabolism assessed in energy balance in premenopausal women

Aerobic exercise training (AET) has been shown to improve mitochondrial bioenergetics and up-regulate proteins related to lipid metabolism. However because exercise often induces acute and chronic energetic deficits, whether these improvements are directly induced by exercise, or occur secondary to negative energy balance (EB), is unclear. The purpose of the study was to assess whether changes in skeletal muscle mitochondrial function induced by AET persist under rigorously controlled EB conditions. In this chapter, we show that 16 weeks of AET induced increases in rates of coupled and uncoupled mitochondrial respiration using a fatty acid substrate. However, when rates were normalized to mitochondrial content, no significant differences were

observed, suggesting that enhancements in mitochondrial oxidative capacity due to AET are mediated by mitochondrial biogenesis.

ASSOCIATIONS OF MITOCHONDRIAL FATTY ACID OXIDATION WITH BODY FAT IN PREMENOPAUSAL WOMEN

by

JONATHAN L. WARREN, BARBARA A. GOWER, GARY R. HUNTER, SAMUEL T. WINDHAM, DOUGLAS R. MOELLERING, AND GORDON FISHER

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Abstract

Higher *in vivo* fatty acid (FA) oxidation rates have been reported in obese individuals compared to lean counterparts, however whether this reflects a shift in substrate-specific oxidative capacity at the level of the skeletal muscle mitochondria has not been examined. The purpose of this study was to test hypothesis that *in situ* measures of skeletal muscle mitochondria FA oxidation would be positively associated with total body fat. Particpants were 38 premenopausal women (BMI=26.5±4.3 kg/m^2). Total and regional fat was assessed by dual-energy X-ray absorptiometry (DXA). Mitochondrial FA oxidation was assessed in permeabilized myofibers using high-resolution respirometry and a palmitoyl carnitine substrate. There were positive associations of total fat mass with State 3 (ADP-stimulated respiration) ($r=0.379$, $p<0.05$) and the respiratory control ratio (RCR, measure of mitochondrial coupling) ($r=0.348$, $p<0.05$). When participants were dichotomized by high or low body fat percent, participants with high total body fat displayed a higher RCR compared to those with low body fat ($p<0.05$). There were no associations between any measure of regional body fat and mitochondrial FA oxidation independent of total fat mass. In conclusion, greater FA oxidation in obesity may reflect molecular processes that enhance FA oxidation capacity at the mitochondrial level.

Introduction

A number of studies have shown enhanced FA oxidation in obese individuals compared to lean counterparts using *in vivo* approaches to assess FA oxidation capacity and anthropometric measures of obesity [1-3]. Whether this observation reflects a shift in substrate-specific oxidative capacity at the level of the skeletal muscle mitochondria has not been examined.

The process of FA oxidation is dependent on a number of factors that cannot be disentangled using *in vivo* approaches. FA must first be transported into cells via fatty acid translocase (CD36), followed by FA transport into the mitochondria via carnitine palmitoyltransferase 1B (CPT1B), and ultimately β-oxidation and oxidative phosphorylation within the mitochondria [4]. Potential alterations at any of these regulatory steps that may be responsible for promoting FA oxidation in obesity cannot be adequately identified by *in vivo* measures of FA oxidation. In contrast, studying FA oxidation in skeletal muscle mitochondria measured *in situ* may provide insight into whether its regulation is controlled at the mitochondrial level or processes further upstream. Thus, these *in situ* measures can be used to examine whether the mechanisms promoting increased whole-body FA oxidation in obese individuals are reflected in changes in mitochondrial capacity.

Mitochondrial capacity is modifiable in order to respond to changes in both acute and chronic metabolic demands [5]. In the context of the chronic metabolic condition of obesity, increases in mitochondrial FA oxidative capacity may be mediated in part by peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), a protein that promotes the transcription of genes associated with FA metabolism and is upregulated by

a high-fat diet and obesity in rodent models [6, 7]. Elevated FA oxidation can interfere with the oxidation of glucose [8], which may lead to a continued reliance on FA substrate [9]. Identifying whether enhanced FA oxidation observed in obese humans is a phenotype regulated in part by the mitochondria will provide a novel mechanism in which to target to prevent and treat obesity.

The overall purpose of this study was to test the hypothesis that *in situ* measures of skeletal muscle mitochondria FA oxidation would be positively associated with total body fat in a diverse cohort of premenopausal women. We hypothesized that total fat mass would be associated with higher skeletal muscle mitochondrial FA oxidation. Additionally, given the inter-individual variability in body fat distribution of the cohort, we examined whether regional fat distribution patterns were independent predictors of mitochondrial FA oxidation.

Methods

Participants

Participants were 38 premenopausal female participants self-identifying as European-American (EA) or African-American (AA). Inclusion criteria were BMI of 18.5-35.0 kg/m^2, sedentary (<30 minutes of structured activity per week), non-diabetic, and no use of medication known to affect body composition or metabolism. All testing was conducted during the first 10 days of the follicular phase of the menstrual cycle. All participants provided informed consent. This study was approved by the Institutional Review Board at the University of Alabama at Birmingham.

Total and regional body fat quantification

Body composition was assessed by dual-energy X-ray absorptiometry (DXA) using a Lunar iDXA densitometer with enCORE™ software (GE-Lunar Corporation, Madison, WI). Participants were scanned in light clothing lying supine with arms at their sides. The scans were assessed for total body fat-free mass (kg), total body fat mass (kg), total body fat percentage (%), leg fat mass (kg), android region fat mass (kg), and IAAT volume (cm^3).

Laboratory analyses

Sera were analyzed by the Core Laboratory of the UAB Nutrition Obesity Research Center, Diabetes Research Center, and Center for Clinical and Translational Science. Fasting glucose, total cholesterol, HDL-cholesterol, triglycerides, and circulating free FA were measured using a SIRRUS analyzer (Stanbio Laboratory, Boerne, TX). Fasting LDL cholesterol was calculated using the method of Friedewald [10]. Fasting insulin was measured using a TOSOH AIA-II analyzer (TOSOH Corp., South San Francisco, CA).

Preparation of permeabilized myofiber bundles

This technique has been adapted from previously published methods [11]. After an overnight fast, each participant came to the UAB Clinical Research Unit for a skeletal muscle biopsy of the vastus lateralis under local anesthesia. The tissue was cleaned of adipose and connective tissue and a tissue bundle of approximately 20 mg was selected for mitochondrial respirometry experiments. The tissue was transferred to the laboratory

on ice in Buffer X containing 50 mM MES, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.7 mM ATP, 14.3 mM PCr, and 6.56 mM $MgCl_2$-6 H_2O (pH 7.1, 290 mOsm). Once in the laboratory, the tissue was mechanically dissected into several smaller muscle bundles (of approximately 3-5 mg wet weight). Each bundle was gently separated with a pair of antimagnetic needle-tipped forceps under magnification. Bundles were treated with 30 µg/ml saponin in Buffer X and incubated on a rotator for 30 min at 4°C. The tissue bundles were washed of saponin for 15 minutes in Buffer Z containing 105mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4, and 5 mM $MgCl_2$-6 H_2O, 5 µM glutamate, 2 µM malate, and 5.0 mg/ml BSA (pH 7.4, 290 mOsm). Finally, the samples were transferred to Buffer Z containing 20 mM creatine hydrate and 5 µM of blebbistatin for 10 minutes prior to respirometry experiments [11].

Mitochondrial respiration measures

High-resolution respirometry experiments were performed using an Oroboros Oxygraph O2K (Oroboros Instruments, Innsbruck, Austria) containing 2 mL of Buffer Z with creatine and blebbistatin, constantly stirred at 37°C. We assessed mitochondrial O_2 consumption using a FA substrate protocol. Coupled respiration (State 3) was measured using malate (2.5 mM) and palmitoyl carnitine (40 µM) after the addition of a submaximal concentration of ADP (1mM) [12]. Cytochrome *c* (10 µM) was added to assess mitochondrial membrane integrity after preparation of the myofibers. There was no significant increase in respiration following the addition of cytochrome *c*. Uncoupled respiration (State 4) was induced by the administration of oligomycin (2 µg/mL). Oxygen

flux was normalized to the wet weight of each fiber bundle, a standard method for normalizing respiration rates [13]. The respiratory control ratio (RCR) was calculated as State 3/State 4. The RCR is a commonly used metric for assessing mitochondrial integrity and coupling [14].

Statistical analyses

All descriptive characteristics are reported as mean ± SD. Pearson correlations were calculated between total and regional body fat and mitochondrial measures. Partial correlations between mitochondrial measures and leg fat (kg), android fat (kg), and IAAT volume (cm^3) controlling for the effect of total fat mass (kg). Participants were dichotomized into high body fat and low body fat groups at the median value for tissue percent body fat (39.8%). Differences between groups were assessed using two-tailed *t*-tests for independent samples. An alpha level of $P<0.05$ was used to determine statistical significance. All statistical analyses were conducted using SPSS Statistics for Macintosh Version 22.0 (IBM Corp., Armonk, NY).

Results

Demographic, physical, and metabolic characteristics of the study sample are shown in **Table 1**. BMI ranged from 19.7 to 34.5 kg/m^2 (mean of 26.9 ± 4.9 kg/m^2), age from 19 to 44 years (mean of 28.3 ± 7.0 years), and 54% were AA. There were no differences between EA and AA in age, BMI, total body and regional fat measures, or measures of mitochondrial FA oxidation (data not shown).

We observed significant associations between total fat mass and both State 3 (r=0.379, p=0.021) (**Figure 1A**) and the RCR (r=0.348, p=0.035) (**Figure 1B**) from the *in situ* studies. There was no association between circulating FA and total body fat. When participants were dichotomized by the median percent body fat, State 3 trended towards being higher (p=0.086) (**Figure 2A**) and the RCR was significantly higher (p=0.024) (**Figure 2B**) in those with high body fat (n=18) compared to those with low body fat (n=19). There were no differences in circulating FA between groups.

Android fat and IAAT were positively associated with State 3 and RCR ($p<0.05$) (**Table 2**). However, after adjusting for total fat mass, no associations between regional fat measures and State 3 or the RCR (**Table 2**) were observed. Leg fat mass was not associated with any measure of mitochondrial function before or after adjusting for total fat mass.

Discussion

The primary finding from this study was that total fat mass was associated with higher ADP-stimulated FA oxidation capacity assessed in skeletal muscle mitochondria in a diverse cohort of premenopausal women. These data corroborate previous investigations that have described elevated FA oxidation in obese subjects using *in vivo* methods (e.g. uptake of tracer-labeled intravenous FA) [2, 3], suggesting that this is regulated in part by a programmed mitochondrial phenotype that is preserved *in situ*. Additionally, we found mitochondrial coupling was positively associated with total fat mass and that women with higher total body fat had greater mitochondrial efficiency compared to those with low total body fat. If obese persons display a chronically coupled

phenotype, this may promote further fat gain and oxidative stress, which may play a role in the onset of comorbidities associated with obesity.

There are a number of hypotheses that may implicate an altered mitochondrial capacity in the increased FA oxidation observed in obese persons measured *in vivo*. Individuals with obesity are known to have greater intramyocellular lipid [15], a phenotype that chronically elevates FA substrate availability and promotes substrate competition that may ultimately impair substrate flexibility [8, 9]. Others have suggested that enhanced FA oxidation is a mitochondrial response to a lipid-induced stress by elevated intramyocellular triglyceride (IMTG), ceramide, and diacylglycerol [16]. Additionally, PGC1α is shown to be induced by high-fat feeding and obesity in animal models [6, 7] and plays an important role in regulating mitochondrial FA oxidation [17]. Identifying the precise mitochondrial mechanism responsible for this enhanced capacity may allow us to better treat and prevent obesity in the future.

In contrast to our findings, impaired mitochondrial function has been observed in participants with obesity in other studies, although these studies did not use FA-derived substrates [18] or observed these effects in cultured tissues [19] and in participants with extreme obesity (BMI>40kg/m^2) [20]. Hulver *et al*. reported significantly lower skeletal muscle FA oxidation in participants exhibiting extreme obesity (BMI>40 kg/m^2) compared to lean controls, but FA oxidation appeared to be modestly increased in a group of overweight and obese subjects (BMI between 25.0 to 34.9 kg/m^2) (non-significant finding, only 8 participants per group) [20]. Taking into account that all participants in our study had a BMI less than 35.0 kg/m^2, our data in conjunction with the study of Hulver *et al*. suggests that greater FA oxidation capacity is indeed elevated in

obesity, but that the association with FA oxidative capacity may no longer exist in morbid obesity (BMI>40 kg/m^2).

The positive relationship between total fat mass and the RCR suggests greater adiposity may contribute to a mitochondrial phenotype marked by greater mitochondrial coupling, or alternatively that greater coupling is a contributing factor determining fat accumulation. Greater coupling of mitochondrial respiration reduces proton re-entry in the mitochondrial matrix independent of ATP synthesis when energy demand is low, which may promote accumulation of FA within the mitochondria, a phenotype associated with insulin resistance [21]. Additionally, greater coupling could promote the production of reactive oxygen species (ROS) [22, 23], which may catalyze the oxidative stress often observed in obesity [24]. Interestingly, mechanisms of mitochondrial proton leak are induced by FA [25]. Thus, it is possible that these mechanisms become impaired in the obese state.

Although previous reports have supported that FA oxidation is elevated in android obesity, our findings showed no independent effects of android fat distribution on FA oxidation. A study by Carey *et al*. reported greater lipid oxidation in women with the highest levels of abdominal fat (a composite measure of IAAT and abdominal subcutaneous fat) using respiratory gas exchange, but may be confounded by total fat [26]. There may be a number of reasons that we did not observe an independent relationship between android fat and FA oxidation. IAAT is known to be more lipolytically active, however the total volume of IAAT relative to other fat depots within the body is still relatively low [27]. Additionally, the present cohort of women may

represent a relatively healthy, young population in which metabolic effects of IAAT may not yet have manifested.

Both metabolic efficiency and FA oxidation are shown to differ between EA and AA individuals [28-32]. However, in the present study, we found no differences in FA oxidation assessed in skeletal muscle mitochondria between EA and AA women. Differences in FA oxidation between EA and AA are thought to be due primarily to FA transport, a mechanism known to differ between races. Lower skeletal muscle activation and transport of FA has been observed in morbidly obese AA women when compared to EA [29]. However, in the same study, no difference was observed between groups in the oxidation of palmitoyl carnitine, suggesting no differences in FA oxidation after activation and translocation of substrate into the mitochondria. It will be important in future studies to recruit sufficient numbers of both lean and obese women of both races to clarify if and under what circumstances potential racial differences in mitochondrial FA oxidation exist.

Strengths of this study included robust measures of body composition and the sizeable sample for which human skeletal muscle biopsies were obtained. Mitochondrial function was assessed using permeabilized human myofibers, which allowed us to analyze mitochondrial function within the native cellular system while also preserving interactions with the cytoskeleton and various cellular components. This method may more closely resemble the *in vivo* environment compared to isolated mitochondria. We acknowledge as a limitation that our data may not reflect FA oxidation in other populations, such as men or participants with impaired glucose metabolism. Future

studies in these populations are needed to better understand the relationship between FA oxidation and fat mass.

Conclusions

In conclusion, this study demonstrates that the positive relationship between measures of *in vivo* FA oxidation and obesity is a phenotype preserved at the level of the skeletal muscle mitochondria. Furthermore, greater mitochondrial coupling under elevated FA substrate in conjunction with obesity could contribute to an environment prone to continued fat deposition. Further research is needed to determine the molecular mechanisms responsible for promoting this mitochondrial phenotype and if this increase in FA oxidation is associated with any obesity-related comorbidities.

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Author Contributions

JLW, BG, GH, DM, and GF were involved in the study conception and design and interpretation of the data. JLW, STW, DM, and GF participated in the data collection. JLW, BG, and GF conducted statistical analyses. All authors have critically revised and approved the submitted manuscript.

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Table 1. Participant characteristics.

	n=38
Age (years)	28.3 ± 7.0
Race	17 EA, 21 AA
BMI (kg/m^2)	26.5 ± 4.3
Total Fat Mass (kg)	27.5 ± 8.8
Leg Fat Mass (kg)	11.1 ± 3.5
Android Fat Mass (kg)	2.0 ± 0.9
IAAT volume (cm^3)†	435.2 ± 374.0
Fat Free Mass (kg)	45.0 ± 6.1
Total Cholesterol (mg/dL)	174.1 ± 30.6
Triglycerides (mg/dL)	78.0 ± 43.4
HDL (mg/dL)	62.6 ± 14.4
LDL (mg/dL)	95.8 ± 23.3
Free Fatty Acids (mEq/L)‡	0.6 ± 0.2
Fasting Glucose (mg/dL)†	88.6 ± 7.5
Fasting Insulin (μIU/mL)†	8.7 ± 3.3
State 3 (pmol/s/mg)†	7.2 ± 3.0
State 4 (pmol/s/mg)	3.5 ± 1.3
RCR (State 3/State 4)†	2.1 ± 0.6

†n=37, ‡n=35. AA, African-American; BMI, body mass index; EA, European-American; IAAT, intra-abdominal adipose tissue.

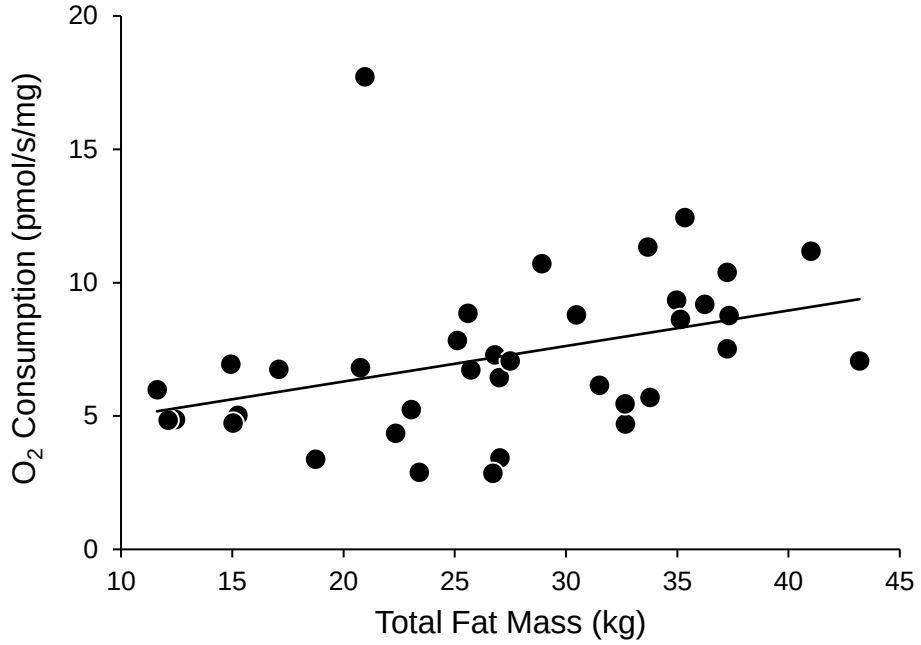
Table 2. Pearson correlation coefficient (P-value) and partial correlation coefficient adjusted for Total Fat (kg) (P-value). *P<0.05.

	State 3	State 4	RCR	State 3 (adj.)	State 4 (adj.)	RCR (adj.)
Total Fat Mass (kg)	0.379 (**0.021***)	0.216 (0.193)	0.348 (**0.035***)	-	-	
Leg Fat Mass (kg)	0.256 (0.126)	0.147 (0.379)	0.286 (0.086)	-0.208 (0.232)	-0.108 (0.537)	-0.097 (0.578)
Android Fat Mass (kg)	0.440 (**0.006***)	0.253 (0.125)	0.360 (**0.028***)	0.283 (0.099)	0.175 (0.314)	0.110 (0.530)
IAAT volume (cm^3)	0.424 (**0.010***)	0.215 (0.201)	0.399 (**0.016***)	0.235 (0.175)	0.091 (0.605)	0.158 (0.363)

IAAT, intra-abdominal adipose tissue.

Figure 1. State 3 respiration is positively associated with total fat mass (r=0.379, p=0.021) (A) and RCR is positively associated with total fat mass (r=0.348, p=0.035) (B).

A



B

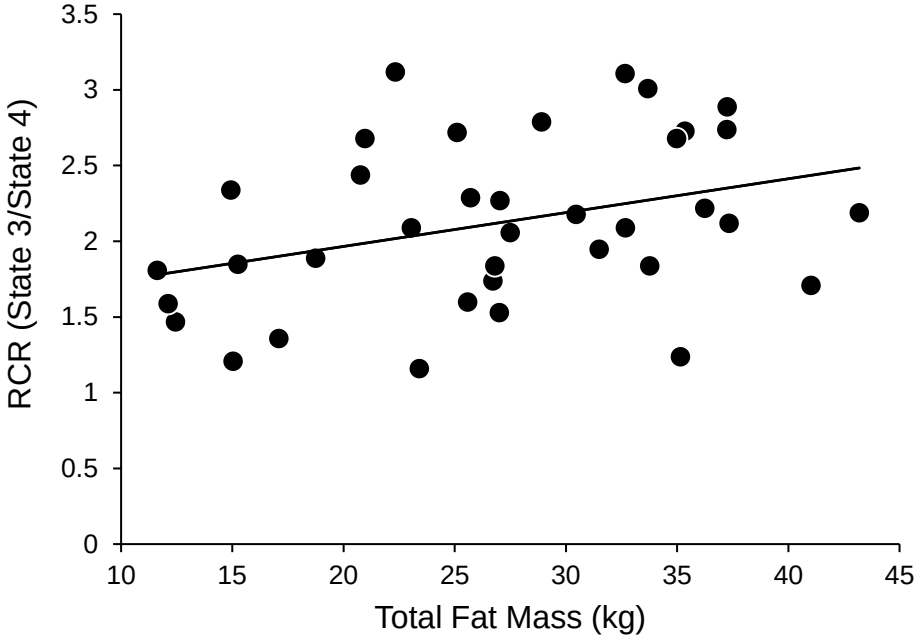
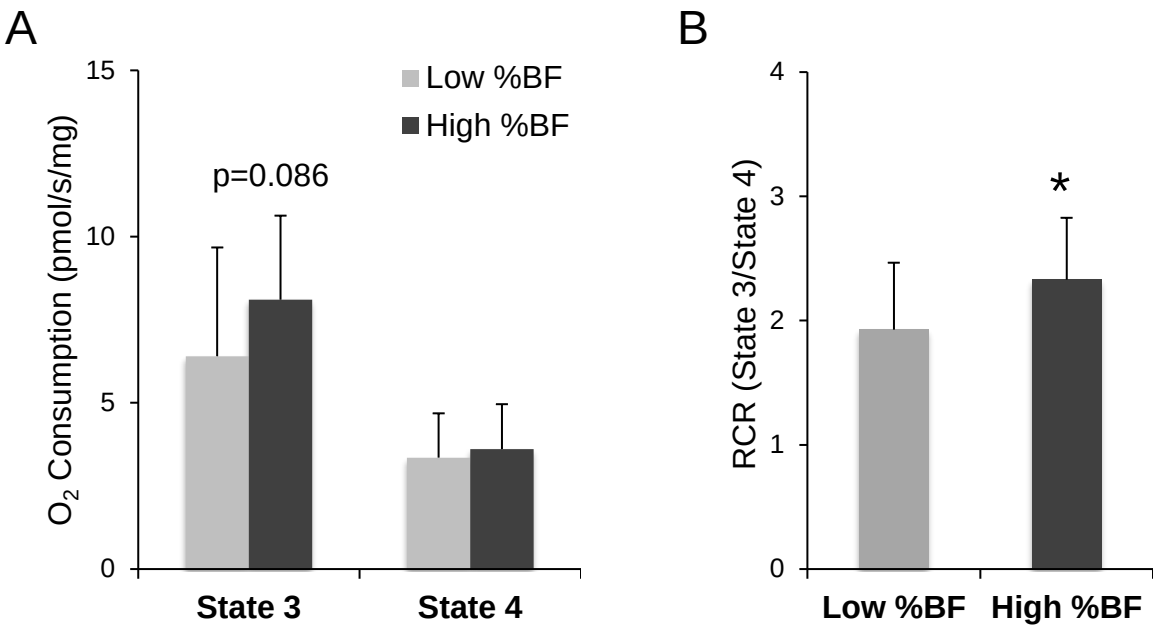


Figure 2. There were no differences in State 3 or State 4 between women with high body fat and women with low body fat (A). RCR was significantly higher in women with high body fat (p=0.024) (B). *P<0.05.



EFFECTS OF ACUTE HYPERINSULINEMIA ON SKELETAL MUSCLE MITOCHONDRIAL FUNCTION, REACTIVE OXYGEN SPECIES PRODUCTION, AND METABOLISM IN PREMENOPAUSAL WOMEN

by

JONATHAN L. WARREN JR., SULE BULUR, FERNANADO OVALLE, SAMUEL T. WINDHAM, BARABARA A. GOWER, AND GORDON FISHER

Format adapted and errata corrected for dissertation

Abstract

Background: Acute metabolic demands that promote excessive and/or prolonged reactive oxygen species production may stimulate changes in mitochondrial oxidative capacity.

Purpose: To assess changes in skeletal muscle H_2O_2 production, mitochondrial function, and expression of genes at the mRNA and protein levels regulating energy metabolism and mitochondrial dynamics following a hyperinsulinemic-euglycemic clamp in a cohort of 11 healthy premenopausal women

Methods: Skeletal muscle biopsies of the vastus lateralis were taken at baseline and immediately following the conclusion of a hyperinsulinemic-euglycemic clamp. Mitochondrial production of H_2O_2 was quantified fluorometrically and mitochondrial oxidation supported by pyruvate, malate, and succinate (PMS) or palmitoyl carnitine and malate (PCM) was measured by high-resolution respirometry in permeabilized muscle fiber bundles. mRNA and protein levels were assessed by real time PCR and Western blotting.

Results: H_2O_2 emission increased following the clamp ($P<0.05$). Coupled respiration (State 3) supported by PMS and the respiratory control ratio (index of mitochondrial coupling) for both PMS and PCM were lower following the clamp ($P<0.05$). IRS1 mRNA decreased, whereas PGC1α and GLUT4 mRNA increased following the clamp ($P\leq0.05$). PGC1α, IRS1, and phosphorylated AKT protein levels were higher after the clamp compared to baseline ($P<0.05$).

Conclusions: This study demonstrated that acute hyperinsulinemia induced H_2O_2 production and a concurrent decrease in coupling of mitochondrial respiration with ATP

production in a cohort of healthy premenopausal women. Future studies should determine if this uncoupling ameliorates peripheral oxidative damage, and if this mechanism is impaired in diseases associated with chronic oxidative stress.

Introduction

The incidence of obesity and associated cardiometabolic diseases, such as insulin resistance (IR) and type 2 diabetes (T2D), continues to rise throughout the westernized world [1]. Skeletal muscle mitochondrial dysfunction and chronic overproduction of reactive oxygen species (ROS) have been considered as primary mediators in their development [2]. In contrast, it has been shown in a rodent model that acute increases in superoxide ($O_2{\cdot}^-$) and hydrogen peroxide (H_2O_2) from the electron transport system (ETS) may actually have beneficial effects on skeletal muscle insulin sensitivity [3]. Thus, assessing mitochondrial responses to acute metabolic challenges (such as hyperinsulinemia) may be a valuable way to differentiate between normal physiological responses following nutrient load and chronic disturbances (i.e. excessive caloric intake, inactivity, etc.) that have been linked to cardiometabolic diseases.

Mitochondria are known to exhibit a great deal of plasticity in order to respond adequately to chronic stimuli that may alter metabolic demands. For example, exercise training can increase mitochondria content [4] and less than two weeks of exercise training can increase mitochondrial content, enzyme activity, and capacity [5]. Additionally, chronic inactivity has been shown to decrease mitochondria content and oxidative phosphorylation capacity [6], whereas caloric restriction increases substrate oxidation that is linked to ATP production (coupled respiration) and cold stress increases substrate oxidation that is uncoupled from ATP production (uncoupled respiration) [7]. Mitochondrial responses to acute stimuli are less well characterized, but it is thought that mitochondria exhibit a number of adaptations to acute metabolic signals that reflect the present environment. For example, uncoupling mitochondrial respiration from ATP

synthesis, by allowing protons to leak across the inner mitochondrial membrane independent of ATP synthase, may be a mechanism to create inefficiency in the presence of abundant substrate and low energetic demand [8, 9]. Uncoupled respiration ensures continued utilization of substrate, promotes the continued movement of electrons through the ETS, and alleviates an elevated proton motive force despite decreased ATP demand [10]. This process is known to be stimulated by ROS production [11] and is thought to be a mechanism to prevent excessive ROS production that may lead to oxidative damage. However, whether this uncoupling action occurs following an acute metabolic insult known to promote ROS production in humans is unclear. Additionally, the morphology of the dynamic mitochondrial reticular network is altered by acute stimuli, which may also be a function of ROS production [12]. However, morphological properties of mitochondria relative to their function are not well characterized as ROS production has been associated with both fusion [13] and fission [14] processes. A better understanding of the regulation of acute mitochondrial function and dynamics in response to ROS production may yield therapeutic approaches to prevent or mitigate oxidative damage.

The hyperinsulinemic-euglycemic clamp technique has been used to assess acute mitochondrial responses to elevated substrate load [2, 7, 15]. Earlier investigations have shown lower insulin-stimulated ATP production in isolated mitochondria from individuals with T2D compared to controls [15], and lower ATP synthetic rates *in vivo* in patients with T2D [16] and insulin-resistant offspring [2] compared to controls. Thus, insulin-stimulated ATP production appears to be impaired in individuals with impaired insulin sensitivity, however no measure of oxidative phosphorylation efficiency was assessed in these studies. The ability to uncouple mitochondrial respiration from ATP

production may be a key response to ROS production that protects against prolonged increases in ROS.

The purpose of the present study was to test the hypothesis that the mitochondria of healthy (premenopausal, non-diabetic) women would display an increase in uncoupled respiration following acute hyperinsulinemia, in conjunction with an increase in ROS production. To test this hypothesis, we examined changes in skeletal muscle H_2O_2 production, mitochondrial bioenergetics using high-resolution respirometry, and expression of genes at the mRNA and protein levels regulating energy metabolism and mitochondrial dynamics in permeabilized fiber bundles from skeletal muscle biopsies obtained before and following a hyperinsulinemic-euglycemic clamp.

Materials and Methods

Participants

Participants were 11 generally healthy premenopausal women. Inclusion criteria were a body mass index (BMI) of 18.5-35 kg/m^2, sedentary (<30 minutes of structured activity per week), and fasting serum glucose <100 mg/dL. Participants were excluded if they reported use of oral contraceptives, use of any medication known to affect metabolism or glucose tolerance, use of anti-hypertensive agents, history of eating disorder, use of tobacco, change in weight greater than 5 lbs in the previous 6 months, active engagement in unusual dietary practices (e.g. "low-carb" diets), or participation in extreme exercise. All testing was conducted in the first 10 days of the follicular phase of the menstrual cycle. All women provided written informed consent before participating in

the study. This study was approved by the Institutional Review Board at the University of Alabama at Birmingham (UAB).

Study design

Each participant presented after a 12 hr overnight fast. An initial baseline percutaneous needle biopsy of the vastus lateralis was obtained. A portion of tissue was immediately prepared for mitochondrial respirometry and H_2O_2 emission experiments, and remaining tissue was snap frozen in liquid N_2 to be used for mRNA and protein analyses conducted later. Following the baseline biopsy, each participant underwent a hyperinsulinemic-euglycemic clamp lasting approximately 2 hr. A second post-clamp biopsy in the contralateral leg was obtained immediately following the conclusion of the clamp procedure and prepared in the same manner as the baseline biopsy.

Total and regional body fat quantification

Body composition was assessed during the screening visit by total body scan from dual-energy X-ray absorptiometry (DXA) using a Lunar iDXA densitometer and Core Scan software (GE-Lunar Corporation, Madison, WI). Participants were scanned in light clothing lying supine with arms at their sides. The scans were assessed for total body fat mass, leg fat mass, android fat mass, and intra-abdominal adipose tissue (IAAT) volume.

Laboratory analyses

The Core Laboratory of the UAB Nutrition Obesity Research Center, Diabetes Research Center, and Center for Clinical and Translational Science analyzed all serum

samples. Serum glucose, total cholesterol, HDL cholesterol, triglycerides, and circulating free fatty acids were assessed by SIRRUS analyzer (Stanbio Laboratory, Boerne, TX). Fasting LDL cholesterol was calculated using the method of Friedewald [17]. Serum insulin was measured using a TOSOH AIA-II analyzer (TOSOH Corp., South San Francisco, CA).

Hyperinsulinemic-euglycemic clamp

A hyperinsulinemic-euglycemic clamp was administered providing a continuous infusion of regular insulin (Humulin, Eli Likely & Co., Indianapolis, IN) at 40 mU/m^2 body surface area/min through the brachial vein. Body surface area was calculated using the method of Du Bois [18]. Blood glucose was monitored at bedside at 5 min intervals using a YSI 2300 STAT Plus (YSI Life Sciences, Yellow Springs, OH) and a 20% dextrose solution was infused at a variable rate to maintain euglycemia (targeting 90 mg/dL). Serum samples were collected every 10 minutes for future analysis. The procedure lasted approximately 2 hr. The steady state period was defined for each individual as a period of 30 min or longer (at least 1 hr after beginning the insulin infusion) during which the coefficient of variation for serum glucose, serum insulin, and the recorded dextrose infusion rate was less than 5%. The glucose disposal rate (GDR) was calculated during steady state conditions and is presented as milligrams of dextrose infused per kg of fat-free body mass per minute. The Insulin Sensitivity Index (ISI) is presented as the GDR of each individual normalized to her average steady state serum insulin concentration [19]. Insulin resistance was assessed for each participant using

homeostatic model assessment of insulin resistance (HOMA-IR), which was calculated as the following: fasting glucose (mg/dl) × fasting insulin (μU/ml)/405 [20].

Muscle biopsies

Skeletal muscle samples were obtained at baseline and immediately following the conclusion of the clamp. Samples were obtained from the vastus lateralis under local anesthesia (1% lidocaine) using a 5 mm Bergstrom biopsy needle with suction. Muscle tissue collected at baseline and following the clamp was processed immediately following each biopsy. The tissue was cleaned of adipose and connective tissue. A bundle of approximately 20 mg was selected for mitochondrial experiments and transported in Buffer X (50 mM K-MES, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 5.7 mM ATP, 14.3 mM phosphocreatine, and 6.56 mM $MgCl_2$ (pH 7.1, 290 mOsm)) on ice [21]. The remaining tissue was frozen immediately in liquid N_2 and subsequently stored at -80°C for mRNA and protein quantification.

Preparation of permeabilized muscle fiber bundles

This technique has been adapted from previously published methods [21]. The biopsied tissue was dissected into several smaller muscle bundles (of approximately 1.0-5.0 mg wet weight). Each bundle was gently separated longitudinally with a pair of antimagnetic needle-tipped forceps under magnification in Buffer X. Bundles were weighed and then treated with 30 µg/ml saponin in Buffer X on a rotator for 30 min at 4°C. Next, the tissue bundles were washed for 15 min in Buffer Z (105mM K-MES, 30

mM KCl, 1 mM EGTA, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 5 µM glutamate, 2 µM malate, and 5.0 mg/ml BSA (pH 7.4, 290 mOsm)). Finally, the samples were transferred to Buffer Z supplemented with 5 µM blebbistatin and 20 mM creatine hydrate for 10 minutes prior to experiments. Blebbistatin was present during all respirometry and H_2O_2 experiments to prevent contraction of the myofibers [21].

H_2O_2 quantification

Using a permeabilized fiber bundle of approximately 1.0-1.5 mg, H_2O_2 emission was measured fluorometrically at 37°C in Buffer Z containing 5 µM blebbistatin, 20 mM creatine hydrate, 10 µM Amplex Ultra Red, and 3 U/mL horseradish peroxidase. Oxidation of Amplex Ultra Red to resorufin was monitored using a Fluoromate SF-2 spectrofluorometer (SCINCO, Seoul, South Korea) with temperature control and magnetic stirring at more than 1,000 rpm detecting at excitation/emission λ = 568/581 nm. For each experiment, resorufin fluorescence was converted to pmol H_2O_2 via an H_2O_2 standard curve generated under identical substrate conditions with the exception of the permeabilized fiber bundles. H_2O_2 emission during coupled respiration (State 3) was measured after the addition of 9 mM pyruvate, 4 mM malate, 2.5 mM succinate and 1 mM ADP. H_2O_2 emission during uncoupled respiration (State 4) was measured after the addition of 2 µg/mL oligomycin. H_2O_2 emission was normalized to the wet weight of the fiber bundle taken prior to each experiment.

Mitochondrial respiration

High-resolution respirometry experiments were performed using an Oxygraph O2K (Oroboros Instruments, Innsbruck, Austria) containing 2 mL of Buffer Z with 5 μM blebbistatin and 20 mM creatine hydrate, constantly stirred at 37°C under conditions of O_2 saturation, containing a prepared permeabilized fiber bundle of approximately 3.0-5.0 mg. Two substrate protocols were used: 9 mM pyruvate, 4 mM malate, and 2.5 mM succinate (PMS) to drive convergent electron input to complexes I and II of the ETS or 40 μM palmitoyl carnitine and 2 mM malate (PCM) to examine mitochondrial fatty acid oxidation. State 3 in each substrate condition was measured after the addition of 1 mM ADP. Cytochrome *c* (10 μM) was added to assess the mitochondrial membrane integrity. There was no significant difference in State 3 respiration assessed after cytochrome *c* addition. State 4 was induced by the addition of 2 μg/mL oligomycin. Oxygen flux was normalized to the wet weight of each fiber bundle taken prior to experiments [22]. The respiratory control ratio (RCR) was calculated as State 3/State 4, providing a measure of mitochondrial coupling.

Quantitative PCR

Muscle samples of approximately 30 mg were powdered in a liquid nitrogen-cooled mortar and pestle and homogenized in Trizol Reagent (Life Technologies; Carlsbad, CA) following manufacturer's procedure to obtain mRNA. mRNA concentration was determined using a NanoDrop Lite (Thermo Scientific, Wilmington, DE). mRNA quality was determined using the A_{260}/A_{280} ratio. iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA) was used to convert

mRNA to double-stranded cDNA. Quantitative real-time PCR was performed using SsoAdvanced SYBR Green 2x Supermix (Bio-Rad Laboratories) in a 20 μL reaction using a CFX Connect RT-PCR Detection System (Applied Biosystems, Foster City, CA), following manufacturer's instructions. All samples were analyzed in triplicate. Relative quantification values (ΔCT) were calculated for each target gene relative to Human GAPDH Endogenous Control (Applied Biosystems), which was run on each individual plate. ΔCT values were used for correlation analyses with other study variables. Results depicted are shown as the mean and range of relative fold differences compared to baseline [23]. Expression was assessed using the following primers (Applied Biosystems by Life Technologies, Warrington UK): glucose transporter type 4 (GLUT4) (Hs 00168966 m1), AKT substrate of 160 kDa (AS160) (Hs 00207999 m1), Protein kinase B (AKT1) (Hs 00178289 m1), insulin receptor substrate 1 (IRS1) (Hs 00178563 m1), pyruvate dehydrogenase kinase 4 (PDK4) (Hs 01037712 m1), uncoupling protein 3 (UCP3) (Hs 01106052 m1), fatty acid translocase (CD36) (Hs 01567185 m1), carnitine palmitoyltransferase 1B (CPT1B) (Hs 03046298 s1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (Ms 01016719 m1), sarcolipin (SLN) (Hs 00161903 m1), Cu-Zn superoxide dismutase (SOD1) (Hs 00533490 m1), and Mn superoxide dismutase (SOD2) (Hs 00167309 m1).

Immunoblotting

Muscle samples of approximately 30 mg were powdered in a liquid N_2-cooled mortar and pestle and homogenized in 6μL/mg muscle of ice-cold lysis buffer (150mM NaCl, 50 mM Tris-HCl, 5mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS,

and 0.5% NP-40 at pH 7.4) with protease and phosphatase inhibitors. Each sample was centrifuged at 15,000 g for 15 min at 4°C and the supernatant was collected. Protein content of the supernatant was quantified using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Thirty-seven micrograms of protein were treated with 4x NuPAGE LDS Sample Buffer (Novex, Carlsbad, CA) and 10x NuPAGE Reducing Agent (Novex) and incubated at 70°C for 10 min. The samples were electrophoresed in an SDS polyacrylamide gel (4-20%) at 100 V in an ice bath. The gels were blotted to polyvinylidene fluoride membranes using a semi-dry transfer method at 25 V for 12 min using a Pierce Power Blotter (Thermo Fisher Scientific). The membranes were blocked under conditions optimized for each antibody (2-5% milk and/or 2-5% BSA in 1 × PBS with 0.1% Tween 20 (PBST)) for 1 hr at room temperature with gentle agitation. Primary antibody was added at an appropriate dilution for incubation overnight at 4°C with gentle agitation. We probed for the following: rabbit polyclonal against total acetyl-CoA carboxylase (ACC) (1:1000, Cell Signaling Technology, #3662), P-ACC (1:500, Cell Signaling Technology, #3661), P-AKT (Ser473) (1:1000, Cell Signaling Technology, #9271), P-AS160 (1:1000, Cell Signaling Technology, #4288), CD36 (1:1000, Santa Cruz Biotechnology, sc-9154), GLUT4 (1:2000, Abcam, ab654), IRS1 (1:1000, Abcam, ab66153), and UCP3 (1:500, Abcam, ab3477); mouse monoclonal against dynamin-related protein 1 (DRP1) (1:500, Abcam, ab56788), Mitofusin 1 (MFN1) (1:1000, Abcam, ab57602), and Mitofusin 2 (MFN2) (1:2000, Abcam, ab56889); rabbit monoclonal against α-tubulin (1:1000, Cell Signaling, #2125), CPT1B (1:1000, Abcam, ab134135), and glycogen synthase 1 (GS1) (1:10000, Abcam, ab40810); and a goat polyclonal against PGC1α (1:1000, Abcam, ab106814) and lipoprotein lipase (LPL) (1:1000, R&D

Systems, AF7197). Horseradish peroxidase-conjugated secondary antibodies were used at 1:50,000 in 0.5% of selected blocking agent in PBST for 1 hr at RT with gentle agitation. Bands were visualized by chemiluminescent detection using ECL Western Blotting Substrate (Pierce Biotechnology) in a ChemicDoc XRS (Bio-Rad Laboratories). Band densitometry was quantified by Image Lab software (Version 4.1) (Bio-Rad Laboratories). Values shown were obtained after normalization to the reference protein α-tubulin. P-ACC was further normalized to total ACC density.

Statistical analyses

All statistical analyses were conducted using SPSS Statistics for Macintosh Version 22.0 (IBM Corp., Armonk, NY). Descriptive characteristics are reported as mean ± standard deviation and outcome variables are depicted as mean ± standard error of the mean. Differences for dependent variables between baseline and post-clamp were assessed using the Wilcoxon signed-rank test. Relationships between variables were determined using Spearman rank-order correlations. An alpha level of 0.05 was used to determine statistical significance.

Results

Participant characteristics

Baseline demographic, body composition, and biochemical data are presented in **Table 1.** One participant had a fasting glucose of 114 mg/dL indicating impaired fasting glucose, however she displayed no other outlying biochemical, body composition, or mitochondrial outcome measures. Analyses were performed including and excluding this

participant. There were no differences in statistical outcomes for any variables; therefore we included her data in the final analyses. Body composition data and GDR (normalized to FFM) are limited to 10 participants, as one participant did not complete her DEXA.

H_2O_2 production and mitochondrial respirometry

We first assessed skeletal muscle ROS production by quantifying H_2O_2 production in permeabilized fiber bundles from samples obtained at baseline and post-clamp. Compared to baseline values, post-clamp State 3 H_2O_2 emission increased by 59% and post-clamp State 4 H_2O_2 emission increased by 57% using PMS ($P<0.05$) (**Figure 1A**).

Following the clamp procedure, State 3 respiration decreased by 30% ($P<0.01$) with no significant change during State 4 using PMS (**Figure 1B**), demonstrating a decrease in oxidative phosphorylation capacity. There were no significant changes in mitochondrial respiration under PCM (**Figure 1C**). The RCR decreased by 37% using PMS and 31% using PCM following the clamp ($P<0.05$) (**Figure 1D**), which suggests greater relative mitochondrial uncoupling.

There were no associations between any measures of mitochondrial respiration and H_2O_2 production (data not shown). We further examined a number of correlations between participant characteristics and mitochondrial respiratory and H_2O_2 experiments. BMI and Total Fat Mass exhibited significant negative correlations with pre-clamp State 3 ($r=-0.865$, $P=0.001$ and $r=-0.638$, $P=0.047$, respectively) and State 4 ($r=-0.734$, $P=0.010$ and $r=-0.679$, $P=0.031$, respectively) oxygen consumption under the PMS condition. Additionally, BMI alone was associated with post-clamp State 3 ($r=-0.834$,

P=0.001) and State 4 (r=0.657, P=0.013) oxygen consumption under the PMS substrate condition. We found no associations between participant characteristics and any measure of H_2O_2 production.

mRNA and protein quantification

Skeletal muscle mRNA levels of target genes associated with energy metabolism and mitochondrial morphology are shown in **Figure 2**. Skeletal muscle IRS1 mRNA was lower following the clamp, whereas there was an increase in PGC1α mRNA levels ($P<0.05$). Additionally, there was a significant increase in skeletal muscle GLUT4 mRNA ($P\leq0.05$). There were no significant changes for the remaining target genes. There were a number of significant associations between H_2O_2 emission and mRNA levels (**Table 2**). There was a significant negative correlation between baseline and post-clamp UCP3 mRNA levels and baseline State 3 and State 4 H_2O_2 emission ($P<0.05$). Baseline and post-clamp UCP3 mRNA levels were negatively associated with baseline State 3 and State 4 H_2O_2 emission ($P<0.05$). Furthermore, baseline SOD1 mRNA levels were negatively correlated with baseline State 3 and State 4 H_2O_2 emission ($P<0.05$). Only baseline SOD2 mRNA levels were negatively correlated with baseline State 3 and State 4 H_2O_2 emission ($P<0.05$).

Skeletal muscle quantity of target proteins is shown in **Figures 3-5.** Following the clamp, we observed an increase in the following proteins: IRS1 by 53.9% (**Figure 3B**), phosphorylated AKT by 24.6% (**Figure 3E**), and PGC1α by 25.6% (**Figure 5E**) ($P<0.05$). There were no other significant changes for the remaining target proteins. We observed several significant correlations between H_2O_2 emission and proteins related to

mitochondrial fusion (**Table 3**). Baseline MFN2 protein density was positively associated with baseline State 3 and State 4 H_2O_2 emission ($P<0.05$). Post-clamp MFN1 protein density was positively associated with post-clamp State 3 and State 4 H_2O_2 emission ($P\leq0.05$). Although they did not reach the level of significance, the relationship between MFN2 protein density and post-clamp State 3 and State 4 H_2O_2 emission displayed a similar trend ($P<0.1$). No associations were found between H_2O_2 emission and DRP1 protein density.

Discussion

Shifts in energy balance can influence the cellular redox environment in favor of greater oxidative stress [24]. This relationship has been observed chronically following changes in habitual dietary intake [24] and acutely following a single meal [25]. However, whether acute changes in ROS production occur concurrently with the uncoupling of mitochondrial respiration from the production of ATP had not previously been shown. The purpose of this study was to identify potential factors within skeletal muscle that respond acutely to regulate fuel metabolism and the redox environment. The primary finding in this study was that there was an increase in the production of H_2O_2 and an increase in mitochondrial uncoupling in skeletal muscle following a hyperinsulinemic-euglycemic clamp, suggesting a possible link between these processes. Furthermore, we found changes in the expression of genes at the mRNA and protein levels that are intricately involved in insulin signaling and in mediating mitochondrial biogenesis and dynamics. We also found that mRNA levels of genes associated with uncoupling mitochondrial oxidative phosphorylation and antioxidant enzymes were inversely related

to H_2O_2 production. Additionally, key proteins known to promote mitochondrial fusion processes were related to higher skeletal muscle H_2O_2 production. These observations support the hypothesis that the ability of the mitochondria to adapt readily to a metabolic demand, specifically by altering efficiency, may be an important mechanism induced by ROS production in healthy individuals for the purpose of regulating the ROS production.

Our data contrast with a previous investigation that found an increase in mitochondrial efficiency using rodent and human myoblasts treated with insulin for 20 minutes [26]. There are a number of differences between the approaches taken in these studies, including the tissues examined and length of the insulin treatment, which may explain the discrepant findings. However, when taken together, data from these two studies may offer further insight into the effect of insulin and glucose on mitochondrial function. The duration of the clamp procedure is approximately two hours, thus we speculate that the present study has characterized mitochondrial function when energy reserves within the muscle have become saturated, electron transport has slowed, and ROS production has increased to promote an uncoupling response in the mitochondria. We hypothesize that our findings would corroborate those of Nisr and colleagues (higher coupling efficiency following 20 minutes of insulin stimulation) if an additional biopsy sample had been obtained during the early phases of the clamp procedure, as restoring ATP concentrations following a fasting period would be a priority.

Prior investigations have shown that acute hyperinsulinemia can increase ROS production [27]. Additionally, postprandial hyperglycemia can promote the production of ROS and oxidative stress especially when carbohydrate and lipid sources are consumed in abundance [28, 29]. The results from this study confirm these findings as we have

demonstrated an increase in skeletal muscle H_2O_2 production of greater than 50% following the clamp. Thus, an increase in ROS in the postprandial state over several months or years may lead to chronic oxidative stress and exacerbate pathophysiological processes associated with the onset of cardiometabolic diseases. It is important to mention that some level of ROS is necessary to activate redox-signaling pathways that are known to have a role in a number of homeostatic cellular processes, including insulin signaling [30]. However, when ROS are produced in excess of the antioxidative capacity of the cell, these molecules may oxidize nearby lipids, proteins, nucleic acids, and enzymes, altering the function of these cellular components [31]. Thus, the ability of the mitochondria to regulate ROS production combined with antioxidant defense mechanisms is imperative to maintaining homeostatic redox environment and preserving cardiometabolic health.

Uncoupling electron transport and proton re-entry from ATP synthesis is thought to limit ROS production and in fact seems to be stimulated by ROS [32]. It is possible that increased uncoupled respiration following the clamp observed in this study is an adaptive mechanism to reduce acute ROS production during the postprandial state and thus prevent chronic oxidative stress. Therapeutic compounds (e.g. 2,4-dinitrophenol (DNP)) that alter mitochondrial efficiency have been investigated as a means to combat obesity, cardiometabolic diseases, and fatty liver disease [33]. However, to date these compounds have yielded equivocal results. In humans, DNP in particular promotes weight loss [34], while others have shown in rodents that uncoupling agents targeting hepatic mitochondria can resolve hypertriglyceridemia and IR [35]. However, use in humans has been limited due to numerous deleterious side effects (e.g. tachycardia,

hyperthermia) [34]. These symptoms are primarily due to an increase in chronic and systemic mitochondrial uncoupling. The present study supports the need for future studies to identify uncoupling agents and/or lifestyle modifications that can increase mitochondrial uncoupling in a timed and targeted manner as a possible means to attenuate ROS production during shifts in energy balance in contrast to chronic and indiscriminate uncoupling agents [36].

Changes at the molecular level provided additional evidence for a shift in processes that regulate energy metabolism following the clamp. Although IRS1 mRNA decreased following the clamp, IRS1 protein increased. Similarly, decreases in IRS1 mRNA following hyperinsulinemia have been demonstrated in several previous studies [37, 38]. It is possible that increased translation of mRNA to protein during hyperinsulinemia is responsible for the imbalance between mRNA and protein changes. We also observed an increase in both the gene transcript and protein levels of PGC1α. PGC1α mRNA increases in response to acute metabolic stressors [39] and has been shown in a number of studies to regulate mechanisms that mediate mitochondrial dynamics [40, 41].

Mitochondrial dynamics, the balance between fusion and fission events, are thought to promote the preservation of mitochondrial health [42] and these processes appear to be regulated by transient metabolic demands. However, we saw no change in mitochondrial fission and fusion proteins (MFN1, MF2, DRP1) in this study. It is possible that changes in these proteins may not occur within the timeframe of our sampling period or they may be chronically regulated. Indeed, a previous study using an exercise model has shown that increases in PGC1α were observed 2 hr post-exercise, but

that MFN1 and MFN2 were only significantly elevated after 24 hr [43]. Despite not observing any changes in MFN1 and MFN2, these proteins did exhibit associations with H_2O_2 production, such that higher MFN1 (post-clamp) and MFN2 (baseline) were associated with greater H_2O_2 emission. Previous investigations do not appear to agree on whether an environment favoring fission or fusion is more 'protective' against ROS production and oxidative damage. Some have found increased mitochondrial fusion in response to mitochondrial ROS production [13] (likely in order to reduce ROS, which is supported by the positive associations presented in this study between MFN1/MFN2 and H_2O_2 production) and/or hyperglycemia [44]. However, others consider ROS production to be associated with a pro-fission environment [14] and when fission was inhibited in a transgenic mouse model, uncoupled respiration increased to prevent oxidative stress under hyperglycemic conditions [45]. While only correlative, our data support the idea that increased fusion may be linked to increased mitochondrial ROS production. Finally, protein levels of UCP3, SOD1 and SOD2 assessed at baseline and UCP3 and SOD1 following the clamp were inversely associated with H_2O_2 emission at baseline. UCP3 is a critically important facilitator of uncoupling action in skeletal muscle [46]. Our data suggest that a greater capacity for uncoupling respiration from ATP production contributes to an overall lower fasted level of ROS production. SOD1 is responsible for the production of H_2O_2; thus the observed inverse relationship may reflect an enhanced antioxidative capacity within the intermembrane and cytosolic spaces. Cross-sectional data must be interpreted with caution, as they do not capture dynamic changes that may lend insight into cause and effect.

T2D is associated with reduced mitochondrial capacity, plasticity, and content [47] and greater ROS production [48]. Futhermore, a previous study by Bloomer et al. has shown that a meal challenge can increase markers of oxidative stress in healthy and obese women similarly, but that the obese cohort exhibited elevations in these markers for a longer period following the meal [25]. This may be a function of a reduced oxidative capacity as BMI increases, observed in the present study as a negative association between body fat and mitochondrial oxidative capacity as well as the work of others [49, 50]. Thus, we hypothesize that if the present study design were to be employed in obese participants with obesity and cardiometabolic diseases such as T2D, these participants may exhibit less dramatic or no uncoupling and greater overall ROS production (contributing to oxidative stress) following hyperinsulinemia compared to healthy counterparts. If the mitochondria from individuals with metabolic disease do not exhibit the plasticity necessary to prevent excessive ROS production, this could contribute to a chronic perturbation of the redox environment and disease progression. Future studies should evaluate mitochondrial bioenergetics and ROS production in individuals that present with obesity and cardiometabolic diseases.

Strengths of this study include the use of the hyperinsulinemic-euglycemic clamp as a well-controlled model of the hyperinsulinemic state in contrast to a mixed meal in which the digestion, absorption, and metabolism of macronutrients may have contributed to greater individual tissue variability. Mitochondrial respiration was assessed using high-resolution respirometry in permeabilized fiber bundles, a technique that may more closely mimic an *in vivo* state in contrast to experiments performed using isolated mitochondria. We also acknowledge there are limitations to the present study design. Our conclusions

are limited to a study population of healthy premenopausal women, and results may differ in older women, men, or individuals with cardiometabolic diseases. Additional pre- and post-transcriptional changes, namely those related to mitochondrial dynamics, may have been observed if muscle samples had been obtained at different time points following to the clamp (e.g. 2 hr – 24 hr post-clamp). Last, non-parametric statistical analyses were utilized due to the small sample size, thus limiting our ability to adjust for potential confounding variables.

Conclusions

This study demonstrated that acute hyperinsulinemia induced H_2O_2 production and a concurrent decrease in coupling of mitochondrial respiration with ATP production in a cohort of healthy premenopausal women. We believe this decrease in coupling contributes to the attenuation of ROS production in healthy individuals, preventing excess ROS that may contribute to oxidative damage in the postprandial state. Future research is needed to identify if uncoupled mitochondrial respiration can mediate the peripheral oxidative state and if this mechanism is impaired in diseases associated with chronic oxidative stress, such as T2D.

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Disclosure

The authors have declared no conflict of interest.

Author Contributions

GF designed the study, participated in data collection, conducted statistical analysis, and drafted the manuscript. JW drafted the manuscript, participated in data collection, and assisted with data analysis. FO assisted with study design and oversaw the clamp experiments. SB participated in data collection. SW conducted the muscle biopsies and assisted with the study design. BG assisted with study design and participated in data collection. All authors made substantial contributions to the intellectual content of the manuscript and have approved the final submitted version.

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Table 1. Participant characteristics (n=11, Mean±SD).

Age (years)	24.7 ± 4.4
Race	7 EA / 4 AA
BMI (kg/m^2)	25.3 ± 4.5
Total Fat Mass (kg)‡	23.2 ± 8.7
Leg Fat Mass (kg)‡	9.1 ± 3.2
Trunk Fat Mass (kg)‡	10.8 ± 5.1
Fat Free Mass (kg)‡	42.3 ± 4.7
IAAT volume (cm^3)‡	261.6 ± 294.0
Total Cholesterol (mg/dL)	168.9 ± 23.4
Triglycerides (mg/dL)	80.9 ± 54.9
HDL Cholesterol (mg/dL)	59.1 ± 13.0
LDL Cholesterol (mg/dL)	93.2 ± 13.0
Free Fatty Acids (mEq/L)	0.7 ± 0.3
Fasting Glucose (mg/dL)	87.9 ± 11.7
Fasting Insulin (μIU/mL)	9.1 ± 4.1
HOMA-IR	2.0 ± 1.3
GDR (mg/kgFFM/min)‡	13.7 ± 7.3
ISI	15.4 ± 8.7

‡n=10. AA, African-American; BMI, body mass index; EA, European-American; GDR, glucose disposal rate; HOMA-IR, homeostatic model assessment of insulin resistance; IAAT, intra-abdominal adipose tissue; ISI, Insulin Sensitivity Index.

Table 2. Spearman correlations between H_2O_2 production data and mRNA levels. Correlation coeffieicient, P-value. **P<0.05.

		Baseline mRNA			**Post-Clamp mRNA**		
		UCP3	SOD1	SOD2	UCP3	SOD1	SOD2
Baseline H_2O_2	State 3	**-0.688, 0.019***	**-0.827, 0.002***	**-0.664, 0.026***	**-0.691, 0.019***	**-0.609, 0.047***	0.064, 0.853
	State 4	**-0.688, 0.019***	**-0.827, 0.002***	**-0.664, 0.026***	**-0.691, 0.019***	**-0.609, 0.047***	0.064, 0.853
Post-Clamp H_2O_2	State 3	-0.340, 0.336	-0.442, 0.200	-0.370, 0.293	-0.442, 0.200	-0.139, 0.701	-0.152, 0.676
	State 4	-0.322, 0.364	-0.455, 0.187	-0.406, 0.244	-0.430, 0.214	-0.127, 0.726	-0.115, 0.751

Table 3. Spearman correlations between H2O2 production data and protein density. Corrleation coeffieicient, P-value. *P<0.05.

		Baseline Protein Density			**Post-Clamp Protein Density**		
		MFN1	MFN2	DRP1	MFN1	MFN2	DRP1
Baseline H_2O_2	State 3	0.418, 0.229	**0.673, 0.033***	-0.336, 0.312	0.430, 0.214	0.091, 0.803	-0.176, 0.627
	State 4	0.418, 0.229	**0.673, 0.033***	-0.336, 0.312	0.430, 0.214	0.091, 0.803	-0.176, 0.627
Post-Clamp H_2O_2	State 3	0.350, 0.356	0.633, 0.067	0.176, 0.627	**0.683, 0.042***	0.633, 0.067	0.450, 0.224
	State 4	0.350, 0.356	0.633, 0.067	0.236, 0.511	**0.683, 0.042***	0.633, 0.067	0.450, 0.224

Figure 1. Changes in skeletal muscle mitochondrial H_2O_2 production and respiration from baseline to post-clamp. Values represent mean ± SEM. *P≤0.05. (A) H_2O_2 emission during State 3 and State 4 respiration supported by pyruvate, malate, and succinate (PMS); (B) State 3 and State 4 respiration supported by PMS; (C) State 3 and State 4 respiration supported by malate and palmitoyl carnitine (PCM); (D) Respiratory control ratio (RCR) for PMS and PCM.

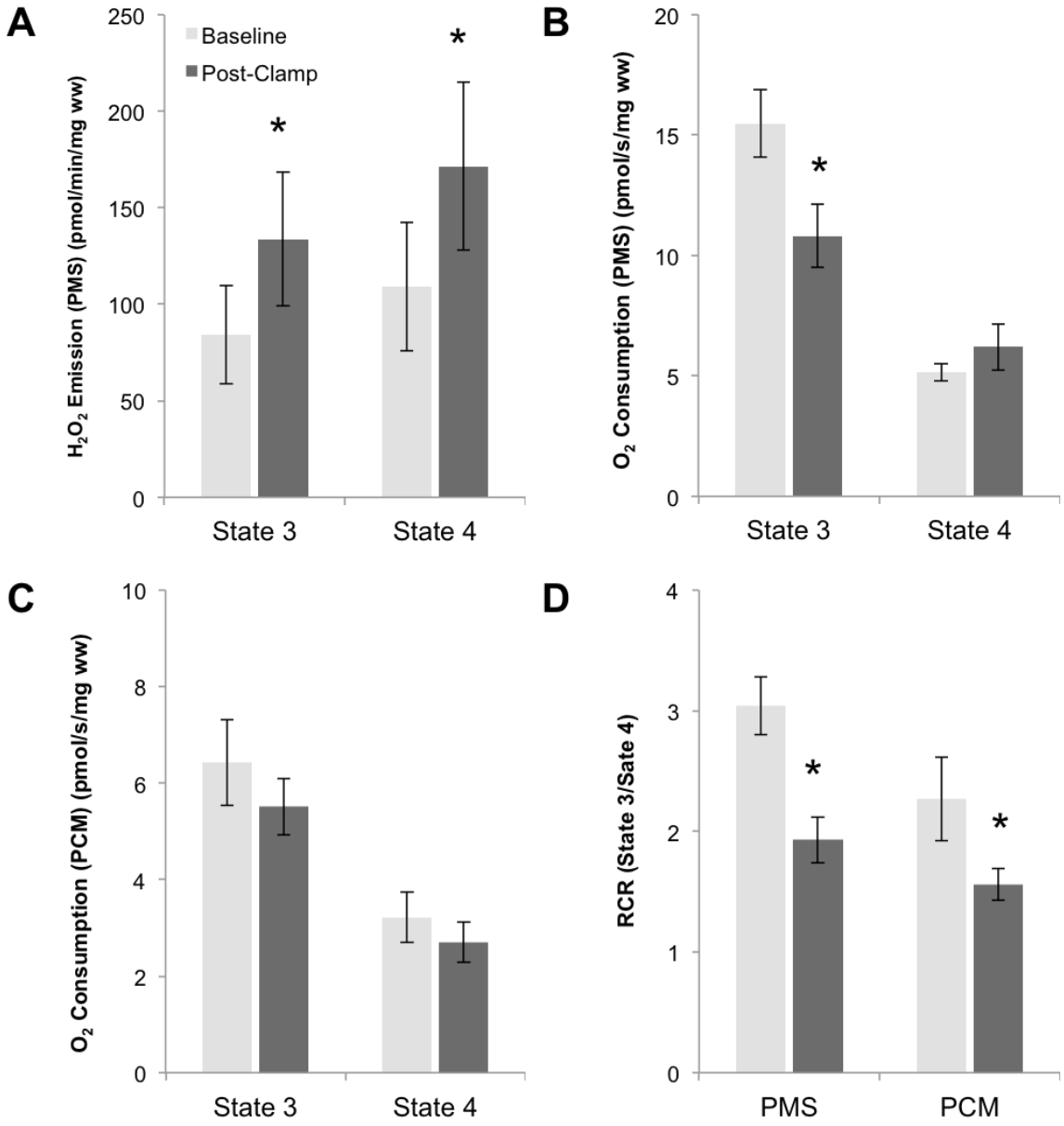


Figure 2. Changes in expression of genes related to energy metabolism and mitochondrial morphology. Mean fold change (relative to baseline values depicted by dashed line) in skeletal muscle mRNA levels from baseline to post-clamp are presented as $2^{-\Delta\Delta CT}$. Error bars represent the possible range of fold differences. *P≤0.05.

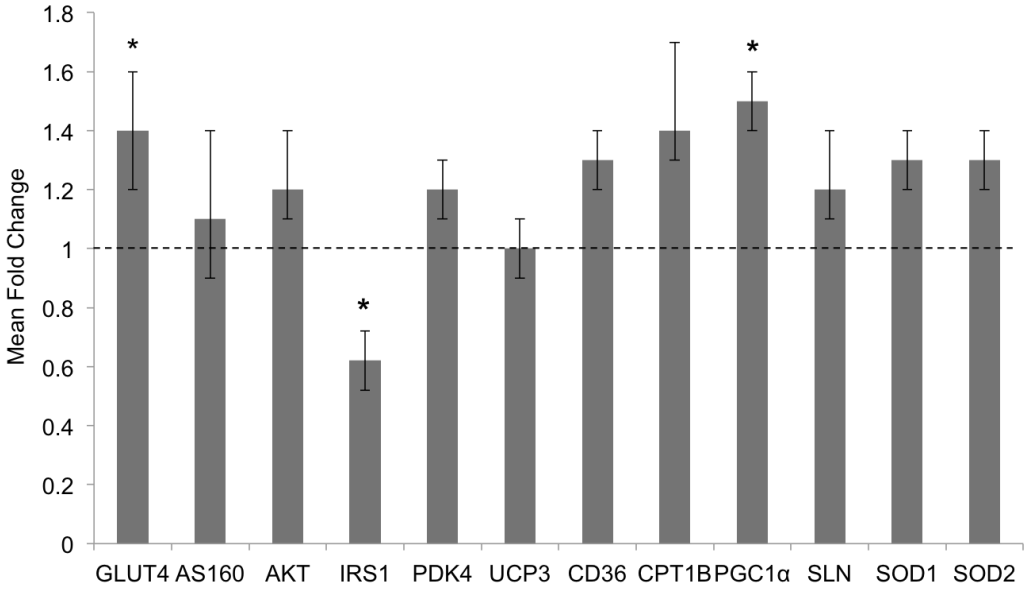


Figure 3. Muscle content of proteins related to glucose metabolism and insulin signaling. Baseline and post-clamp contents were measured were measured using Western blotting normalized to α-tubulin. Values represent mean ± SEM. *P≤0.05. (A) GLUT4, n=11; (B) IRS1, n=11; (C) P-AS160, n=10; (D) E) GS1, n=11; (E) P-AKT, n=10.

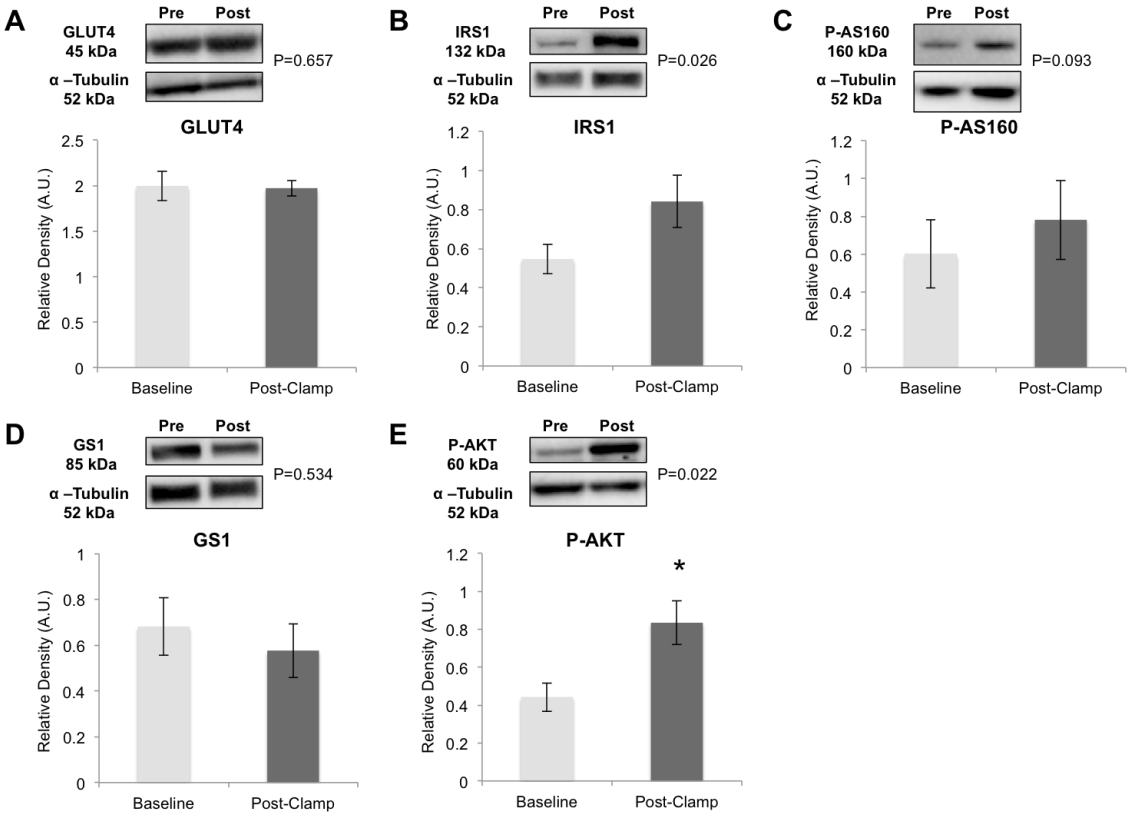


Figure 4. Muscle content of proteins related to fatty acid transport and metabolism. Baseline and post-clamp contents were measured were measured using Western blotting with α-tubulin as a loading control. Values represent mean ± SEM. *P≤0.05. (A) CPT1B, n=10; (B) CD36, n=10; (C) LPL, n=10; and (D) P-ACC, n=11.

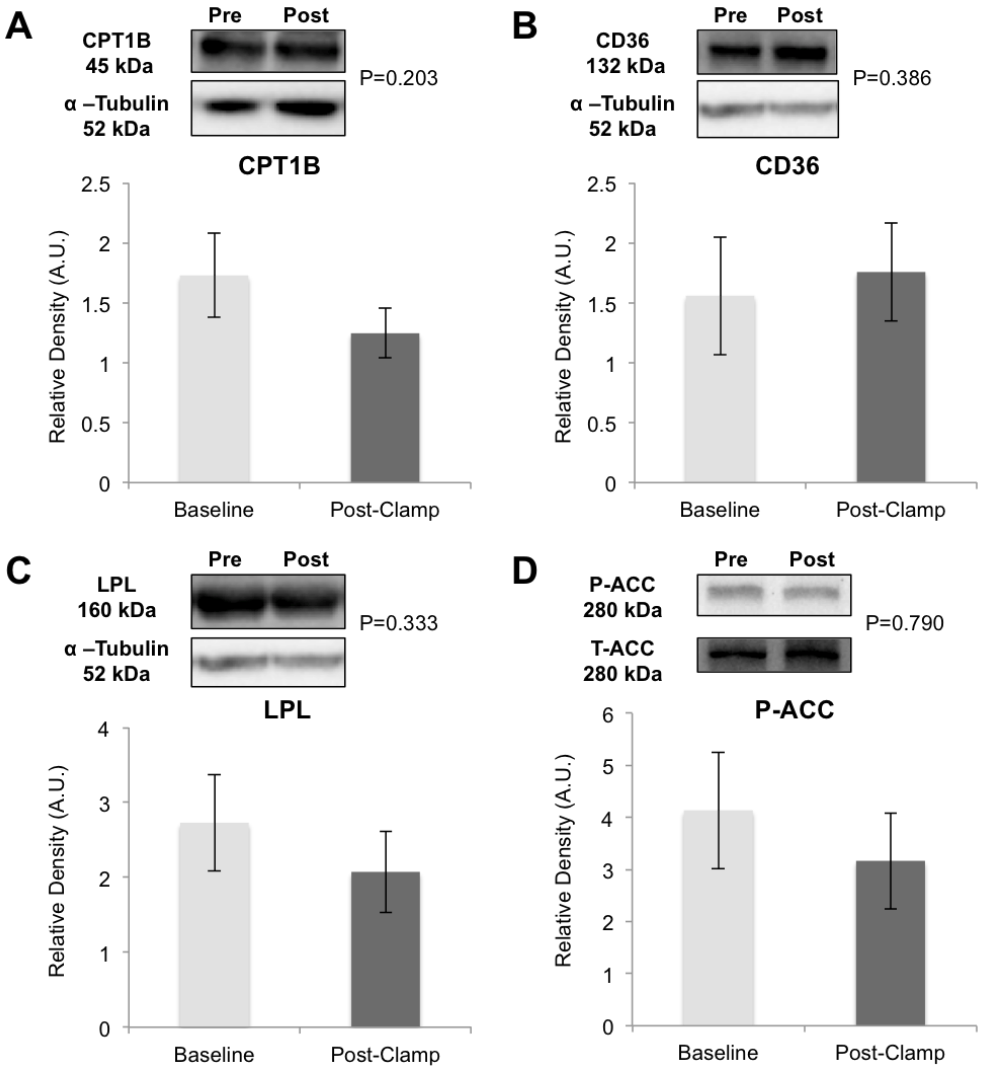
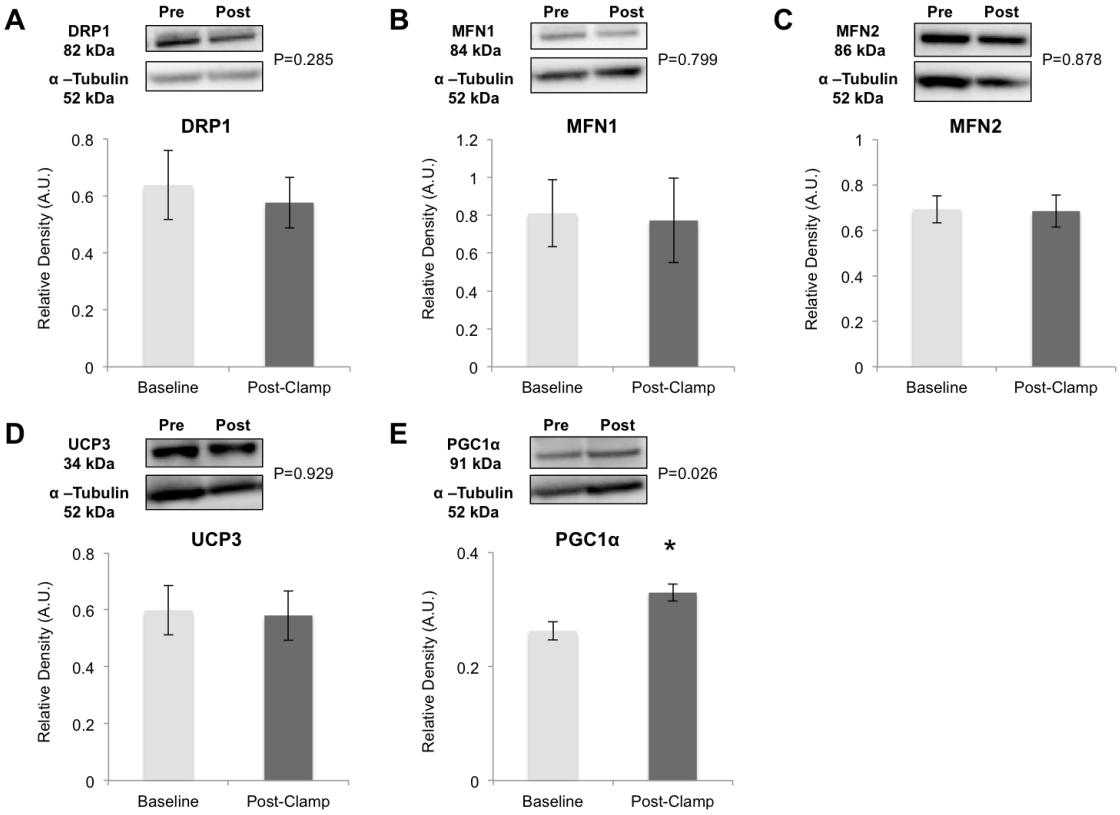


Figure 5. Muscle content of proteins related to mitochondrial morphology and function. Baseline and post-clamp contents were measured were measured using Western blotting normalized to α-tubulin. Values represent mean ± SEM. *P≤0.05. (A) DRP1, n=10; (B) MFN1, n=10; (C) MFN2, n=10; (D) UCP3, n=11; (E) PGC1α, n=11.



EFFECTS OF AEROBIC EXERCISE TRAINING ON SKELETAL MUSCLE MITOCHONDRIAL FUNCTION AND LIPID METABOLISM ASSESSED IN ENERY BALANCE IN PREMENOPAUSAL WOMEN

by

JONATHAN L. WARREN, GARY R. HUNTER, BARBARA A. GOWER, SAMUEL T. WINDHAM, DOUGLAS R. MOELLERING, AND GORDON FISHER

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Abstract

Aerobic exercise training (AET) has been shown to improve mitochondrial bioenergetics and up-regulate proteins related to lipid metabolism. However, whether these alterations occur independent of negative energy balance (EB) associated with AET is unclear. The purpose of the study was to test the hypothesis that improvements in skeletal muscle mitochondrial function induced by AET observed in previous literature would persist under rigorously controlled EB conditions. Participants were 14 premenopausal women (age = 31.2 ± 6.4 years, BMI = 27.0 ± 5.2 kg/m^2). The AET program required 3 monitored training sessions per week for 16 weeks. Skeletal muscle biopsies were obtained at baseline and following 16 weeks of AET (≥72 hrs after the last exercise bout). Energy balance was controlled for 24 hrs prior to testing within ±100 kcal of calculated energy requirements using a whole-room calorimeter. Mitochondrial oxidative capacity was quantified in permeabilized muscle fibers from the vastus lateralis. We found that AET increased coupled respiration (154%) and uncoupled respiration (90%) rates using a fatty acid substrate (palmitoyl carnitine) ($P<0.05$). However, when rates were normalized to mitochondrial content, no significant differences were observed. Additionally, there were no changes in proteins known to mediate mitochondrial biogenesis or lipid transport and metabolism following AET. In conclusion, 16 weeks of AET improved mitochondrial capacity under fatty acid substrate when assessed in EB, which appears to be due to mitochondrial biogenesis.

Introduction

Aerobic exercise training (AET) is well known to confer numerous cardiovascular and metabolic health benefits. These beneficial effects may be mediated in part by improvements in mitochondrial biogenesis and oxidative capacity (16, 33) and up-regulation of proteins related to lipid metabolism (22). These findings have implications in the treatment of chronic cardiometabolic diseases, as improved mitochondrial function has been linked to improved insulin sensitivity (18) and lower oxidative stress (36). However, AET can promote both acute and chronic energetic deficits and there are a number of beneficial effects of negative energy balance (EB) alone on mitochondrial function, including reduced oxidative stress and improvements in mitochondrial efficiency and quality (27). Thus, whether improvements in mitochondrial function observed after AET occur independent of negative EB is unclear.

Numerous studies have provided evidence to suggest the mechanisms by which AET can induce improvements in mitochondrial function. AET improves the activity of the electron transport system (ETS) in rodents (31) and humans (23, 24). Additionally, a key adaptation to AET is increased mitochondrial content, which is thought to occur in order to meet the energetic demands of endurance training (16) and contribute to increased mitochondrial capacity. Mitochondrial biogenesis is induced in part by peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) (29), a key protein also implicated in mitochondrial function (38) and dynamics (3, 4), thus regulating mitochondrial quality. AET is also known to enhance mitochondrial capacity to oxidize fatty acid (FA) (22) and can increase the storage of intramyocellular triacylglycerol (13). mRNA levels of key regulatory proteins in FA transport and metabolism, fatty acid

translocase (CD36) and carnitine palmitoyltransferase 1B (CPT1B), have been shown to be elevated after a short exercise program (35) and CD36 appears to be an important mediator in the increase in FA metabolism following exercise (21). However, many of the samples in these studies are obtained ≤24 hrs following the last bout (19, 35) or following weight loss during the course of the AET (24). Given these limitations, it is unknown whether changes in mitochondrial oxidative capacity due to AET persist when measured in carefully controlled EB.

The acute and chronic energetic deficits created by AET interventions may play a role in the improvements observed in mitochondrial function attributed to exercise. Chronic caloric restriction is known to promote greater coupled respiration (1), likely to maximize efficiency of ATP production despite limited substrate availability. The acute depletion of energy reserves from an acute bout of aerobic exercise is known to promote increased O_2 consumption, increased ATP synthesis (6), and decreased uncoupled mitochondrial respiration likely driven by decreased uncoupling protein 3 (UCP3) (7). Whether weight loss alone is sufficient to alter mitochondrial oxidative capacity is debated (24), however weight loss is well understood to decrease energy expenditure (9).

The purpose of this study was to assess the effects of 16 weeks of AET on skeletal muscle mitochondrial function and markers of lipid metabolism when assessed under rigorously controlled EB conditions in a cohort of premenopausal women. The overall hypothesis was that exercise induced improvements in skeletal muscle mitochondrial function commonly reported in previous literature would persist when negative energy balance is controlled. A secondary aim was to assess proteins associated with mitochondrial bioenergetics and lipid metabolism in order to determine mechanisms that

may be mediating potential changes in skeletal muscle mitochondrial function following AET.

Methods

Participants

Participants were 14 generally healthy premenopausal women (BMI = 27.0±5.2 kg/m^2,) between the age of 21 and 45 years who self-identified as either of European-American (EA) or African-American (AA) descent. Participants were not taking oral contraceptives or any medication known to influence blood pressure or metabolism. All participants were normotensive, non-smokers, normoglycemic, and sedentary (participating in <1 exercise activity per week). All testing was conducted in the first 10 days of the follicular phase of the menstrual cycle. All participants provided written, informed consent and the Institutional Review Board at the University of Alabama at Birmingham approved this study.

Study design

The study design is depicted in **Figure 1** and each method is outlined in further detail below. A skeletal muscle biopsy of the vastus lateralis was collected from each participant prior to beginning an AET program. All food was provided to the participants for 72 hours prior to the tissue collection. The participants spent the 23 hours immediately prior to the tissue collection in our whole-room indirect calorimeter. Skeletal muscle tissue was used to measure mitochondrial oxidative function and protein levels. Each participant then completed 16 weeks of AET. Following the AET, a similar design

was implemented to collect post-AET muscle biopsies. Again, all food was provided for 72 hours and the last 23 hours prior to tissue collection were spent in the whole-room calorimeter. Post-AET tissue was collected at least 72 hours removed from the last exercise bout to assess the chronic effect of AET on mitochondrial oxidative function and protein levels.

Energy balance and room calorimetry

Participants were provided with all food during the 72 hrs prior to each tissue collection. Caloric needs were calculated using the Harris-Benedict equation (11). Meals were prepared by the metabolic kitchen of the UAB Clinical Research Unit in order to provide ≈60% energy as carbohydrates, ≈25% energy as fat, and ≈15% energy as protein. Participants spent the last 23 hrs before each biopsy in a whole-room calorimeter previously described in detail (2), during which time all food provided continued to be adjusted to ensure EB in order to isolate the chronic effect of exercise on mitochondrial variables.

Aerobic exercise training (AET) program

Each participant trained 3 days per week for 16 weeks and was monitored by trained exercise physiologists during each training session. During the first week, participants began at 67% heart rate max (HRM) for 20 minutes, progressing time and intensity each week until the beginning of the fifth week, when each participant was training at 80% HRM for 40 minutes. This intensity was maintained for the duration of the AET program. Each exercise session included a 3-5 minute warm-up and cool-down

period of light activity. Participants were able to complete the AET using a treadmill, recumbent bike, or stationary bike.

VO_2peak

This protocol has been adapted from previously published work (14, 15). The testing was completed on a cycle ergometer and each participant pedaled at 25 watts. Resistance increased every 2 minutes thereafter by 25 watts until volitional exhaustion. Oxygen uptake and carbon dioxide production were continuously monitored using a MAXX-II metabolic cart (Physio-Dyne Instrument Corp., Massapequa, NY). Heart rate was measured using a POLAR Vantage XL heart rate monitor (Gays Mills, WI).

Body fat quantification

Body composition was assessed by dual-energy X-ray absorptiometry (DXA) using a Lunar iDXA densitometer and Core Scan software (GE-Lunar Corporation, Madison, WI). Participants were scanned in light clothing lying supine with arms at their sides. The scans were assessed for fat-free mass (kg) and total body fat mass (kg).

Laboratory analyses

Sera were analyzed by the Core Laboratory of the UAB Nutrition Obesity Research Center, Diabetes Research Center, and Center for Clinical and Translational Science. Fasting glucose, total cholesterol, HDL cholesterol, triglycerides, and circulating free FA were measured using a SIRRUS analyzer (Stanbio Laboratory, Boerne, TX).

LDL cholesterol was calculated using the Friedewald method (8). Fasting insulin was measured using a TOSOH AIA-II analyzer (TOSOH Corp., South San Francisco, CA).

Mitochondrial respiration measures

This technique has been adapted from previously published methods (26). Immediately following each stay in the room calorimeter (baseline and post-AET), a skeletal muscle biopsy of the vastus lateralis was collected under local anesthesia from each participant. The tissue was immediately cleaned of adipose and connective tissue and a bundle of approximately 20 mg was selected for mitochondrial respirometry experiments. This tissue was transferred to the laboratory on ice in Buffer X buffer containing 50 mM MES, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.7 mM ATP, 14.3 mM PCr, and 6.56 mM $MgCl_2$-6 H_2O (pH 7.1, 290 mOsm). The tissue was then dissected into several smaller muscle bundles (of approximately 3-5 mg wet weight) and each was gently separated with a pair of antimagnetic needle-tipped forceps under magnification. Bundles were treated with 30 µg/ml saponin in Buffer X and incubated on a rotator for 30 min at 4°C. The tissue bundles were washed of saponin for 15 minutes in Buffer Z containing 105mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4, and 5 mM $MgCl_2$-6 H_2O, 5 µM glutamate, 2 µM malate, and 5.0 mg/ml BSA (pH 7.4, 290 mOsm). The samples were transferred to Buffer Z containing 20 mM creatine hydrate and 5 µM of blebbistatin for 10 minutes prior to respirometry experiments (26).

High-resolution respirometry experiments were performed using an Oroboros Oxygraph O2K (Oroboros Instruments, Innsbruck, Austria) containing 2 mL of Buffer Z

with creatine and blebbistatin, constantly stirred at 37°C. We assessed mitochondrial O_2 consumption using two substrate protocols: 4 mM malate, 9 mM pyruvate, and 2.5 mM succinate (PMS) to drive convergent electron input to complexes I and II of the ETS or 2 mM malate and 40 μM palmitoyl carnitine (PC) to examine mitochondrial fatty acid oxidation. State 3 in each substrate condition was measured after the addition of 1 mM ADP. Cytochrome *c* (10 μM) was added to assess mitochondrial membrane integrity following the dissection of the myofibers. There was no significant increase in respiration following the addition of cytochrome *c*. Uncoupled respiration (State 4) was induced by the administration of oligomycin (2 μg/mL). Maximal complex IV activity was assessed by the addition of ascorbate (2 mM) and tetramethyl-*p*-phenylenediamine (TMPD) (0.5 mM), providing a measure of mitochondrial content. The respiratory control ratio (RCR) was calculated as State 3/State 4. The RCR is a commonly used metric for assessing mitochondrial integrity and is highly correlated with coupling efficiency. Oxygen flux was normalized to either the wet weight or complex IV activity of each fiber bundle.

Immunoblotting

A portion of muscle tissue collected at baseline and following the completion of the 16 week AET protocol was snap frozen in liquid N_2 and stored at -80°C until analysis. Muscle samples of approximately 30 mg were powdered in a liquid N_2-cooled mortar and pestle and homogenized in 6 μL/mg muscle of ice-cold lysis buffer (150mM NaCl, 50 mM Tris-HCl, 5mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 0.5% NP-40 at pH 7.4) with protease and phosphatase inhibitors. The samples were centrifuged at 15,000 g for 15 min at 4°C and the supernatant was collected. Protein content of the

supernatant was quantified using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Thirty-seven micrograms of protein was treated with 4x NuPAGE LDS Sample Buffer (Novex, Carlsbad, CA) and 10x NuPAGE Reducing Agent (Novex) and incubated at 70°C for 10 min. The samples were electrophoresed in an SDS polyacrylamide gel (4-20%) at 100 v on ice. The gels were blotted to polyvinylidene fluoride membranes using a semi-dry transfer method at 25 v for 12 min using a Pierce Power Blotter (Thermo Fisher Scientific). The membranes were blocked under conditions optimized for each antibody (2-5% milk and/or 2-5% BSA in 1 × PBS with 0.1% Tween 20 (PBST)) for 1 hr at room temperature (RT) with gentle agitation. An appropriate dilution of primary antibody (Ab) was added for incubation overnight at 4°C with gentle agitation. We probed for the following: rabbit polyclonal Ab against CD36 (1:1000, Santa Cruz Biotechnology, sc-9154), and UCP3 (1:500, Abcam, ab3477); rabbit monoclonal Ab against α-tubulin (1:1000, Cell Signaling, #2125), and CPT1B (1:1000, Abcam, ab134135); and a goat polyclonal Ab against PGC1α (1:1000, Abcam, ab106814). Horseradish peroxidase-conjugated secondary Abs were used at 1:50,000 in 0.5% of selected blocking agent in PBST for 1 hr at RT with gentle agitation. Bands were visualized by chemiluminescent detection using ECL Western Blotting Substrate (Pierce Biotechnology) in a ChemicDoc XRS (Bio-Rad Laboratories). Band densitometry was quantified by Image Lab software (Version 4.1) (Bio-Rad Laboratories). Values shown were obtained after normalization to the reference protein α-tubulin.

Statistical analyses

Descriptive characteristics are reported as mean ± SD. Changes in variables between baseline and post-AET were assessed using two-tailed *t*-tests for paired samples and depicted as mean ± SD. An alpha level of 0.05 was used to determine statistical significance. All statistical analyses were conducted using SPSS Statistics for Macintosh Version 22.0 (IBM Corp., Armonk, NY).

Results

Demographic, physical, and metabolic characteristics of the study participants at baseline and following 16 weeks of AET are shown in **Table 1**. Body mass index (BMI) ranged from 20.1 to 35.0 kg/m^2 and age from 21 to 40 years. There were 8 EA participants and 6 AA participants. There were no significant changes in weight, BMI, or body fat between baseline and post-AET conditions. There was an increase in fat-free mass ($P<0.05$). VO_2peak improved significantly following the AET protocol ($P<0.05$). There were no differences in resting energy expenditure (REE) between baseline and post-AET and no differences between energy intake and REE at either baseline or post-AET (**Figure 2**).

Following AET, there were no changes in mitochondrial respiration normalized to fiber weight when supported by PMS (**Figure 3A**), but there were significant increases in both State 3 and State 4 respiration rates supported by PC (**Figure 3B**). There were no changes in the RCR following AET supported by PMS or PC (**Figure 4**). When mitochondrial respiration measures were normalized to maximal complex IV activity,

there were no changes in mitochondrial function following AET supported by either PMS (**Figure 5A**) or PC (**Figure 5B**).

There were no changes in any proteins measured following the AET program when assessed under energetic balance (PGC1α, UCP3, CD36, or CPT1B) (**Figure 6**).

Discussion

Mitochondrial oxidative capacity and content are known to improve following AET (16, 33). This study tested the hypothesis that these improvements would still persist when measured in rigorously controlled EB conditions. There were no differences between caloric intake and energy expenditure in the 23 hours prior to each tissue collection and no changes in weight loss or REE during the course of the AET. We demonstrated that mitochondrial respiratory capacity supported by PMS was unchanged when measured in an energetically balanced state following 16 weeks of AET. However, when mitochondrial oxidation was supported by a fatty acid substrate, we observed an enhanced mitochondrial oxidative capacity and elevated uncoupled respiration. These changes were no longer apparent when these data were normalized for a marker of mitochondrial content. These findings suggest that the improvements in mitochondrial capacity were due to mitochondrial biogenesis induced by AET, and not intrinsic changes to existing mitochondria. Finally, there were no changes in proteins known to mediate mitochondrial biogenesis or lipid transport and metabolism.

The increase in mitochondrial oxidative capacity measured under a FA substrate may reflect a greater capacity to utilize FA substrate prompted by AET. Greater FA oxidative capacity is an adaptation often associated with AET and may be induced in part

by PGC1α (20), which is induced acutely by both exercise (28) and high-fat feeding in animal models (30). Greater mitochondrial FA oxidation may have the potential to decrease cellular storage of FA in the form of intramyoceullar triglyceride (10) or ceramides and diacylglycerols (25), and prevent the cardiometabolic complications associated with them. In addition, State 4 respiration was increased when supported by FA substrate, a measure of mitochondrial respiration uncoupled from ATP synthesis. Uncoupled respiration is commonly thought to be a mechanism to limit the production of mitochondrial-derived reactive oxygen species (ROS), and is known to be induced by both FA substrate (32) and ROS production (5). Given that there were no changes in the RCR, the overall proportion of coupled to uncoupled respiration remained similar before and after AET, and thus overall efficiency was unchanged.

Chronic AET is well understood to induce mitochondrial biogenesis. Improvements in mitochondrial number have been observed after only 7 weeks of training (34), but can be reversed in as little as 6 weeks after the conclusion of an exercise program (12). The present study suggests that this biogenesis is solely responsible for the improvements in mitochondrial capacity to utilize FA substrate. Acute exercise is known to promote enhanced expression of p38 MAPK and thus activation of PGC1α, an important signaling cascade for mediating biogenesis. PGC1α is well characterized to be upregulated following AET (28), however, we observed no change in the expression of PGC1α when measured more than 72 hours following the last exercise bout. We are aware of no study that has observed increased mitochondrial content following an acute bout of exercise and hypothesize that this response is likely regulated in a more chronic manner, independent of acute energetic balance conditions. Thus, the transient increases

in PGC1α from acute AET are likely sufficient to induce mitochondrial biogenesis associated with chronic AET.

In addition to PGC1α, the proteins measured in the present study are known to be induced with AET exercise despite our observations to the contrary. For example, UCP3 expression is increased following AET in rodents, but only as a function of increased mitochondrial content (17). This study did not consider the effect of energy balance on UCP3 expression. Elevated expression of mRNA for both CD36 and CPT1B has been observed when measured after a short exercise training program (35). Additionally, previous investigators suspect that CD36 is critically important in the upregulation of FA oxidation following exercise, independently of mitochondrial biogenesis (21). However, we speculate that the timing of sample collection plays an important role in mediating these results, as others have also found (in agreement with our findings) no changes in CD36 or CPT1B protein expression when measured 24 hours after an exercise bout (37).

There were a number of strengths associated with the present study design. The AET program required participants to attend three sessions per week in our training facility and trained personnel monitored the participants during each session. The provision of all food consumed by the participants for 72 hrs prior to the collection of each biopsy sample and 23 hr energy expenditure measured in a room calorimeter ensured a high degree of control over EB. We acknowledge limitations within this study. There are a number of additional methods with which to quantify mitochondrial content not used in the present study (e.g. mtDNA copy number, citrate synthase activity). These measures may have provided additional insight into the mechanisms of mitochondrial biogenesis that appear to be responsible for the improved mitochondrial capacity.

Additionally, these data may only reflect the population in which they were collected (generally healthy premenopausal women), and not other subgroups, including men, aging persons, or those with cardiometabolic disease.

Conclusions

In conclusion, a 16 week AET program was sufficient to improve mitochondrial respiratory capacity under a fatty acid substrate load even when measured in a rigorously controlled energetically balanced state. After normalization of respiratory capacity to a marker for mitochondrial content, these changes were no longer apparent, suggesting that the improvements in mitochondrial capacity after AET were due primarily to mitochondrial biogenesis.

Acknowledgements

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Author Contributions

JW drafted the manuscript, participated in data collection, and assisted with data analysis. GH designed the study and conducted statistical analysis. SW conducted the

muscle biopsies. BG assisted with study design and data analysis. DRM assisted with the study design and participated in data collection. GF assisted with the study design, participated in data collection, and conducted statistical analysis. All authors reviewed and critically revised the submitted manuscript.

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Table 1. Clinical characteristics of participants (n=14). *P<0.05.

	Baseline	Post-AET	P
Age (years)	31.7 ± 6.7	-	-
Weight (kg)	72.2 ± 12.8	72.5 ± 12.5	0.656
BMI†	26.6 ± 5.1	26.7 ± 5.0	0.717
Total Body Fat (kg)†	39.7 ± 4.0	39.8 ± 4.6	0.373
Fat Free Mass (kg)†	43.0 ± 5.6	43.6 ± 6.0	0.026*
Total Cholesterol (mg/dL)†	180.4 ± 38.2	182.3 ± 36.1	0.649
Triglycerides (mg/dL)†	94.5 ± 39.6	103.0 ± 45.1	0.163
HDL (mg/dL)†	65.2 ± 19.4	65.9 ± 19.7	0.577
LDL (mg/dL)†	96.3 ± 26.5	95.8 ± 25.0	0.885
Fasting Glucose (mg/dL)^	91.3 ± 9.9	90.1 ± 7.8	0.620
Fasting Insulin (μIU/mL)^	10.5 ± 5.9	9.9 ± 4.4	0.680
VO_2peak (L/min)	1.7 ± 0.3	1.9 ± 0.3	0.043*
VO_2peak (mL/kgFFM/min)‡	40.8 ± 5.9	43.7 ± 4.8	0.024*

†n=13, ‡n=12, ^n=11.

Figure 1. Study design.

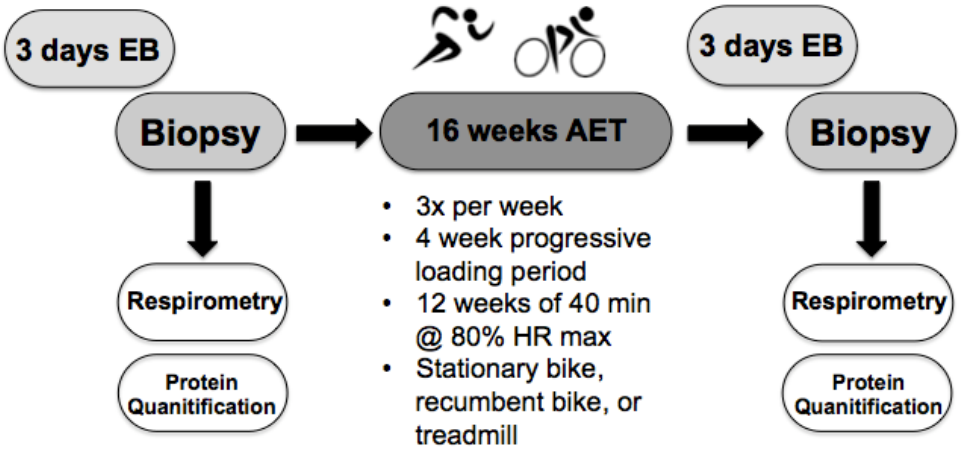


Figure 2. Energy intake, energy expenditure, and difference calculated during room calorimetry. n=12.

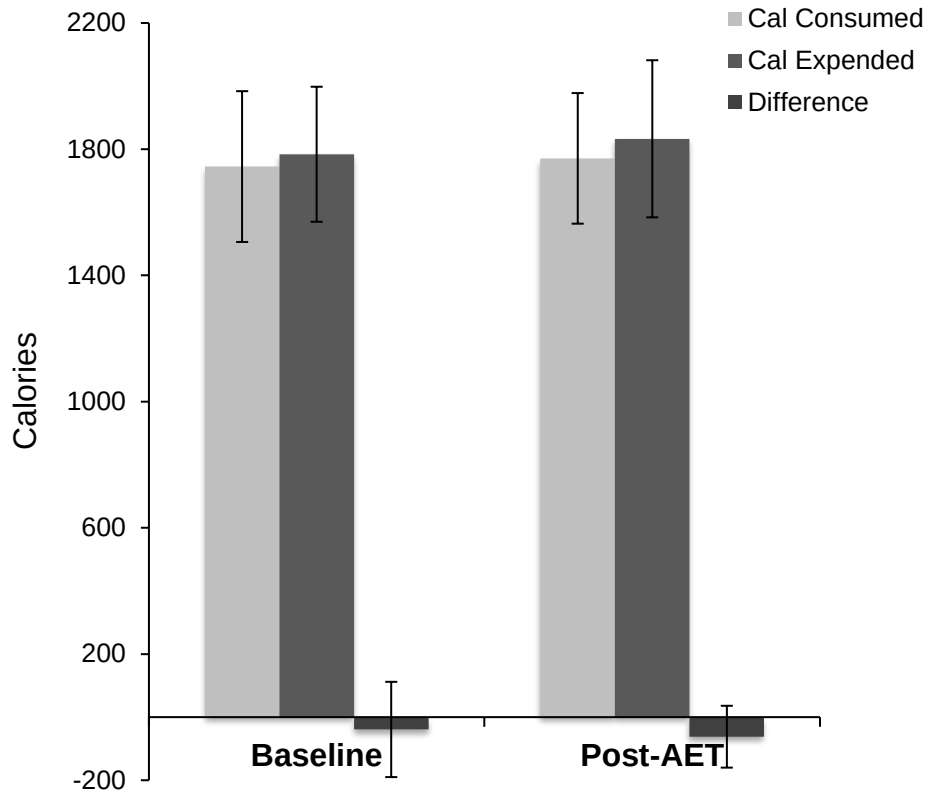


Figure 3. Oxygen consumption under State 3 and State 4 conditions normalized to fiber weight supported by PMS (A) and PC (B). n=11, *P<0.05.

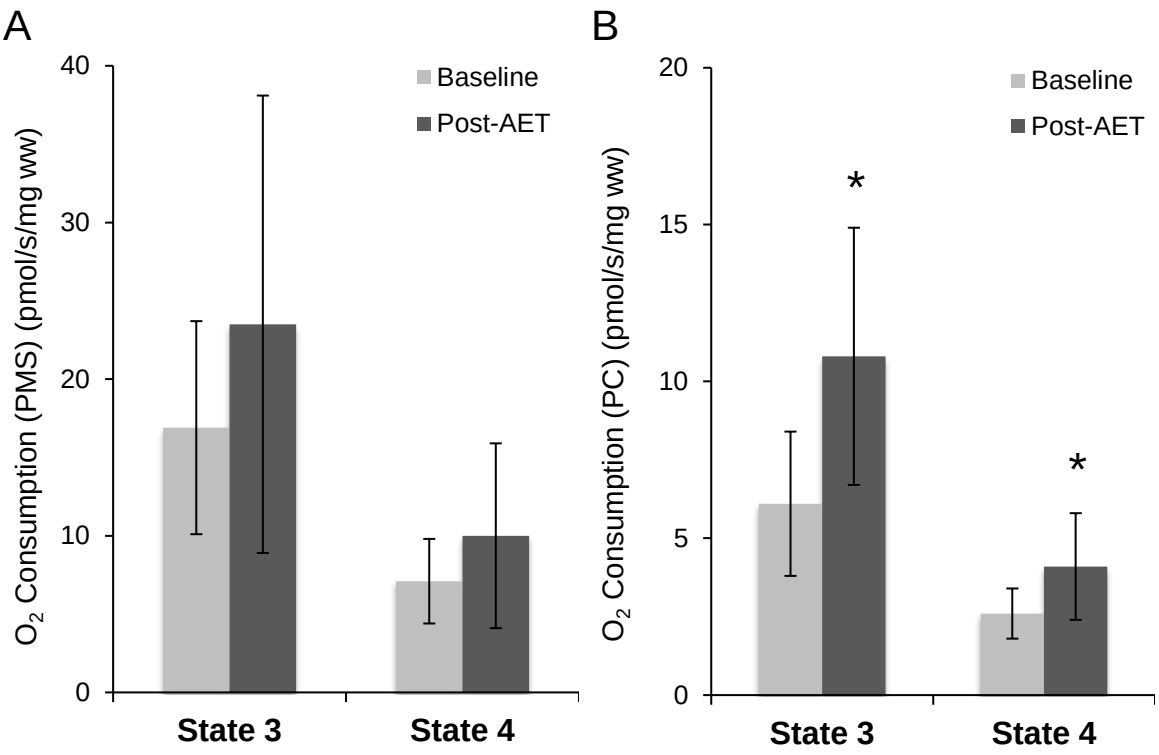


Figure 4. Respiratory control ratio (RCR) supported by pyruvate substrate (PMS) or palmitoyl carnitine (PC). n=11, *P<0.05.

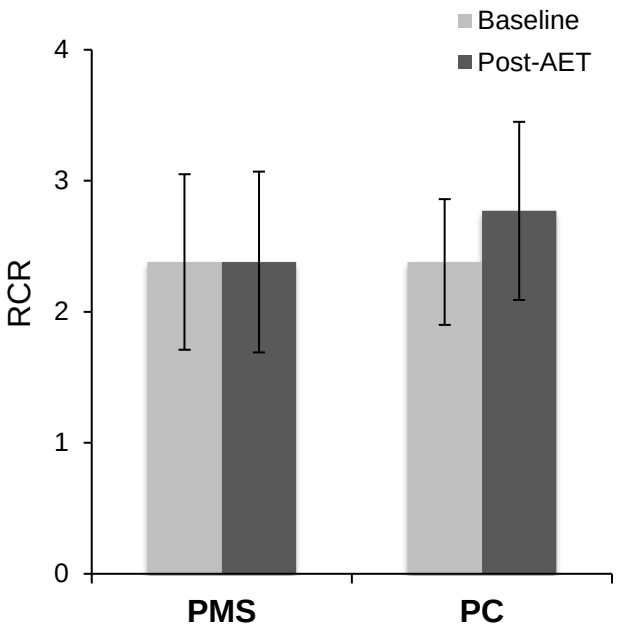


Figure 5. Oxygen consumption under State 3 and State 4 conditions normalized to maximal complex IV activity supported by PMS (A) and PC (B). n=12, *P<0.05.

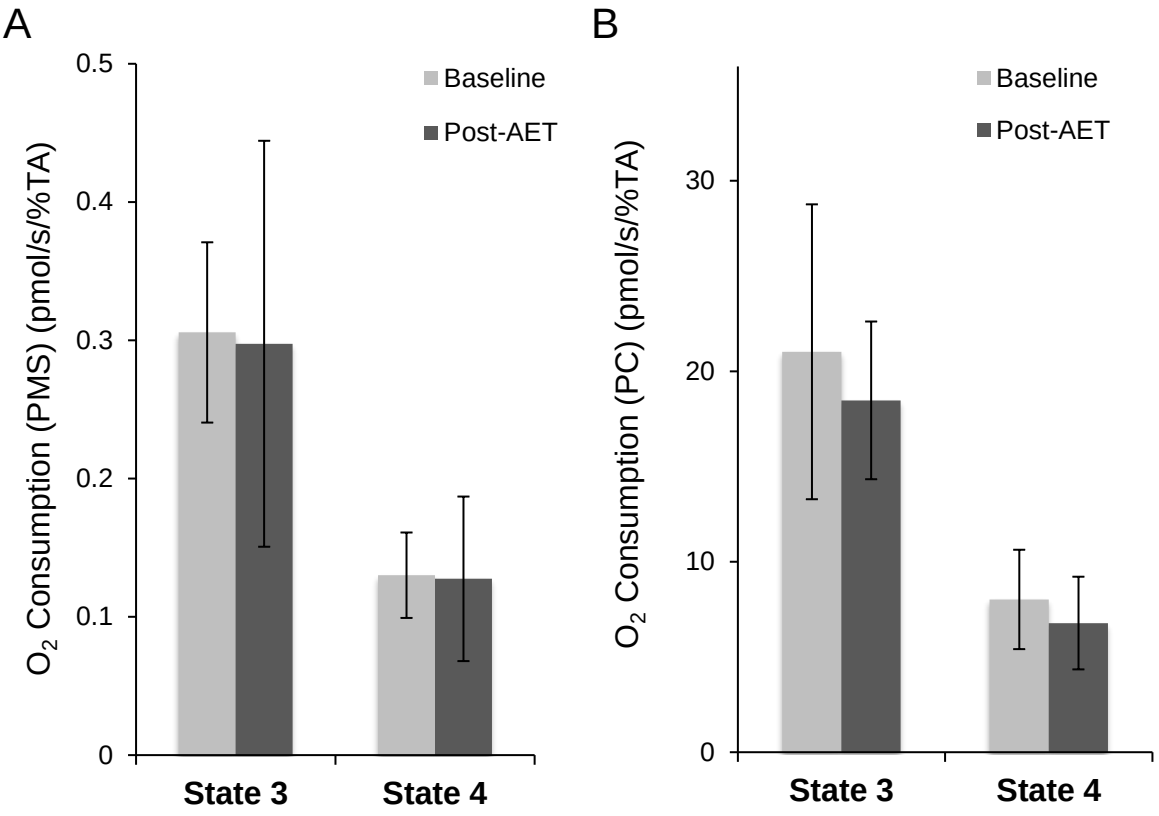
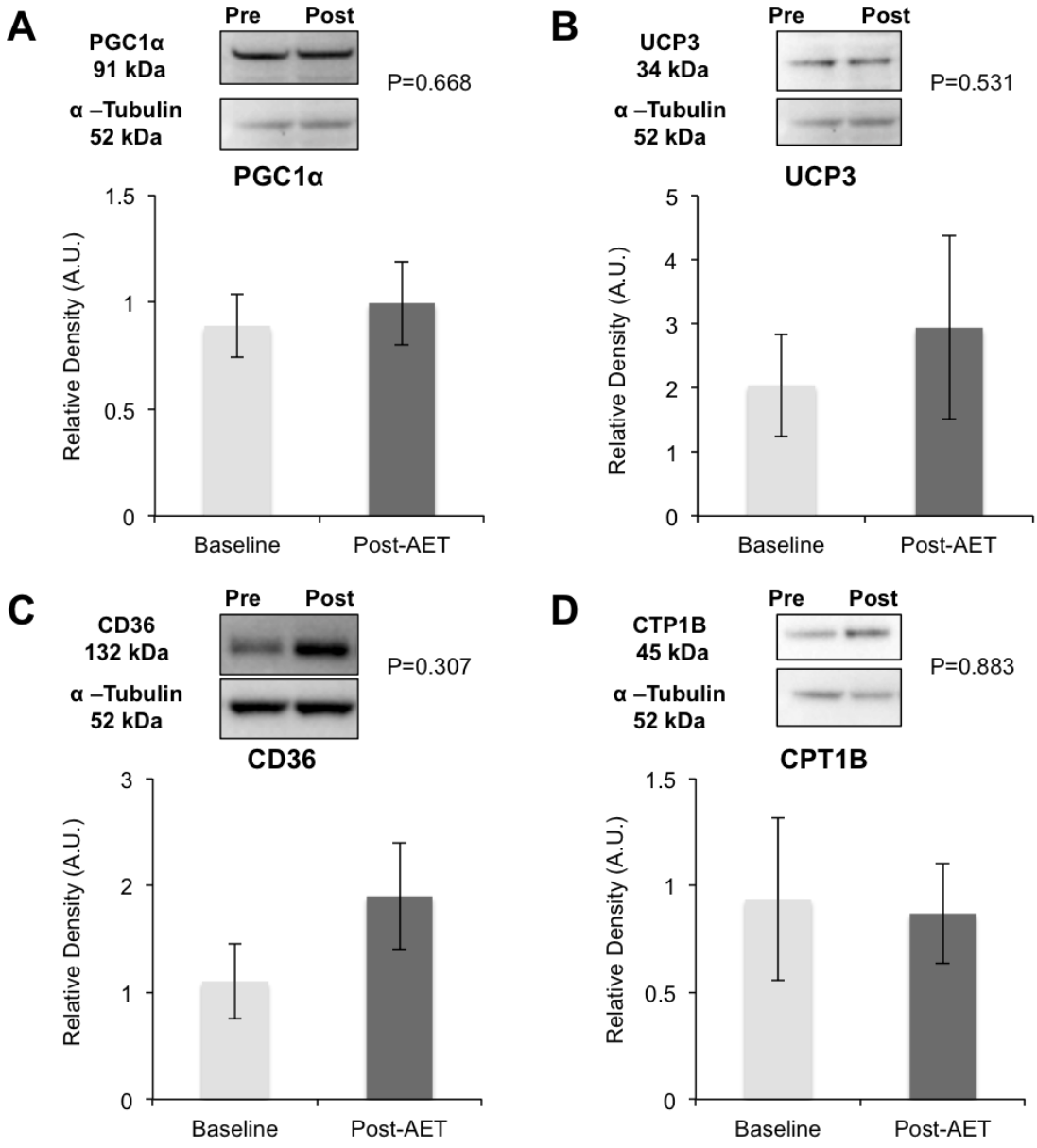


Figure 6. Skeletal muscle protein expression at baseline and post-AET measured using Western blotting. Values are normalized to α-tubulin and are presented as mean ± SEM. *P<0.05. (A) PGC1α, n=11; (B) UCP3, n=11; (C) CD36, n=11; (D) CPT1B, n=5.



SUMMARY AND CONCLUSIONS

The devastating consequences of obesity and T2D within the US and across the world necessitate continued study to identify novel targets for the prevention of these diseases and for treatment strategies that are less onerous, less costly, and more effective. Given that chronic oxidative stress is associated with obesity and has been implicated in the onset of IR, the objective of this dissertation was to characterize mitochondrial adaptations in response to acute and chronic stimuli that may mediate the redox environment and thereby promote or prevent disease.

The mitochondria are responsible for the majority of energy production within the body, but are also a primary site for the production of ROS. ROS play an important role in a variety of cellular signaling processes, but excessive ROS production can contribute to an environment prone to oxidative damage. This oxidative damage is thought to impair peripheral insulin signaling pathways and may consequently mediate the risk for T2D (69). Mitochondria are thought to exhibit a level of plasticity or flexibility in order to balance the energetic needs of the organism while mediating the oxidative environment. This plasticity is characterized by changes in oxidative activity and mitochondrial morphology. Impairments in plasticity may contribute to excessive ROS production and thus the onset and progression of IR, T2D, and diseases associated with oxidative stress.

The preceding chapters have described at length our contribution to the understanding of the remarkable dynamic and plastic nature of mitochondrial phenotypes, how these adaptations may affect the risk for cardiometabolic disease, and how lifestyle

intervention strategies are capable of improving mitochondrial function. The data presented suggest that the maintenance or restoration of mitochondrial plasticity to meet both acute and chronic metabolic demands undoubtedly warrants further investigation as a potential target for the treatment and prevention of obesity, T2D, and other chronic diseases linked to mitochondrial dysfunction and oxidative stress.

FA oxidation and obesity

A number of studies have shown enhanced FA oxidation in obese individuals compared to lean counterparts using *in vivo* approaches to assess FA oxidation capacity and anthropometric measures of obesity (37-39). Whether this observation reflects a shift in substrate-specific oxidative capacity at the level of the skeletal muscle mitochondria in response to the chronic metabolic conditions imparted by obesity had not been examined. Thus, the purpose of this study was to test the hypothesis that *in situ* measures of skeletal muscle mitochondrial FA oxidation would be positively associated with total body fat in a diverse cohort of premenopausal women.

This study demonstrated positive associations of total fat mass with State 3 (ADP-stimulated respiration) and the RCR. When participants were dichotomized by high or low body fat percent, participants with high total body fat displayed a higher RCR compared to those with low body fat. These results suggest that greater FA oxidation in obesity may be reflective of molecular processes that enhance FA oxidation capacity at the mitochondrial level. However, these data were collected in a relatively healthy, young population. It remains to be determined whether this phenotype is a positive adaptive mechanism to prevent the storage of lipid intermediates implicated in poor

cardiometabolic health or one that should be targeted for inhibition in attempt to restore substrate flexibility in obese persons.

Excessive and continued fat accumulation in humans appears to trigger metabolic abnormalities that lead to complications of obesity over time and our results suggest a potential role for the mitochondria in mediating this risk. Greater mitochondrial FA oxidative capacity observed here as total adiposity increases may represent an early mitochondrial adaptation to an environment characterized by chronically elevated FA substrate. However, this adaptation may become dysfunctional over time, as previous investigators have shown FA oxidation is impaired in morbid obesity (BMI>40kg/m^2) (70). Loss of this adaptive mechanism could contribute to the development of IR and cardiometabolic disease given that FA substrate is known to promote ROS production (71) and products of incomplete FA oxidation (e.g. diacylglycerols, ceramides) are thought to impair insulin signaling (72). Future studies should seek to characterize changes in FA oxidation capacity across a spectrum of cardiometabolic disease states to more definitively implicate changes in mitochondrial FA oxidation in the progression of T2D.

Obesity is also often associated with elevated oxidative stress (12). The greater mitochondrial coupling observed in obese persons may contribute to greater ROS production and mitochondrial impairments that further contribute to ROS production in an environment characterized by elevated FA substrate. When substrate is elevated and energetic demands are low, uncoupling mitochondrial respiration from oxygen consumption is thought to be a protective mechanism that can limit ROS production. Whether the coupling we observed in obese persons persists in the midst of an acute

metabolic challenge promoting ROS production remains to be determined. If the mitochondria of obese persons inadequately adapt to changing conditions to mediate ROS produced, this may provide a mechanistic link between obesity and the development of IR.

In the healthy, young cohort examined in this particular study, there was no association between android fat mass and FA oxidative capacity independent of total fat mass, however a trend was still evident ($p=0.099$). IAAT is known to be more lipolytically active (73), and thus may enhance FA substrate availability that may uniquely promote FA oxidation and ROS production (71). We would hypothesize that populations with significant stores of android fat would exhibit this mitochondrial accommodation to utilize an elevated substrate even without elevated subcutaneous stores. This hypothesis may help further explain the relationship between android obesity, oxidative stress, and cardiometabolic risk. Additionally, given the relationship between android fat and IR, there may be an even greater reliance on FA oxidation as IR can impair carbohydrate utilization (74). Future studies should seek to recruit men with significant levels of IAAT fat and older adults with IR that simultaneously exhibit a "healthy" BMI to identify if there is an independent effect of android fat mass and/or IR in FA oxidation capacity.

Mitochondrial responses to acute hyperinsulinemia

The previous study demonstrated that chronic mitochondrial adaptations that may promote ROS production and IR occur in the obese state. However, acute changes in mitochondrial plasticity may also play a critical role in mediating the redox environment.

Uncoupling of mitochondrial respiration from ATP production is hypothesized to be a feedback mechanism initiated by elevated ROS production (14,75). This mechanism may play a role in the mediation of the chronic oxidative state of the body and thus the risk for cardiometabolic disease. However, acute mitochondrial responses to a metabolic challenge known to increase ROS production have not been sufficiently characterized and whether elevated uncoupled respiration occurs concurrent with ROS production had not been shown.

In this study, hyperinsulinemia increased the production of ROS concurrent with a decrease in the RCR. Non-parametric procedures were conducted given the relatively small sample size and non-normal data. Cause and effect cannot be determined from the study design, but these data support the hypothesis that this uncoupling effect is a feedback response to ROS produced to mediate the quantity produced in healthy tissues. Whether this effect plays a role in managing the long-term oxidative state is unclear and could have implications in the onset of IR. Additionally, whether this uncoupling action occurs in individuals with T2D in response to acute hyperinsulinemia has not been examined. If this acute uncoupling mechanism is impaired in obesity and T2D, this may result in elevated ROS production, the accumulation of oxidative damage, and disease progression.

A number of therapeutic compounds and lifestyle modifications designed to initiate uncoupling in the mitochondria are under investigation as a potential means of combating obesity and cardiometabolic disease. However, therapeutics in particular have demonstrated a number of detrimental side effects associated with chronic and indiscriminate uncoupling action (76). Insights gained from the present study highlight

the need for the development of timed and targeted uncoupling agents that restore true mitochondrial plasticity to meet metabolic conditions. Chronic uncoupling agents and antioxidant compounds may depress this plasticity further. Given the cost and social burden associated with medications for T2D, lifestyle modifications that can restore plasticity represent promising strategies for improving mitochondrial function and decreasing oxidative stress.

Mitochondrial responses induced by AET

If obesity and T2D are indeed characterized by derangements in mitochondrial function, the identification of feasible, cost-effective strategies to improve it are of vital clinical importance. Aerobic exercise training (AET) has been shown to improve mitochondrial bioenergetics and up-regulate proteins related to lipid metabolism. However, whether these alterations are evident independent of the acute and chronic negative energy balance (EB) associated with AET (e.g. glycogen depletion, weight loss) is unclear. The purpose of the study was to test the hypothesis that improvements in skeletal muscle mitochondrial function induced by AET observed in previous literature would persist under rigorously controlled EB conditions.

This study demonstrated that mitochondrial respiratory capacity supported by PMS was unchanged when measured in an energetically balanced state following 16 weeks of AET. However, when mitochondrial oxidation was supported by a fatty acid substrate, enhanced mitochondrial oxidative capacity and elevated uncoupled respiration were observed. These results suggest that the mitochondria are adapting to meet the changing demands of the participants as AET relies primarily on oxidative metabolism.

Changes in oxidative capacity were no longer apparent when these data were normalized for a marker of mitochondrial content. These findings support that the improvements in mitochondrial FA oxidative capacity were due to mitochondrial biogenesis induced by AET. Thus, AET is a valuable tool for improving mitochondrial health even in the absence of weight loss and acute energetic deficits. These findings may have implications in the prevention and treatment of T2D since impaired mitochondrial oxidative capacity and ROS production have commonly been associated with IR.

Future studies should seek to validate these findings using additional methods for assessing mitochondrial content (e.g. citrate synthase activity, mtDNA content). Additionally, determining whether AET can restore acute mitochondrial plasticity following metabolic insults known to promote ROS production (like hyperinsulinemia) could further solidify AET as a treatment for obesity, T2D, and other disease states associated with chronic oxidative stress. These lingering questions could be addressed using a longitudinal study designed to characterize the acute responses to a hyperinsulinemic challenge prior to and following an AET program. Animal models could be employed in order to reduce the recruitment burden associated with studies requiring multiple biopsies and to ensure strict adherence to an AET protocol. Conversely, continued investment in non-invasive methods for characterizing these mitochondrial responses in humans (e.g. NIRS, MRS) will decrease participant burden and allow for an increase in the sample population to definitively address these hypotheses.

Final conclusions

The studies presented have been conducted to further characterize the role of mitochondrial health in the onset and treatment of obesity, T2D, and other chronic diseases associated with oxidative stress. This body of work suggests that there are important mitochondrial adaptations to both acute and chronic stimuli that occur that have the potential to affect the redox environment and thus cardiometabolic health. When these adaptations become chronically compromised or fail to be acutely elicited, this may contribute to excessive ROS production, oxidative damage, and manifest as obesity, T2D, and other cardiometabolic disease states.

The evidence presented suggests that there are fundamental differences in mitochondria between obese and lean persons. These mitochondrial adaptations that occur in obesity may promote ROS production and IR, suggesting a possible mechanistic link between obesity and the development of IR. Additionally, ROS production and concurrent uncoupling of mitochondrial respiration induced by acute hyperinsulinemia may reflect an important dynamic response in healthy individuals. This response also likely reflects a physiological response to the postprandial state, a condition we find ourselves in more often than not in modern Western culture. If this uncoupling mechanism is impaired in obesity and T2D, there may be increases in acute ROS production with chronic consequences related to oxidative stress. If these mitochondrial derangements exist that promote ROS production in the disease state, then the development of lifestyle modifications and next-generation therapies that restore acute mitochondrial plasticity may be of vital clinical importance to reduce oxidative stress and disease progression. Simply being content to reduce overall ROS production may no

longer be sufficient to prevent and treat obesity, T2D, and other chronic diseases given this new understanding of the dynamic nature of the mitochondria and the vital role of ROS in cellular signaling. As such, treatment and prevention strategies should be designed to improve both the acute and chronic mitochondrial responses that allow the mitochondria to adjust oxidative efficiency in order to mediate ROS production. Adaptable, cost-conscious lifestyle interventions such as AET represent promising methods for improving (or maintaining) mitochondrial plasticity given the well-characterized ability to promote mitochondrial biogenesis and oxidative capacity even in the absence of weight loss, but deserve further study to ensure acute mitochondrial responses are also improved.

The association between mitochondria and the eukaryotic cells that contain them is thought to be the product of an endosymbiotic relationship established over a billion years ago. This work supports that this relationship requires that the mitochondria not be passive or static passengers, but that these organelles exhibit the capability to alter their physiology and morphology to meet the myriad acute and chronic demands of the "host" cell/organism.

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APPENDIX: INSTITUTIONAL REVIEW BOARD APPROVALS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Office of the Institutional Review Board for Human Use

470 Administration Building
701 20th Street South
Birmingham, AL 35294-0104
205.934.3789 | Fax 205.934.1301 | irb@uab.edu

APPROVAL LETTER

TO: Gower, Barbara

FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance Number FWA00005960

DATE: 26-Jul-2017

RE: IRB-130228002
Race Adiposity Interactions Regulate Mechanisms Determining Insulin Sensitivity

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The IRB reviewed and approved the Continuing Review submitted on 10-Jul-2017 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

Type of Review: Full (Institutional Review Board 01 (UAB))
Determination: Approved
Approval Date: 26-Jul-2017
Approval Period: One Year
Expiration Date: 25-Jul-2018

The following apply to this project related to informed consent and/or assent:

- Waiver (Partial) of HIPAA

Additional Comments:

- The IRB reviewed and approved the changes requested in the Investigator's Progress Report.

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Office of the Institutional Review Board for Human Use

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205.934.3789 | Fax 205.934.1301 | irb@uab.edu

APPROVAL LETTER

TO: Fisher, Gordon

FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance Number FWA00005960

DATE: 27-Jul-2017

RE: IRB-140516004
Mitochondrial Dysfunction, Oxidative Stress, and Risk for Type 2 Diabetes

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The IRB reviewed and approved the Continuing Review submitted on 27-Jun-2017 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

Type of Review: Full (Institutional Review Board 02 (UAB))
Determination: Approved
Approval Date: 19-Jul-2017
Approval Period: One Year
Expiration Date: 18-Jul-2018

Additional Comments:

-Approval of this protocol expired on June 1, 2017. Renewal materials were received in the Office of the IRB on June 26, 2017. The IRB recommends submitting renewal materials 4-6 weeks before the protocol expiration date to avoid a lapse in approval. The IRB noted the memorandum dated June 26, 2017 describing no study related work has been performed during the lapse in approval.

-The IRB reviewed and approved the changes requested in the Investigator's Progress Report (IPR).

-The IRB noted that this protocol was permanently closed to enrollment and may qualify, in the future, for expedited review under Category 8. Expedited review would be appropriate where:
a. (i) the research is permanently closed to the enrollment of new subjects; and (ii) all subjects have completed all research-related interventions; and (iii) the research remains active only for long-term follow-up of subjects; or
b. no subjects have been enrolled and no additional risks have been identified; or
c. the remaining research activities are limited to data analysis.

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205.934.3789 | Fax 205.934.1301 | irb@uab.edu

APPROVAL LETTER

TO: Hunter, Gary R

FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance Number FWA00005960

DATE: 01-Aug-2017

RE: IRB-101018001
Exercise Intensity, Metabolic Rate & Insulin Sensitivity

The IRB reviewed and approved the Continuing Review submitted on 22-May-2017 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

Type of Review: Expedited (Category 8)
Determination: Approved
Approval Date: 01-Aug-2017
Approval Period: One Year
Expiration Date: 31-Jul-2018

Additional Comments:

exp cat 8

The following documents have been reviewed:

- ipr.170522