Effects of High-Insulin Exposure on Mitochondrial Content in Skeletal Muscle Myotubes from Lean & Severely Obese Individuals:

A Time Course Analysis

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Skeletal muscle is the site at which highly regulated energetic processes take place as a means of maintaining both tissue-specific and whole-body metabolism. With the development of severe obesity (BMI \geq 40 kg/m²), skeletal muscle often loses its ability to properly respond to insulin, which aside from regulating glucose, has also been suggested to regulate factors specific to mitochondrial biogenesis. In this study, we classified human skeletal muscle cells (HSkMCs) derived from lean and severely obese individuals. We found them to be strikingly similar with respect to their proliferation and differentiation behavior. Also, to test the hypothesis that insulin would increase proteins associated with mitochondrial biogenesis, mature myotubes were treated with 100nM insulin at time points ranging from 1-24 hours. To validate our model, we incorporated a number of methodologies, including measures of AKT phosphorylation, which we found to be 51% lower in the obese group at the first hour (p = 0.05). However, 100nM insulin produced no changes in protein content of PGC-1α, nor OXPHOS proteins, Complex V or Complex III. This high-insulin treatment did result in increases in Complex IV at 3 hours (p = 0.02), as well as the slow isoform of

MYHC (at multiple time points) in both groups. We also observed trends for decreases in intracellular ATP at 24 hours of high-insulin treatment in both lean and severely obese groups. Collectively, these data demonstrate that insulin produces no consistent increases in mitochondrial biogenesis as defined by PGC-1α and OXPHOS proteins in myotubes from lean and severely obese individuals. We further conclude that the responses to sustained, high-insulin exposure in these groups are alike as it relates to increases in protein synthesis (i.e., MYHC (slow)) at the rather costly expense of ATP.

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Chapter I

Introduction

Currently affecting approximately one-third of the U.S. population [1], obesity, an over-expansion of the adipose tissue resulting in a Body Mass Index (BMI) \geq 30 kg/m², is a disease state that results in skeletal muscle dysfunction. Human skeletal muscle is an organ comprising less than half (roughly 40% for men and 30% for women) of total body mass [2]. It provides the support needed for bodily movement and is known for its key contributions to energy production with respect to athletic performance (i.e., power, force, velocity), thermoregulation and substrate metabolism. In recent years, skeletal muscle has been studied extensively for its role in health and metabolic disease, such as obesity. As mentioned, obesity is far too prevalent in U.S., comprising about one-third of the population, with 1 in 20 Americans being severely obese (BMI \geq 40 kg/m²) [3]. Obesity has been closely linked to the development of cardiovascular disease as well as skeletal muscle insulin resistance and Type 2 Diabetes (T2D) [4]. As a result, understanding how obesity dictates energetic processes in skeletal muscle is of high concern.

Insulin is an anabolic peptide hormone released from pancreatic B-cells for the control of blood glucose levels. In skeletal muscle, the site at which ~80% of glucose uptake occurs [5], this process is defective in the state of obesity [6, 7, 8]. Though insulin sensitivity is primarily thought to be a crucial component of the regulation of glucose, there is a growing body of evidence to suggest insulin is also responsible for the regulation of other components of energy production [9, 10]. Though this relationship is still not fully established with respect to skeletal muscle, there is

evidence to suggest that insulin also regulates aspects of mitochondrial function [9, 10].

The mitochondrion an organelle at which produces a high volume of energy in the form of adenosine triphosphate (ATP). Both the content and function of mitochondria are reduced with severe obesity [11, 12], which contribute to the inflexibility of skeletal muscle in terms of substrate metabolism. Whether this decrement in mitochondrial energetics precedes or is a result of the insulin-resistant state of skeletal muscle from obese individuals is debatable [13]. Nonetheless, insulin has been suggested to somehow be involved in the regulation of the mitochondria and/or subsequent function. Moreover, this contribution to mitochondrial metabolism may be tissue-specific. For example, a study was conducted in which mitochondria were extracted from pancreatic B-cells derived from B-cell-specific insulin receptor knockout mice [14]. Major findings were that there was a decrease of function in factors associated with mitochondria, including an apoptotic protein, Bcl2-associatd death promoter (BAD). These impairments were restored with expression of the same tissue/cell-specific receptors. In mouse hepatocytes, however, it was found that insulin stimulation over a 24-hour time period, decreased mitochondrial content, ATP production and O₂ consumption [15]. These data are in line with the idea of insulin as a regulatory factor of mitochondria that is specific to tissue type.

In terms of skeletal muscle, a 2014 study found that 4 hours of insulin infusion in healthy individuals resulted in a 2-fold increase in phosphorylation of isolated mitochondrial proteins including those indicative of oxidative capacity and tricarboxylic acid (TCA) cycle function, to name few [16]. This is in agreement with

a study from 2003 that showed an 8-hour insulin infusion induced changes in ATP production that were accompanied with increases in measurements of mitochondrial function (citrate synthase (CS) and cytochrome oxidase (COX) activities) [17]. However, it is important to note that the findings from this particular study were observed in patients with T2D [17].

The extent to which the anabolic potential of insulin could enhance mitochondrial content and/or function in skeletal muscle in with obesity remains unclear. Furthermore, there is a lacking in the literature concerning such changes and how they are altered with longer time durations in vitro. The purpose of this study was to investigate if high-insulin exposure alters indices of mitochondrial content/function differently in the skeletal muscle from lean vs. obese individuals with a time course analysis. For these experiments, we utilized a cell culture model in which we maintained a highly controlled environment that eliminated the influence of external factors (i.e., other hormones, exercise/physical activity) on the physiological conditions we aimed to study. As mentioned, human skeletal muscle cells (HSkMCs) derived from severely obese individuals often exhibit reductions in glucose and lipid metabolism [44, 49]. Because of this, HSkMCs serve as a great model for studying metabolic dysfunction in certain populations (i.e., insulin resistant) [47, 48]. However, the authors wanted to evidence whether these differences were due to cellular properties (i.e., cell proliferation or differentiation, muscle cell machinery).

We hypothesized that there would be no differences in proliferation or differentiation in HSkMCs derived from lean and severely obese individuals.

Additionally, we hypothesized that high-insulin would acutely upregulate factors associated with mitochondrial biogenesis in muscle from lean, but not obese individuals. These factors include but are not limited to: peroxisome proliferatoractivated receptor gamma coactivator 1-alpha (PGC-1 α) and proteins involved with the electron transport chain (Complexes I-V). We further hypothesized insulinstimulated improvements in mitochondrial function in lean individuals, particularly citrate synthase activity and ATP content. With these findings, it was our intent to provide additional evidence to the changes in response to insulin that may or may not occur in skeletal muscle with severe obesity.

Chapter II

Review of Literature

Dangers of Obesity

Skeletal Muscle Insulin Resistance

Obesity (BMI \geq 30 kg/m²) is concerning due to its association with conditions such as hypertension and coronary artery disease, as well as the development of insulin resistance and type 2 diabetes. In skeletal muscle, insulin-mediated glucose transport initially requires insulin to bind to the α -subunits on the transmembrane receptor on the cell surface [18]. This binding results in an intracellular signaling cascade that involves the phosphorylation of AKT (also known as Protein Kinase B), which stimulates the translocation of GLUT4 vesicles to the membrane allowing glucose uptake to occur [19-23]. Like many other signaling pathways in the human body, this is a tightly controlled and highly regulated system. However, in the state of obesity, oftentimes glucose levels remain high, as well as the levels of insulin, indicating that this process is impaired.

To determine if insulin signaling is impaired with obesity, intact muscle strips were teased from rectus abdominus muscle biopsy samples from 8 lean (BMI = $25.7 \pm 0.9 \text{ kg/m}^2$) and 8 severely obese (BMI = $52.9 \pm 3.6 \text{ kg/m}^2$) individuals [24]. The participants were all women matched for age and race. The muscle strips were measured for 2-deoxyglucose in both the basal and insulin-stimulated states. Insulinstimulated glucose uptake was reduced by 53% in muscle strips obtained from the severely obese individuals in comparison to their lean counterparts. Additional measures were taken for insulin signaling including the insulin receptor, insulin receptor substrate-1 (IRS-1), and PI3K activity. Muscle strips obtained (from the same participants) were incubated in basal or insulin-stimulated (10⁻⁷M) states for 2, 4, 15 or 30 minutes). These data indicated that at each time point, insulin-stimulated tyrosine phosphorylation of both the insulin receptor and IRS-1 were reduced in muscle strips from obese individuals compared to lean. These differences reached statistical significance at 2 minutes and 15 minutes as well as in the basal state for the insulin receptor. However, only the 15 minute time point was significant for IRS-1. Furthermore, the researchers found that the regulatory subunit of PI3K, p85, was also reduced in muscle strips from severely obese individuals. PI3K activity was reduced as well, suggesting that this reduced activity of PI3K could be due in part to decreased p85 expression [24]. Taken together, these and other findings [25, 26, 27] demonstrate that impairments in steps of the insulin signaling cascade serve as potential molecular mechanisms for skeletal muscle insulin resistance.

Alterations in Muscle Properties & Fiber Type

In addition to deficiencies in insulin signaling, research has suggested that obesity is associated with differences in the muscle cell properties including fiber type. Recent data has shown that these cellular properties in diseased populations (i.e., individuals with T2D, COPD, other inflammatory diseases) are altered in comparison to their healthy counterparts. These changes include, but are not limited to, changes in cell proliferation, differentiation and regeneration [50, 51, 52]. One particular group of researchers provided evidence to the oxidative function of primary muscle cells being retained up to the fifth passaging of the cells in cells from lean and

individuals with T2D [53]. Still, there is a lacking in the research that has been done to address these questions with respect to obesity.

With regards to fiber type, both type I and type II muscle fibers have been shown to respond to insulin [28, 29], though type I muscle fibers have been shown to be more insulin sensitive compared to type II fibers [29]. Additionally, decreased type I fibers have been observed in obese T2D patients [30], suggesting the insulinresistant state often accompanying obesity is related to the glycolytic capacity of muscle associated with fiber type.

A group of researchers sought to further examine this phenomenon in a study in which they hypothesized that a relationship existed between muscle fiber type and obesity [31]. This study consisted of middle-aged Caucasian and African American women who were undergoing abdominal surgery and were either lean (undergoing hysterectomy), or obese (undergoing gastric bypass). Body mass index (BMI) was compared across groups. A rectus abdominus muscle biopsy was obtained from each of the participants who underwent surgery. These samples were mounted, frozen, sliced in 10 µM sections and stained for ATPase for analysis of fiber type. The data indicated that adiposity was positively correlated with muscle fiber type, specifically type IIb. Moreover, all lean participants possessed roughly 55% type I fibers and about 14% type IIb fibers. However, obese participants exhibited a significantly lower percentage of type I fibers as well as a significantly higher percentage of type IIb. Furthermore, African American participants contained lower percentages of type I fibers and subsequently higher percentages of type II fibers (in comparison to lean subjects). This, as the authors mention, could be a plausible explanation as to why

African Americans are generally more likely to be obese and/or develop T2D or similar metabolic complications [31]. Lastly, participants with greater type I fiber percentages who underwent gastric bypass experienced greater reductions in adiposity, suggesting muscle fiber type may also play an important role in facilitating fat metabolism.

Mitochondrial Dysfunction

In addition to skeletal muscle insulin resistance and changes in fiber type, obesity has also been linked to reduced mitochondrial capacity and function. Deficiencies in proteins associated with mitochondrial biogenesis and fat oxidation have been observed [32]. Other complications as a result of obesity (i.e., mitochondrial morphology, dynamics) that are associated with skeletal muscle functionality are also of concern.

Such complications were illustrated when a research group examined leptindeficient mice ((ob/ob), with control littermates) and high-fat diet mice (HFD), with low-fat diet controls [33]. Mouse myoblasts (C2C12s) were obtained and differentiated for experiments aimed to induce a shift in energy balance (i.e., hyperglycemia, palmitate incubation). Measurements that were taken included an assessment of mitochondrial morphology, mtDNA content and mitochondrial protein. The major findings were that since mitochondrial fusion protein levels were unchanged, it was suggested that mitochondrial fission is where the problem may lie, as mitochondrial fragmentation was increased in ob/ob and HFD mice. This fragmentation was associated with palmitate-induced increases in fission proteins, dynamin-related protein 1 (Drp1) and mitochondrial fission protein 1 (Fis1) in ob/ob

and HFD mice [33]. These data indicate that lipid overexposure associated with severe obesity, is damaging to mitochondria and their associated proliferation mechanisms.

These findings are consistent with a 2011 study conducted in humans in which HSkMCs were obtained from young lean and severely obese Caucasian males [34]. These cells were cultured, differentiated to myotubes and incubated with palmitoyl carnitine for 24 hours. Following the lipid substrate incubation, measures for mitochondrial content and function were taken. Results indicated that mitochondrial DNA (mtDNA) copy number increased in myotubes from lean, but decreased in myotubes from obese donors. Also, in myotubes from obese donors, mitochondrial respiration did not increase to the same degree of myotubes from lean individuals. Even after normalization to mitochondrial content (by mtDNA copy number and citrate synthase activity), data show a persistent reduction in fat oxidation after lipid treatment in myotubes from obese individuals [34]. These findings suggest that obese muscle is metabolically inflexible as only myotubes from lean individuals expressed the ability to induce mitochondrial proliferation and increase respiration following lipid exposure. It can be concluded that reduced mitochondrial content and function are plausible explanations for altered fat metabolism in obese individuals.

Lastly, a study conducted with eight severely obese individuals undergoing bariatric surgery also highlights the inherent metabolic defects with mitochondria as a result of severe obesity [35]. In this study, permeabilized muscle fibers from muscle biopsies from each participant were analyzed using high-resolution respirometry to assess mitochondrial function. The findings from these eight severely obese

individuals were compared to ten lean and ten obese individuals. Before surgery, state 3 respiration was significantly lower in severely obese individuals. This particular finding emphasizes the reduced function of mitochondria with severe obesity. However, the remaining findings were that state 3 respiration increased after a mean of 35 kg weight loss by bariatric surgery. Similarly, overall mitochondrial function in the severely obese individuals was comparable to that of lean individuals 1-year post surgery. Furthermore, respiration on a lipid substrate also increased in muscle fibers from obese individuals. These data suggest that though the mitochondria are defective in the obese state, dramatic weight loss can serve as a means to improve them. In addition, this could mean that insulin as a stimulatory factor is also involved, as insulin sensitivity is also rapidly restored with bariatric surgery [35-38].

The Role of Insulin in Obesity/Metabolic Disease

Regulation of Gene Expression & Protein Content by Insulin

Though insulin has long been studied for its responsibilities to regulating small molecules like glucose [39], there is a collection of evidence that illustrates its effects on transcriptional regulation. In the liver, insulin effects (via vanadate administration) have been shown to increase glucokinase, a critical enzyme responsible for the breakdown of glucose in hepatocytes. Glucokinase mRNAs increased by 20-fold after only 45 minutes of vandate treatment in liver nuclei of streptozotocin-treated (STZ) rats [40]. Similar findings are evident in liver in a study investigating effects of insulin on gene expression, particularly glycolytic enzymes like pyruvate kinase [41].

A more recent study was conducted examining this relationship in-vivo. A hyperinsulinemic-euglycemic clamp was performed on 10 young males for 3 hours [42]. Vastus lateralis muscle biopsies taken before and after the clamp procedure indicated that hyperinsulinemia induced significant increases in myosin heavy chain (MYHC) type IIx mRNAs. These data suggest that prolonged insulin exposure (at 600 pmol \cdot m⁻² \cdot min⁻¹) has an effect on the gene expression of type II muscle fibers in vivo. This may explain mechanisms of lower amounts of mitochondria in obese individuals who have higher percentages of these type II fibers. Furthermore, an MYHC analysis in a recent study revealed that severely obese individuals exhibit significantly less of the type I isoform in comparison to their lean counterparts [43]. Taken together, these studies offer evidence to a critical relationship between

obesity and the regulation of genes by insulin in glycolytic as well as oxidative metabolism.

Mitochondrial Content is altered in Skeletal Muscle with Insulin Exposure

The mitochondria, which handle the vast majority of oxidative processes within the cell, have been shown to be involved in regulatory mechanisms that are controlled in part, by insulin. In some cases this is not an energetically favorable phenomenon, as witnessed by the mitochondrial dysfunction that often accompanies obesity as well as insulin resistance.

This idea was demonstrated in a study that examined respiratory function in mitochondria from human skeletal muscle after exposure to insulin and/or fatty acid [44]. Vastus lateralis muscle biopsies were obtained from lean, obese and obese type 2 diabetic (T2D) individuals. Utilizing a cell culture model, HSkMCs were isolated from biopsy samples, grown in culture and differentiated to myotubes for experimentation. On days 5 or 6 of differentiation, myotubes were treated with 1 μ M insulin, 0.6 mM palmitate or co-incubation of both for 4 hours. In isolated myotube mitochondria, 3-hydroxyacyl-CoA (HAD) protein content was measured as an indicator of B-oxidation, whereas citrate synthase (CS) and hexokinase (HK) protein content were taken as a means to assess the ratio of oxidative to glycolytic metabolism. Also, in an attempt to discern whether lower CS in obese T2Ds could be explained by lower mitochondrial content, NADH respiration was measured. Major findings were that CS was significantly lower in myotubes derived from obese T2Ds in comparison to lean and severely obese controls [44]. Insulin stimulation significantly increased CS in myotubes derived from lean and severely obese individuals, however, this effect was

not observed in myotubes from obese T2Ds. Moreover, this effect of insulin stimulation was eradicated with co-incubation of insulin and palmitate. Regardless of the experimental condition, no significant differences in oxygen consumption were observed in any group, until normalized to CS. These findings first highlight that CS is significantly lower in obese T2Ds under normo-physiological conditions that was not improved upon 4 hours of insulin treatment. This suggests a metabolic impairment in oxidative enzymes for mitochondrial function that may be regulated by insulin and thus, defective in the insulin-resistant state [44]. This study also illustrates the dangers high fatty acid as myotubes from lean and severely obese individuals functioned to a level of obese T2Ds upon exposure to only 4 hours of high palmitate [44].

In contrast, a similar, more recent study examined the influence of insulin resistance induced by high insulin and/or saturated fatty acids on mitochondrial function [45]. C2C12 cells were grown in culture and treated with 100 nM of insulin for 24 or 48 hours (on days 3 and 5 of differentiation). For experiments with fatty acid, C2C12 myotubes were treated with palmitate at various concentrations. Findings were that 48 hours of insulin treatment induced insulin resistance through impaired PI3K signaling as proteins associated with this pathway were significantly reduced [45]. Researchers also found that 48 hours of insulin significantly increased oxygen consumption, lowered mitochondrial membrane potential and lowered mitochondrial reactive oxygen species (ROS) production. However, there were no changes in ATP content. In addition, PGC-1α mRNA and uncoupling proteins 2 and 3 (UCP2 and UCP3) mRNAs were significantly reduced upon 48 hours insulin stimulation, though no

significant changes in oxidative phosphorylation complex proteins (Complexes I-V) were observed. Conversely, fatty acid treatment resulted in a significant decrease in oxygen consumption, decrease in mitochondrial membrane potential and increase in electron transport chain (ETC/OXPHOS) subunits and UCP proteins [45]. Collectively, these data suggest that insulin resistance as a result of either prolonged exposure to insulin or saturated fatty acids results in adaptations in mitochondrial function. However, the changes seem to occur by two separate mechanisms [45]. It can be inferred, then, that there may exist a conditioned response of insulin to upregulate factors that can maintain a "normal" of level of mitochondrial function that is lost over time with the development of obesity/severe obesity.

Chapter III

Research Design & Methods

Experimental Purpose

Experiments for this thesis consisted of two components. In the first set of studies, we wanted to examine the proliferation and differentiation characteristics of HSkMCs derived from lean and severely obese individuals under normo-physiological conditions. The second set of studies consisted of examining responses to insulin with respect to mitochondrial biogenesis and other indices of oxidative function.

Human Participants

Before any testing procedures began, approval by the East Carolina University Policy and Review Committee on Human Research was obtained. Participants included lean and severely obese women, matched in age and ethnicity. Table 1 outlines these characteristics.

Muscle biopsies & HSkMCs

Muscle biopsies were taken from the vastus lateralis of each participant using the percutaneous needle biopsy technique [46]. From these biopsy samples, primary muscle cells were isolated and grown in culture [46]. These human skeletal muscle cells (HSkMCs) were transferred and sub-cultured in 12-well or 6-well type I collagencoated plates at densities of 40,000 to 60,000 cells per well. Once the cells were approximately 80-90% confluent, they were switched from growth medium (DMEM [5mM glucose], 10%FBS, 1% BSA, 1% Fetuin, 0.1% Dexamethasone, 0.1% rhEGF, 1% Pen-Strep) to differentiation medium (DMEM [5mM glucose], 1% Horse Serum, 0.3% BSA, 0.5% Fetuin, 1% Pen-Strep). Procedures for primary muscle cell culture have been previously outlined in detail [46].

Determination of Cellular Properties from HSkMCs

Myoblast Proliferation

In a sub-sample of cells derived from lean and severely obese individuals matched for age and ethnicity, proliferation experiments were performed. 24 hours after plating sub-cultured cells, cells were lifted (.05% Trypsin/EDTA) and viability was assessed using an automated cell counter (Beckman Coultre Vi-Cell). Total cell counts were also determined using this cell counter. To validate these proliferation measures, cells were counted using the MTT [3-(4, 5dimethylthiazolyl-2)-2, 5diphenyltetrazolium bromide] Cell Proliferation assay (Life Technologies), which measures the metabolic activity of cells utilizing NAD(P)H-dependent reactions. Thus, the MTT provides somewhat of a combination of the measures obtained from the automated cell counter (Beckman Coultre Vi-Cell), as the MTT indicates the number of viable cells based off of the absorbance. Each of these MTT experiments were also repeated at 48, & 72-hour time points.

Visualization of Myotube Differentiation & Protein Determination

On day 7 of differentiation, myotubes in 6-well plates were fixed with methanol, permeabilized (0.1% Triton-X100 in PBS), stained with DAPI [(2-(4amidinophenyl)-1H -indole-6-carboxamidine,] (Sigma Aldrich) and performed immunofluorescence for total myosin heavy chain, MHC (MF20; DS Hybridoma Bank, University of Iowa). Using the Pierce bicinchoninc acid assay (BCA), protein concentrations of the samples were determined at days 2, 5 and 7 of differentiation. Using ImageJ64, the fusion index and myotube area were obtained. The fusion index was calculated as the ratio of nuclei in myotubes containing \geq 3 nuclei to total nuclei per image. Myotube area was calculated as the amount of myotubes per area of the image [43]. 15 images were analyzed for 2 individuals per group for a total of 30 images analyzed per lean and severely obese groups. Western Blot analyses for specific protein content were also conducted at these time points (methodologies described in detail below).

High-Insulin Treatment in Skeletal Muscle Myotubes

For insulin-stimulation studies, on days 6 or 7 of differentiation, mature myotubes were treated with 100 nM insulin in a starvation medium (DMEM [5mM glucose], 1% BSA, 1% Pen-Strep) for 1, 3, 8, 16 or 24 hours. Basal conditions (starvation media only) at each time point were also included. Myotubes in 6-well plates were washed with ice-cold PBS and treated with 250 µL of a lysis buffer (50mM HEPES, 50mM sodium pyrophosphate, 100mM sodium fluoride, 10mM EDTA, 10mM sodium orthovanadate). Inhibitors (0.5% protease inhibitor cocktail [, 1% triton, and 1% phosphatase cocktail 2 and 3) were added in order to protect protein from degradation (by proteases) and the removal of phosphates (by phosphatases) on proteins of interest. After suspension in lysis buffer solution with inhibitors, cells were fully lysed by sonication and centrifuged at 12000 rpm for 15 minutes at 4°C. The pellets were discarded and cell lysates were analyzed using the Pierce BCA protein assay was performed for determination of protein concentrations in each samples.

Measures of Insulin and Metabolites from Culture Media

Measurements of insulin and related-metabolites was conducted across 1, 3, 8, 16 & 24 hour time points. Directly after insulin-stimulation, 1.0 mL of sample was collected from a single well on a 6-well plate for the basal conditions at each time point for each subject. For the insulin-stimulated conditions, 1.0 mL of sample was collected from two wells on the 6-well plate (1.0 mL per well for a total of 2.0 mL of sample per time point for each subject). Each sample corresponded to the same subject and condition for a total of 2.0 mL per sample. These samples were stored immediately at -80°C in Eppendorf tubes. At the time of analysis, a 500 µL aliquot was inserted into a chemistry analyzer (Unicel DxC 600i; Beckman Coulter) and determinations of insulin, glucose and lactate from the culture media were obtained.

Western Blot Analyses

From the subsample of myotubes used to determine physical cell properties, 18µg from each of the cell lysates were assayed for markers of differentiation (MyoD (M-318); Santa Cruz Biotech) and total myosin content (MHC (MF20); Hybridoma Bank). For the samples that were treated with 100nM insulin at time points between 1 and 24 hours, 18µg from each of these cell lysates were assayed for contents of the following markers of insulin signaling (p-AKT(Ser⁴⁷³) (9271), total AKT (9272); Cell Signaling) mitochondrial biogenesis (PGC-1 α (ab106814); Abcam), electron transport chain (Total OXPHOS cocktail (ab110413); Abcam) and the slow isoform of myosin heavy chain (MYHC (BA-D5); DS Hybridoma Bank). These experiments were performed

using SDS-PAGE and transfer to immobilon-fluorescent polyvinylidene difluoride membranes. Each sample was normalized to a control sample on each gel (β -actin; .

Determination of Intracellular ATP

Immediately following 24 hours of insulin treatment, myotubes in the 6-well plates not being used for other assays were washed twice with ice-cold PBS. PBS was then completely removed and the plates were put on ice. For each well, 200 µL of 0.5 N HClO₄ was added, scraped for cellular content and transferred to chilled centrifuge tubes. This step was repeated to ensure proper recovery of the cellular contents. Samples collected were then sonicated for 10 seconds at 100% power, centrifuged at 12000 rpm for 10 minutes at 4°C. 250 µL of the supernatant was then transferred to new tubes and neutralized with 140 μ L of ice cold 1.0 N KOH. These samples were centrifuged at 12000 rpm for 10 minutes at 4°C, then 300 µL of this supernatant was collected and stored at -80°C until analysis of nucleotides was performed using Ultra-Performance Liquid Chromatography (UPLC) [91]. These included ATP, ADP and AMP. From the previous pellet-containing tubes, residual liquid was completely removed using a pipette and the pellet was re-suspended in 300 µL of 0.2 N NaOH. Protein concentrations of these samples were determined using the Pierce BCA protein assay.

Citrate Synthase Activity

Citrate Synthase (CS) activity was determined using the Citrate Synthase Assay Kit (Sigma Aldrich). Immediately following insulin stimulation, myotubes in 12-well plates not being used for other assays were washed twice with ice cold PBS and

treated with 125 µL of the CelLytic[™] M Cell Lysis Reagent for 15 minutes on a shaker at 4°C. Cells were then scraped for cellular content and transferred to chilled centrifuge tubes that were stored at 80°C until later analyzed for protein concentrations via Pierce BCA. Once protein concentrations were determined, samples of at least 8 µg were combined with a reaction buffer also containing Acetyl Co-A and DTNB [5,5'-Dithiobis-(2-nitrobenzoic acid)], in 96-well plates. Oxaloacetic acid was added last after obtaining background activity from the sample and the rate of the reaction was also measured on a microplate reader (Synergy H1 Hybrid Reader, Biotek) at 412 nm.

Statistical Analyses

Repeated-measures ANOVA, two-way ANOVA, and both paired and unpaired Ttests were used to analyze these data. Values are represented as the mean ± SEM unless stated otherwise.

Chapter IV

Results

Determination of Cellular Properties:

Similar Proliferation and Differentiation Patterns in HSkMCs from Lean and Severely Obese Individuals

Measures of proliferation were carried out in two ways. Using the MTT assay, we found no differences in myoblast cell count between and lean and severely obese groups. With respect to time, we found that myoblast cell count in both groups was significantly increased at 48 (p < 0.001) and 72 hours (p < 0.001) compared to the 24-hour time point (Figure 1A). Using the Beckman Coultre Vi-Cell to obtain total myoblast cell number, we found no differences in total myoblast cell number between lean and severely obese groups. With respect to time, we found that total cell number in both groups was significantly increased at 48 (p < 0.001) and 72 hours (p < 0.001) and 72 hours (p < 0.001) (Figure 1B). Percent cell viability in myoblasts was also measured across 24, 48 and 72-hour time points in both lean and severely obese groups at 48 (p = 0.01) and 72 hours (p = 0.02). However, cell viability did not differ between lean and severely obese groups (Figure 1C).

Measures of differentiation were carried out in two ways. At day seven of differentiation, myotubes were stained with antibodies against DAPI and total MYHC for visualization of nuclei and myotubes (representative image shown in Figure 2A). ImageJ software analysis revealed that at day 7 of differentiation, both the fusion index was and myotube area were not different between lean and severely obese groups (Figure 2B, 2C).

At the protein level, we found that at days 2, 5 and 7 of differentiation, MyoD, nor total MYHC, were different between lean and severely obese groups (Figures 3A, 3B). We also observed no changes in MyoD content in either group over time. However, total MYHC protein content was significantly increased in both groups at day 5 of differentiation (p = 0.01) in comparison to day 2 (Figure 3B). Total MYHC protein content at day 7 also tended to be higher in both groups compared to day 2, however, this did not reach statistical significance (p = 0.06).

Insulin Incubation Study:

Comparable Changes in Insulin, Glucose & Lactate from Culture Media

As anticipated, we were not able to detect substantial amounts of insulin from culture media obtained in CTRL(-) (non-insulin) conditions across time points (i.e., . Thus, our results/data analyses for insulin obtained from culture media consider only the insulin-stimulated states between lean and severely obese groups. We found that insulin obtained from culture media under insulin-stimulated conditions was not different between lean and severely obese groups. We also found that insulin significantly decreased in both groups at 3 hours (p < 0.001), 8 hours (p < 0.001), 16 hours (p < 0.001) and 24 hours (p < 0.001) compared to the 1-hour time point (Figure 4A).

There were no differences in glucose obtained from culture media with insulin treatment between the lean and severely obese groups. Glucose levels obtained from

culture media were significantly decreased over time in both lean and severely obese groups (p < 0.001) (Figure 4B, 4C). Also, glucose levels were significantly decreased at 8 hours (p = 0.05), 16 hours (p < 0.001) and 24 hours (p < 0.001) compared to the 1-hour time point.

We also found that insulin significantly increased lactate levels in culture media from both lean and severely obese groups (p < 0.001). Lactate levels also increased significantly with time (p < 0.001) (Figure 4D, 4E). Furthermore, lactate levels were significantly higher at 16 hours (p = 0.02) and 24 hours (p = 0.03) compared to the 1-hour time point in both lean and severely obese groups (p = 0.02). Still, lactate obtained from culture media was not different between lean and severely obese groups with insulin stimulation.

Insulin Stimulation of AKT Phosphorylation

Across time points, there were no differences in protein content in CTRL(-) conditions between lean and severely obese groups for AKT phosphorylation. At the 1, 8, 16 and 24-hour time points, we found that insulin significantly increased AKT phosphorylation (Ser⁴⁷³) in both lean and severely obese groups (p = 0.02, 0.03, 0.01 and 0.02, respectively) (Figure 5). At the 1-hour time point, however, AKT phosphorylation with insulin stimulation was 51% lower in the obese group compared to lean (p = 0.05).

Insulin Upregulates Myosin Heavy Chain Content (slow form)

Across time points, there were no differences in protein content in CTRL(-) conditions between lean and severely obese groups for the protein content MYHC (slow isoform). Surprisingly, at the 1, 8, 16 and 24-hour time points, insulin significantly increased MYHC (slow isoform) protein content in both lean and severely obese groups (p = 0.03, 0.01, 0.002 and 0.05, respectively) (Figure 6).

PGC-1a & Mitochondrial Content (OXPHOS Proteins) are Unchanged with High-Insulin Treatment

Across time points, there were no differences in protein content in CTRL(-) conditions between lean and severely obese groups for PGC-1α protein content. We also found no changes in PGC-1α protein content with insulin treatment in either lean or obese groups (Figure 7). Across time points, there were no differences in protein content in CTRL(-) conditions between lean and severely obese groups for Complex V (ATP Synthase). At the 16-hour time point, insulin significantly decreased Complex V protein content (Figure 8). Across time points, there were no differences in protein content in CTRL(-) conditions between lean and severely obese groups for Complex III protein content. Additionally, we found no changes in Complex III protein content with insulin treatment in either the lean or obese groups (Figure 9). Across time points, there were no differences in protein content in CTRL(-) conditions between lean and severely obese groups for Complex IV protein content. Additionally, we found no changes in Complex III protein content with insulin treatment in either the lean or obese groups (Figure 9). Across time points, there were no differences in protein content in CTRL(-) conditions between lean and severely obese groups for Complex IV protein content. At the 1-hour time point, there were trends for increased Complex IV protein content with insulin treatment in both lean and severely obese groups though this measure did not reach

statistical significance (p = 0.07) (Figure 10). At the 3-hour time point, however, insulin significantly increased Complex IV protein content in both lean and severely obese groups (p = 0.02). Furthermore, we found that at the 8-hour time point, Complex IV protein content was 39% higher in the obese group compared to lean (p = 0.02). Complexes I and II were probed in these experiments, but were not consistently detected in our samples. Therefore, they were excluded from these analyses.

Downward Shifts in Nucleotides ATP, ADP & AMP with High-Insulin

For these experiments, only the 24 hour time point was examined. In myotubes derived from lean individuals, there was no effect of 24 hours of insulin on the levels of ATP, ADP or AMP (Figure 11A, 11B, 11C). In myotubes derived from obese individuals, we found that ADP levels decreased 78% from CTRL(-) with 24 hours of insulin treatment (p = 0.04) (Figure 11B). Also in the obese group, AMP levels decreased 83% from CTRL(-) with 24 hours of insulin treatment (p = 0.04) (Figure 11C). In CTRL(-) conditions, ADP levels were 38% higher in obese compared to lean (p = 0.04). We also observed no differences in the ratios of ADP:ATP (Figure 11D) or AMP:ATP (Figure 11E) with insulin treatment within or between lean and severely obese groups. We were also unable to detect any IMP levels from these measures.

High-Insulin may Stimulate Changes in Citrate Synthase Activity

For these experiments, insulin stimulation was conducted in a sub-sample of individuals from the experiments mentioned above for 3 hours and repeated at a 24hour time point. At the 3-hour time point, there were no effects of insulin on CS activity in either lean or obese groups (Figure 12A). There were also no differences between groups in CS activity in CTRL(-) conditions. In myotubes obtained from obese individuals, CS activity was 21% higher with 24-hours insulin treatment (p = 0.05) (Figure 12B).

Chapter V

Discussion

In this study, skeletal muscle myotubes derived from lean and severely obese humans were challenged with 100nM insulin over the course of 24 hours and examined for mitochondrial biogenesis. Before conducting these experiments, we classified the cellular properties of these human skeletal muscle cells (HSkMCs) and to our knowledge, we are the first to report these data as it relates to lean and severely obese individuals.

In regards to myoblast proliferation, our data suggests that lean and severely obese individuals exhibit similar patterns of growth up through 72 hours, with the most growth occurring between the first 24-48 hours (Figures 1A, 1B). This is consistent with data obtained from time-lapse image analyses in recent years [54]. At present, when utilizing this HSkMC model, we begin the differentiation process once myoblasts are 80-90% confluent, which typically occurs between 48 & 72 hours at densities ranging from 30,000 - 50,000 cells [46]. For this study, we chose a seeding density of ~40,000 cells per well in 6-well plates. Our proliferation data suggest that at these densities, beginning the differentiation process at either 48 or 72-hour time points is methodologically valid.

In regards to myotube differentiation, which is of increasing importance due to evidence suggesting that myogenic potential of skeletal muscle is impaired with obesity [55-58], we find a story similar to that of proliferation. In the present study, at day 7 of differentiation, immunofluorescent analyses revealed that myotubes from lean and severely obese individuals exhibit similar morphology as indicated by total nuclei and total MYHC content (Figure 2A). The fusion index has been used to investigate similar questions regarding the formation and/or alteration of skeletal muscle *in vitro* [59]. However, this index has not been extensively used to examine differences as it relates to obesity-related issues, as findings from one study indicate no differences between healthy/lean and severely obese groups [60]. Similarly, our protein data (Figures 3A, 3B) are aligned with our imaging data suggesting that myotubes from lean and severely obese individuals express similar levels of myogenic proteins over a differentiation period of 7 days. In our HSkMC experiments, we terminate the differentiation process and begin experimentation between days 5 and 7. These data would suggest that doing so is appropriate as myotubes from lean and severely obese individuals demonstrate similar differentiation patterns/muscle protein levels at this time.

There are other known markers that contribute to skeletal muscle proliferation and differentiation [61, 62, 63]. MyoD is suggested to be the most significant transcriptional regulator of muscle differentiation [64] with its largest role in fast/glycolytic fibers [65]. Downstream of MyoD is myogenin, which is considered a late marker of myogenesis. Myf5 has been shown to greatly contribute to the development of skeletal muscle as well [66, 67]. In addition, Myf5 is thought to work in conjunction with MyoD and/or other myogenic factors to regulate muscle differentiation. The roles of these proteins were substantiated, when a group of researchers found that in a triple knockout mouse model (lacking MyoD, MRF4 and Myogenin), Myf5 alone was inadequate to upregulate skeletal muscle differentiation
in vitro [68]. Our study could have provided even more robust data about muscle regeneration with obesity if Myf5, Myogenin and MRF4 were included. We would anticipate that these proteins, similar to MyoD, would be similar between lean and severely obese individuals and may be reduced in type 2 diabetes (or the combination of severe obesity and type 2 diabetes). We have already observed some dysregulation in the myogenic program *in vitro* as indicated by MyoD (and total MYHC) content in severely obese type 2 diabetic populations (data not shown).

From the findings in the present study, we conclude that human skeletal muscle cells derived from lean and severely obese individuals demonstrate patterns of proliferation and differentiation over time that are nearly identical. This study provides additional evidence to support the use of HSkMCs from these populations to address questions concerning skeletal muscle function, metabolism and/or disease.

The work in this thesis also addressed the question of whether high-insulin treatment would upregulate proteins associated with mitochondrial biogenesis *in vitro*, across time. As a means to be sure this experimental approach kept our system intact, we monitored the changes is insulin as well as glucose and lactate from the extracellular environment (culture/starvation media [DMEM (5mM glucose), 1% BSA, .01% Pen-Strep]). We found that insulin gradually decreased over time in both lean and severely obese groups (Figure 4A). Though not statistically significant, we noted a trend for insulin levels to be lower in the obese groups at the 1 and 3-hour time points. However, it is difficult to speculate why this occurred, which is one of the limitations of our study. The maximal detection limit for this method is 319 µIU/mL and as a result, we diluted our samples when measuring insulin (40x dilution). The

data shown (Figure 4A) accounts for these dilutions, and it is possible these diluted samples may be associated with these discrepancies between groups at the early time points. Still, it is of note that insulin levels are decrease over time (as anticipated) and are maintained at supra-physiological levels at the "chronic," 24-hour time point in both groups (~4,000 μ IU/mL or ~28 nM).

For glucose obtained from culture media, we observed noticeable decreases over time in both lean and severely obese groups (Figures 4B, 4C). Though these changes are not completely reflective of the intracellular environment, these data imply that skeletal muscle myotubes are exceptional at preserving glucose with insulin treatment over time. Lactate obtained from culture media increased with both time and insulin both groups (Figures 4D, 4E), suggesting that the little glucose that was taken up by the myotubes was utilized (and utilization was highest when glucose levels were lowest).

With a lack of current research using similar methodological techniques, it is difficult to compare these data with existing literature. However, these findings demonstrate that the extracellular adaptations to high-insulin in both lean and severely obese groups are comparable.

Following high-insulin exposure in these myotubes derived from lean and severely obese individuals, we found that insulin stimulated phosphorylation of AKT in both lean and severely obese groups (Figure 5). Furthermore, we found that at the earliest (1-hour) time point, AKT phosphorylation (Ser⁴⁷³) in myotubes from the obese group was half (51%) that of AKT phosphorylation in the lean group. This data provide

further evidence to the extensive reviews on the relationship between obesity and insulin resistance [69-72]. Why these group differences are not sustained at later time points in our study is uncertain. However, there is evidence that this prolonged high-insulin exposure reduces AKT phosphorylation at this serine residue and also reduces glucose metabolism in muscle cells [73]. Moreover, the 24-hour time duration of 100nM insulin has been utilized as a means to induce insulin resistance *in vitro* [74]. In our study, 100nM insulin reduced a 3 fold increase in AKT phosphorylation at the first hour to only a ~50% increase at 24 hours (in myotubes from lean individuals). From this perspective, these data are reflective of the idea that these myotubes can be pushed to adopt an insulin resistant phenotype with supra-physiological insulin levels in as little as 24 hours.

The slow isoform of MYHC increased with 100nM insulin in both lean and severely obese groups nearly all time points, including 24 hours (Figure 6). It has been established that MYHC can be transcriptionally regulated [75] and/or post-translationally modified [76] as a result of insulin action. However, our data slightly contrasts what has been reported previously *in* vivo, as 3-hours insulin exposure increased fast MYHC (type IIx) mRNAs while MYHC type I mRNAs were unchanged [42]. These discrepancies may exist due to the fact that the present study was conducted *in vitro*, with a much higher stimulus of insulin. Still, keeping in mind that slow fibers contain more insulin receptors [29], and have been positively associated with insulin sensitivity [77, 78], it is practical that high levels of insulin would upregulate the slow isoform of MYHC in this study. Additionally, research has long established that mammalian target of rapamycin (mTOR), is a crucial regulator of protein synthesis in

skeletal muscle [79, 80, 81]. Though we did not measure mTOR and/or mTOR phosphorylation, the increases in AKT phosphorylation observed in this study would support that the stimulus provided by insulin would be associated with increases in protein synthesis [81, 82, 83] as indicated by increases in the slow form of MYHC.

With respect to intracellular nucleotides, previous literature has shown that ATP synthesis rates are increased with acute high-insulin treatment in skeletal muscle (and that this effect is reduced in an excessive lipid environment) [84, 85]. In contrast, chronic, insulin-stimulated ATP synthesis in skeletal muscle has not been thoroughly investigated. In this study, ATP trended to decrease in myotubes from both lean and severely obese individuals. We also found no differences in CTRL(-) ATP levels, which is consistent with previous research [86]. In addition, the ratios of ADP:ATP and AMP:ATP remained constant with insulin treatment in both groups. The significant decreases in ADP and AMP with insulin treatment that were also observed (in obese groups only), as well as the group differences in CTRL(-) ADP levels are puzzling and could be (re)investigated in larger samples sizes or utilizing other methodologies (i.e., isolated mitochondria) [86, 87]. Still, these data along with the non-detectable levels of IMP (in both groups) suggest a shift in the entire nucleotide pool as well as a balance between ATP synthesis and degradation. Taken together with our previous (MYHC) data, we conclude that 24 hours high-insulin exposure may be associated with decreased nucleotide levels as a result of increased protein synthesis, and that this process occurs in a similar fashion in myotubes from both lean and severely obese individuals.

As mentioned, a recent study examining the effects of insulin on ATP synthesis in skeletal muscle with obesity found no differences between lean and obese groups [86]. Interestingly, these similarities were observed in myotubes from lean and obese groups with comparable mitochondrial mass. These data provide additional evidence to the idea that altered/reduced mitochondrial-specific functions, rather than mitochondrial content alone, are an integral part of the metabolic problems observed with obesity and/or obesity-related disorders (i.e., insulin resistance). This may also explain why we observed no changes in PGC-1 α , or ETC/OXPHOS proteins (Complexes III & V). We do not deny, however, the possibility that gene expression and/or posttranslational modifications of mitochondrial proteins are occurring as these phenomena have been established [16, 17]. Our findings do, however, illustrate the significance of Complex IV, as it was higher and significantly increased with highinsulin treatment at the 1-hour and 3-hour time points, respectively. This effect was witnessed in both lean and severely obese groups, though we did find that this effect was statistically higher in the obese group at the 8-hour time point. Why this difference exists is also unclear. Still, the acute responses in Complex IV content with high-insulin treatment is logical, as OXPHOS genes have been shown to be insulin-sensitive in skeletal muscle (and adipose tissue as well) [88, 89, 90].

It is difficult to interpret our measures of CS activity with high-insulin treatment with such small sample sizes in comparison to other experiments (n = 2 per group vs. n = 5 to 6). If these experiments are repeated, it is likely that insulinstimulated increases in CS activity at 3 hours would be significant in both lean and

severely obese groups, as that has been shown to occur in myotubes/isolated mitochondria [44].

In closing, we conclude from this study that myotubes from lean and severely obese individuals behave similarly with respect to their proliferative behavior, and are morphologically alike at the time of differentiation. These HSkMCs are valuable experimental tools that aided in filling the gap(s) in current research about the hormonal regulation of mitochondrial biogenesis, specifically by insulin. Given the data from the present study, it is suggested that increases in muscle protein, rather than mitochondrial protein, are favored under circumstances in which an overload of anabolic stimuli (supra-physiological insulin levels) are present. We deem that continued use of the HSkMC culture would be beneficial for expanding our knowledge about the relationship between insulin and more functional outcomes relevant to the mitochondria (i.e., lipid oxidation, ATP synthesis rates, oxygen consumption). Such findings would also assist in advancing even further our understanding of how metabolic adaptations in skeletal muscle are disrupted in obese and/or insulinresistant populations.

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Figures











Figure 3. Markers of differentiation in myotubes from lean and severely obese individuals.

(3A) MyoD and (3B) total myosin heavy chain (MYHC) protein content in myotubes at days 2, 5 and 7 of differentiation. Across time points, the protein content was not different between lean and severely obese groups for MyoD or total MYHC. Data are expressed as the mean \pm SEM. #Statistical difference from day 2 at $p \le 0.05$.



4B

Glucose (mg/dL)



Figure 4. Insulin and metabolites from culture media after 100nM insulin exposure. One hour after insulin exposure, (4A) insulin (only insulin-stimulated states are shown), (4B, 4C) glucose and (4D, 4E) lactate were collected from the starvation media in which myotubes were incubating in. This process was repeated at 3, 8, 16 and 24-hour time points and assayed using a chemistry analyzer (Unicel DxC 600i; Beckman Coulter). Across time points, the fold change from CTRL(-) was not different between lean and severely obese groups for insulin, glucose or lactate. Data are expressed as the mean \pm SEM. *Seffect of insulin at* $p \leq 0.05$.



Figure 5. Phosphorylation of AKT(Ser⁴⁷³) after 100nM insulin exposure in myotubes from lean and severely obese individuals. Western Blot quantifications for p-AKT at 1, 3, 8, 16 and 24-hr time points in lean and severely obese groups. Data are expressed as the fold change from CTRL \pm SEM. *SEffect of insulin at* $p \le 0.05$. *Statistical difference from lean at $p \le 0.05$.



Figure 6. MYHC (slow isoform) protein content after 100nM insulin exposure in myotubes from lean and severely obese individuals. Western Blot quantifications for MYHC (slow) at 1, 3, 8, 16 and 24-hr time points in lean and severely obese groups. Across time points, the fold change from CTRL(-) was not different between lean and severely obese groups for MYHC (slow isoform). Data are expressed as the fold change from CTRL \pm SEM. *SEffect of insulin at* $p \le 0.05$.



Figure 7. PGC-1a protein content after 100nM insulin exposure in myotubes from lean and severely obese individuals. Western Blot quantifications for PGC-1a at 1, 3, 8, 16 and 24-hr time points in lean and severely obese groups. Across time points, the fold change from CTRL(-) was not different between lean and severely obese groups for PGC-1a.



Figure 8. Complex V (ATP Synthase) protein content after 100nM insulin exposure in myotubes from lean and severely obese individuals. Western Blot quantifications for Complex V at 1, 3, 8, 16 and 24-hr time points in lean and severely obese groups. Across time points, the fold change from CTRL(-) was not different between lean and severely obese groups for Complex V. Data are expressed as the fold change from CTRL \pm SEM. *SEffect of insulin at p* \leq 0.05.



Figure 9. Complex III protein content after 100nM insulin exposure in myotubes from lean and severely obese individuals. Western Blot quantifications for Complex III at 1, 3, 8, 16 and 24-hr time points in lean and severely obese groups. Across time points, the fold change from CTRL(-) was not different between lean and severely obese groups for Complex III. Data are expressed as the fold change from CTRL ± SEM.



Figure 10. Complex IV protein content after 100nM insulin exposure in myotubes from lean and severely obese individuals. Western Blot quantifications for Complex IV at 1, 3, 8, 16 and 24-hr time points in lean and severely obese groups. Data are expressed as the fold change from CTRL \pm SEM. *SEffect of insulin at* $p \le 0.05$. *Statistical difference from lean at $p \le 0.05$.



Figure 11. Intracellular nucleotides ATP, ADP & AMP after 100nM insulin exposure in myotubes from lean and severely obese individuals. Levels of (A) ATP, (B) ADP, and (C) AMP after 24 hours of 100nM insulin exposure in lean and severely obese groups. Ratios of (D) ADP:ATP and (E) AMP:ATP after 100nM insulin exposure in lean and severely obese groups. Data are expressed as the mean \pm SEM. *Seffect of insulin at* $p \le 0.05$. *Statistical difference from lean at $p \le 0.05$.



Figure 12. Citrate Synthase activity after 100nM insulin exposure in myotubes from lean and severely obese individuals. CS activity after 100 nM insulin exposure at (A) 3 and (B) 24-hour time points in lean and severely obese groups. Data are expressed as the mean \pm SEM. **§**Effect of insulin at $p \le 0.05$. **Table 1. Participant Characteristics.** Participant characteristics are outlined for the participants of these studies. Data are expressed as the mean \pm standard deviation. **Statistical difference from lean at* $p \le 0.05$.

		r
Participant Characteristics	Lean (n = 6)	Obese (n = 6)
Age (yrs.)	32.0 ± 7.2	35.6 ± 7.1
Ethnicity (AA/Caucasian)	2/4	2/4
Height (in.)	66.3 ± 2.5	65.1 ± 1.7
Weight (lbs.)	155.1 ± 19.7	268.5 ± 57.3 *
BMI (kg/m²)	25.0 ± 3.9	44.5 ± 8.1*
Fasting Glucose (mg/dL)	90.8 ± 6.7	92.2 ± 7.6
Insulin (µIU/mL)	9.6 ± 5.1	14.9 ± 6.2
HOMA-IR	2.1 ± 1.2	3.3 ± 1.3

Supplemental Figure 1. Representative western blots. Western blots are shown for various proteins examined in both starved (-) and insulin-treated (+) conditions at 1, 3, 8, 16 or 24 hour time points (from days 6 to 7 of differentiation). Proteins were normalized to B-actin (and p-AKT(Ser⁴⁷³) normalized to total AKT).



























LEAN







Supplemental Figure 2. Quantifications of Normalization Proteins. Histograms are shown for (S2-A) total AKT and (S2-B) B-actin in both starved (-) and insulin-treated (+) conditions at 1, 3, 8, 16 or 24 hour time points. Across time points, the fold change from CTRL(-) was not different between lean and severely obese groups for AKT B-actin. There were also no effects of time for total AKT or B-actin.





Appendix A. IRB Letters



EAST CAROLINA UNIVERSITY University & Medical Center Institutional Review Board Office 4N-70 Brody Medical Sciences Building Mail Stop 682 600 Moye Boulevard · Greenville, NC 27834 Office 252-744-2914 · Fax 252-744-2284 · www.ecu.edu/irb

Notification of Continuing Review Approval

From:	Biomedical IRB
То:	Joseph Houmard
CC:	
	Gabriel Dubis
Date:	10/29/2015
Re:	CR00003321
	UMCIRB 06-0080
	Lipid Metabolism in Obesity, Weight Loss and Exercise (2): Muscle Cell Studies

I am pleased to inform you that at the convened meeting on 10/28/2015 of the Biomedical IRB, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 10/28/2015 to 10/27/2016.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Document 06-0080 HIPAA Authorization.pdf(0.01) Acute Exercise Training(0.05) Advertisments(0.04) Authorization-for-Future-Effect of Weight Loss.High-Fat Diet(0.02)

Brief Protocol(0.02)

Diabetic Ad 06-0080.docx(0.01) ECU-Designated-Health-Care-Components-April-2014.pdf(0.01) Effect of Exercise Training(0.02) Effect of Exercise Training.High Fat Diet(0.02)

rate.ecu.edu/app/Doc/0/EC46CFD1FGT45B8UNDUCQ71A6B/fromString.html

Description HIPAA Authorization Consent Forms Recruitment Documents/Scripts HIPAA Authorization Study Protocol or Grant Application Recruitment Documents/Scripts HIPAA Authorization HIPAA Authorization HIPAA Authorization

Effect of Weight Loss.High Fat Diet(0.03)	HIPAA Authorization
Full Protocol(0.02)	Study Protocol or Grant
	Application
Full Protocol Version 4 Track Changes.docx(0.01)	Study Protocol or Grant
Handessen - Konzel-Galandes aufstellt werkenden. Im Lindenservers der konzellt – Bakenden werkend zu Konzellerung	Application
Full Protocol Version 4.docx(0.01)	Application
Genetics Consent(0.03)	Consent Forms
HIPAA-Authorization-Effect of Acute Exercise Training $(0, 02)$	HIPAA Authorization
HIPAA-Authorization-for-Future-High Fat Diet Study(0.03)	HIPAA Authorization
HIPAA-Authorization-for-Future-Research Genetics Database(0.02)	HIPAA Authorization
HIPAA-Authorization-for-Future-Research-Effect of Acute Exercise Training(0.02)	HIPAA Authorization
HIPAA-Authorization-for-Future-Research-Effect of Exercise Training(0.02)	HIPAA Authorization
HIPAA-Authorization-for-Future-Research-Effects of Exercise Training.High Fat	HIDAA Authorization
Diet(0.02)	HIPAA Authonization
HIPAA-Authorization-for-Future-Research-Muscle Cell Studies(0.01)	HIPAA Authorization
HIPAA-Authorization-Genetics Database(0.02)	HIPAA Authorization
HIPAA-Authorization-High Fat Diet Study(0.03)	HIPAA Authorization
HIPAA-Authorization-Muscle Cell Studies(0.01)	HIPAA Authorization
Lipid metabolism Ad Women 18-45.docx(0.01)	Recruitment Documents/Scripts
Lipid metabolism Ad Women 28-44.docx(0.02)	Recruitment Documents/Scripts
Medical History(0.01)	Data Collection Sheet
Version 5 exercise training.doc(0.03)	Consent Forms
Version 5 cell culture studies.doc(0.02)	Consent Forms
Version 5 cell culture studies.doc- tracked changes(0.01)	Consent Forms
Version 5 exercise training.doc- tracked changes(0.01)	Consent Forms
Version 5 HFD and exercise.doc(0.02)	Consent Forms
Version 5 HFD and exercise.doc- tracked changes(0.01)	Consent Forms
Version 5 HFD and weight loss.doc(0.02)	Consent Forms
Version 5 HFD and weight loss.doc- tracked changes(0.01)	Consent Forms
Version 5 HFD.doc(U.02)	Consent Forms
Version 5 HFD.doc- tracked changes(0.01)	Consent Forms

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:

R. Hickner & M. Pories

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting:

None

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IRB00000705 East Carolina U IRB #1 (Biomedical) IORG0000418 IRB00003781 East Carolina U IRB #2 (Behavioral/SS) IORG0000418


EAST CAROLINA UNIVERSITY University & Medical Center Institutional Review Board Office 4N-70 Brody Medical Sciences Building Mail Stop 682 600 Moye Boulevard · Greenville, NC 27834 Office 252-744-2914 @ · Fax 252-744-2284 @ · www.ecu.edu/irb

Notification of Amendment Approval

From: Biomedical IRB To: Joseph Houmard CC: Gabriel Dubis Date: 4/25/2016 Re: Ame16 UMCIRB 06-0080 UMCIRB 06-0080 Lipid Metabolism in Obesity, Weight Loss and Exercise (2): Muscle Cell Studies

Your Amendment has been reviewed and approved using expedited review for the period of 4/23/2016 to 10/27/2016. It was the determination of the UMCIRB Chairperson (or designee) that this revision does not impact the overall risk/benefit ratio of the study and is appropriate for the population and procedures proposed.

Please note that any further changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. A continuing or final review must be submitted to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Document KineWeightLossAd.pdf(0.01) Weight Loss Study Flyer 06-0080(0.01) Description Recruitment Documents/Scripts Recruitment Documents/Scripts

The Chairperson (or designee) does not have a potential for conflict of interest on this study.