Functional and Proteome Differences in Skeletal Muscle Mitochondria Between Lean and Obese Humans

by

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ABSTRACT

Skeletal muscle (SM) mitochondria generate the majority of adenosine triphosphate (ATP) in SM, and help regulate whole-body energy expenditure. Obesity is associated with alterations in SM mitochondria, which are unique with respect to their arrangement within cells; some mitochondria are located directly beneath the sarcolemma (i.e., subsarcolemmal (SS) mitochondria), while other are nested between the myofibrils (i.e., intermyofibrillar (IMF) mitochondria). Functional and proteome differences specific to SS versus IMF mitochondria in obese individuals may contribute to reduced capacity for muscle ATP production seen in obesity. The overall goals of this work were to (1) isolate functional muscle SS and IMF mitochondria from lean and obese individuals, (2) assess enzyme activities associated with the electron transport chain and ATP production, (3) determine if elevated plasma amino acids enhance SS and IMF mitochondrial respiration and ATP production rates in SM of obese humans, and (4) determine differences in mitochondrial proteome regulating energy metabolism and key biological processes associated with SS and IMF mitochondria between lean and obese humans.

Polarography was used to determine functional differences in isolated SS and IMF mitochondria between lean (37 ± 3 yrs; n = 10) and obese (35 ± 3 yrs; n = 11) subjects during either saline (control) or amino acid (AA) infusions. AA infusion increased ADP-stimulated respiration (i.e., coupled respiration), non-ADP stimulated respiration (i.e., uncoupled respiration), and ATP production rates in SS, but not IMF mitochondria in lean (n = 10; $P < 0.05$). Neither infusion increased any of the above parameters in muscle SS or IMF mitochondria of the obese subjects.

Using label free quantitative mass spectrometry, we determined differences in proteomes of SM SS and IMF mitochondria between lean (33 ± 3 yrs; n = 16) and obese (32 ± 3 yrs; n = 17) subjects. Differentially-expressed mitochondrial proteins in SS versus IMF mitochondria of obese subjects were associated with biological processes that regulate: electron transport chain ($P<0.0001$), citric acid cycle ($P<0.0001$), oxidative phosphorylation ($P<0.001$), branched-chain amino acid degradation, ($P<0.0001$), and fatty acid degradation ($P<0.001$). Overall, these findings show that obesity is associated with redistribution of key biological processes within the mitochondrial reticulum responsible for regulating energy metabolism in human skeletal muscle.

DEDICATION

I am grateful for the support of family and friends who helped me throughout this process. To my incredible parents John and Evelyn, raising me to always try my best and being the amazing role models of, unconditional love, hard work and commitment. To my loving sister Cindy, always providing motivation to keep on keeping on. Thank you Michella, for everything. I know this journey would not have been possible without you. You truly are an amazing wife, mother, my best friend, and I love you. My two children, Kenzie and Logan who were babies when I started, have grown with me through this process.

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CHAPTER 1: INTRODUCTION

Obesity results from an energy surplus and is characterized by an accumulation of excess body fat. Incidence of obesity has increased in the United States where the age-adjusted prevalence of obesity in 2013-2014 was 35.0% among men and 40.4% among women (Flegal, Kruszon-Moran, Carroll, Fryar, & Ogden, 2016). A key biological process associated with obesity is a reduced capacity of ATP production in skeletal muscle (Abdul-Ghani et al., 2009). The reduced capacity of ATP production in skeletal muscle mitochondria from obese has been associated with the term mitochondrial dysfunction (Hojlund et al., 2010; Hwang et al., 2010; Karakelides et al., 2010; Koves et al., 2008; Menshikova et al., 2005; Minet & Gaster, 2010; Ritov et al., 2005; Toledo et al., 2008). In turn, defining mitochondrial dysfunction has been difficult. Conflicting reports suggest skeletal muscle mitochondria from obese individuals have a reduced ability to oxidize fatty acids (Koves et al., 2008; Razak & Anand, 2004). Consequently this has been suggested to lead to lipid accumulation within skeletal muscle (Adams et al., 2004; Boyle, Zheng, Anderson, Neufer, & Houmard, 2012; Koves et al., 2008). Alternatively, skeletal muscle mitochondria from obese individuals have been reported as comparable to lean controls (Fisher-Wellman et al., 2014; Holloway et al., 2007). Normally, skeletal muscle mitochondria generate more than 90% of the available adenosine triphosphate (ATP) in skeletal muscle, which helps to regulate whole body energy metabolism. In addition, skeletal muscle, one of the primary tissues for regulating whole-body energy metabolism, contains one of the largest collections of proteins in the body (Chevalier, Marliss, Morais, Lamarche, & Gougeon, 2005; Wolfe, 2006).

Maintenance of the skeletal muscle mitochondrial protein pool is important to the overall function of myocytes and muscle metabolic health.

Healthy mitochondrial proteins support the maintenance of energy metabolism through the generation of ATP. Previous studies have shown differences in skeletal muscle mitochondria from obese individuals with respect to size and abundance (Kelley, He, Menshikova, & Ritov, 2002; Ritov et al., 2005). Consequently, the reduced size and abundance of mitochondria from muscle of obese individuals, might predict a reduced capacity, along with lower rates, of ATP production in obesity (Abdul-Ghani et al., 2009; Chanseaume et al., 2010). In turn, lower rates of ATP production in skeletal muscle of obese individuals may lead to early fatigability during exercise resulting in reduced levels of activity (Cooper, Page, Fox, & Misson, 2000; Ravussin et al., 1988). Lower activity in obese individuals leads to a reduced daily expenditure of energy and ultimately an energy surplus (Ravussin et al., 1988) when caloric intake is maintained.

Expected medical care costs associated with obesity treatment are rising annually (Finkelstein, Trogdon, Cohen, & Dietz, 2009; Jacob, 2015) Additionally, risks of diabetes, heart disease, and cancer are linked to obesity (Hossain et al., 2016; Ritov et al., 2005; Sverdlov et al., 2014). The financial burden on the healthcare system to treat obesity continues to rise as access to food increases in parallel with the adoption of a more sedentary lifestyle. Furthermore, there is no evidence of a decline in the prevalence of obesity in any age groups, despite substantial efforts to target the issue (Skinner, Perrin, & Skelton, 2016).

Practical treatments for obesity include nutritional and exercise interventions. Lifestyle interventions to treat obesity such as nutrition, specifically the increased intake

of protein/amino acids, and exercise have been shown to effect skeletal muscle mitochondrial function (Guillet et al., 2009; Tatpati et al., 2010). Furthermore, increased availability of amino acids has also been shown to promote protein synthesis in skeletal muscle at the total (Bohé, Low, Wolfe, & Rennie, 2003; Guillet et al., 2009; Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe, 2005, 2006; Volpi, Kobayashi, Sheffield-Moore, Mittendorfer, & Wolfe, 2003) as well as mitochondrial protein levels (Guillet et al., 2009; Tatpati et al., 2010), which results in higher skeletal muscle mitochondrial ATP production (Tatpati et al., 2010).

A growing body of evidence has revealed that skeletal muscle mitochondria are arranged in a reticulum within the muscle fiber (Amchenkova, Bakeeva, Chentsov, Skulachev, & Zorov, 1988; Bakeeva, Chentsov YuS, & Skulachev, 1978; Dirksen, 2009; Glancy et al., 2015, 2017; Kirkwood, Munn, & Brooks, 1986; Ogata & Yamasaki, 1997; Patel, Glancy, & Balaban, 2016). This unique arrangement has been suggested to facilitate the transmission of energy from the periphery of the muscle fiber to the interior regions (Amchenkova et al., 1988; Glancy et al., 2015, 2017; Skulachev, 2001). Cellular mitochondrial networks permit the sharing of metabolites, proteins as well as mitochondrial deoxyribose nucleic acid (DNA) (Bach et al., 2003; Elachouri et al., 2011). The mitochondrial reticulum also provides the architecture to facilitate the rapid conductive path for the distribution of potential energy (Glancy et al., 2015). This conductive pathway is the major mechanism (Glancy et al., 2015; Patel, Glancy, & Balaban, 2016) in skeletal muscle under normal conditions, and the facilitated diffusion of metabolites becomes significant as maximum performance levels are approached (Glancy et al., 2015). Therefore, it is possible that proteins and metabolites associated

with energy metabolism in skeletal muscle during obesity is disrupted, reorganized or redistributed in such a way as to match the energy demands of the myocyte mitochondrial reticulum network.

The biochemically distinct subpopulations of skeletal muscle mitochondria localize in the periphery of the cell directly beneath the sarcolemma, historically termed subsarcolemmal (SS) mitochondria or are nested in between the myofibrils, intermyofibrillar (IMF) mitochondria (Palmer, Tandler, & Hoppel, 1977). Palmer et al. were one of the first to demonstrate that these two populations of mitochondria could be independently isolated from muscle fibers (Palmer et al., 1977; Palmer, Tandler, & Hoppel, 1985). However, the isolation of IMF mitochondria from skeletal muscle requires the addition of protease to liberate the mitochondria tethered to myofibrillar elements (Idell-Wenger, Grotyohann, & Neely, 1982; Palmer, Tandler, & Hoppel, 1977; Thakar & Ashmore, 1975).

Using the protease Nagarse to isolate mitochondria significantly improves indices of functional integrity and specific activities of isolated mitochondria from skeletal muscle (Kras et al., 2016). Studies from chapter 3 and 4 emphasize the differences in SS and IMF mitochondria between lean and obese; due to this confounding factor when isolating SS and IMF mitochondria from muscle from lean and obese individuals, the mitochondrial subpopulations are compared independent of one another in those studies. However, the ability to independently study distinct regional fractions of skeletal muscle mitochondrial reticulum, i.e. SS and IMF mitochondria, may provide insight into the proteome, function and mechanisms, which contribute to changes in energy metabolism of skeletal muscle mitochondria in obese.

Previously reported differences in skeletal muscle mitochondrial ATP production observed in muscle from obese (Abdul-Ghani et al., 2009) could be specific to the regions of biochemically distinct skeletal muscle mitochondria studied. Furthermore the SS mitochondria are more responsive to metabolic changes, compared to IMF mitochondria (Bizeau, Willis, & Hazel, 1998; Chomentowski, Coen, Radiková, Goodpaster, & Toledo, 2011; Cogswell, Stevens, & Hood, 1993; Crescenzo et al., 2006; Koves et al., 2005). Consequently, metabolic changes associated with obesity may particularly affect the SS mitochondria.

There are reported differences in the proteome of skeletal muscle mitochondria from obese individuals (Hojlund et al., 2010; Højlund et al., 2003; Hwang et al., 2010; Lefort et al., 2010), which may contribute to a reduced capacity of ATP production in muscle from obese (Abdul-Ghani et al., 2009). Earlier studies have observed significant increases in the abundance of adenylate kinase (KAD2), an enzyme which catalyzes the near equilibrium interconversion of two molecules of adenosine diphosphate (ADP) to an ATP and an adenosine monophosphate (AMP); a key enzyme in maintenance of intracellular adenine nucleotide ratios (Hittel, Hathout, Hoffman, & Houmard, 2005). In this same study, they concluded that reduced mitochondrial content and function with increasing obesity, would lower the total cellular ATP yield. This in turn would necessitate the need to increase the abundance of adenylate kinase and other glycolytic enzymes; helping to maintain intracellular free energy of ATP (Hittel et al., 2005). The reorganization of muscle enzymes during specific physiologic conditions, (i.e. exercise and fasted states) is well established in the literature (Bizeau et al., 1998; Leek, Mudaliar,

Henry, Mathieu-Costello, & Richardson, 2001; Saleem & Hood, 2013; Smith et al., 2013; Wibom et al., 1992).

Differences in the skeletal muscle mitochondrial proteome of obese have also revealed changes in the enzymes responsible for the catalysis of ADP phosphorylation, specifically the beta-F1-ATPase subunit. This critical enzyme is reported as lower in muscle from obese individuals (Tran et al., 2016). Furthermore, previous groups have shown that beta-F1-ATPase is altered in obesity (Hojlund et al., 2010; Hwang et al., 2010). The changes in skeletal muscle in obesity are also associated with differences in rates of protein synthesis (Guillet et al., 2009; Tran et al., 2016). The impairments of protein synthesis and mitochondrial ATP production observed in muscle from obese individuals could be specific to the population of skeletal muscle mitochondria studied. As a result, SS mitochondria in the periphery of the myocyte may be particularly tailored for the generation of the proton-motive force (PMF), while IMF mitochondria, proximal to myofibrils, effectively utilizes the PMF to generate ATP. This concept agrees with the model suggested by Skulachev (Bakeeva et al., 1978) and recently supported by Glancy et al. (Glancy et al., 2015). Others have also demonstrated that obese subjects show blunted insulin stimulated mitochondrial protein turnover and breakdown compared to lean controls (Guillet et al., 2009), further supporting the idea of a disruption in the mitochondrial protein turnover, and possibly abundance, within skeletal muscle of obese individuals. The exact mechanisms responsible for the reduced capacity of ATP production in response to increased availability of energy such as amino acids, in skeletal muscle from obese individuals are still unknown.

Therefore the goal of this dissertation was to: (1) better understand differences in the function of isolated skeletal muscle mitochondria; (2) determine the effects in mitochondrial function in response to the stimulatory effect of plasma amino acids; (3) determine differences in the proteomes of muscle mitochondria from lean versus obese humans; and (4) identify key differences with respect to biological processes associated with the regulation of energy metabolism in both subsarcolemmal and intermyofibrillar mitochondria between healthy lean and healthy obese individuals. The work included herein involves the functional assessment of isolated SS and IMF mitochondria from skeletal muscle between healthy lean and apparently healthy obese individuals, and the underlying mechanisms responsible for the reduced capacity of skeletal muscle mitochondrial ATP production from obese individuals. Therefore, to better understand the differences in the function and proteome of SS and IMF mitochondria in skeletal muscle of obese individuals, I have investigated the following: (1) total and specific activity of enzymes associated with regulating energy metabolism; (2) the capacity of ATP production under basal conditions and during a stimulus such as amino acids; and (3) differences in the proteomes of SS and IMF mitochondria between lean and obese humans. Assessment of SS and IMF mitochondrial function was carried out by oxygen consumption assays using oxygen electrode polarography, while identification and quantification of differentially expressed proteins in SS and IMF mitochondrial assigned proteins was carried out using label free quantitative mass spectrometry and total spectra counts between relatively healthy lean and obese participants.

Overall, the work in this dissertation revealed the following with respect to differences in SS and IMF mitochondria between lean and obese individuals: as expected,

the function of SS and IMF mitochondria were not different between lean and obese at basal when subjects were matched for age and physical activity; our results further described that SS (but not IMF) mitochondria were stimulated by amino acids and surprisingly, enhanced uncoupled respiration while sustaining increased capacity for ATP production from lean subjects only; none of these effects of plasma amino acids were observed in IMF mitochondria of either lean or obese subjects. In alignment with expectations, quantitative proteomics revealed that the total abundance of mitochondrial assigned proteins in both SS and IMF mitochondria were reduced in obese individuals, but unexpectedly, the reduced abundance of total spectra in SS mitochondria from obese individuals could not be attributed to mitochondrial proteins assigned to the citric acid cycle, electron transport chain or ATP synthase. Further investigation revealed that the reduced abundance of spectra in SS mitochondria of obese was primarily the result of a lower total abundance of spectra assigned to proteins involved in the metabolism of proteins. Unlike SS mitochondria, the abundance of mitochondrial proteins assigned to electron transport chain and ATP synthase were reduced, while proteins from the citric acid cycle and succinate-coenzyme Q reductase (i.e. complex II) were increased in IMF mitochondria of obese subjects. Collectively, this dissertation provides novel findings in obesity with respect to the function and distribution of protein composition in myocytes related to regulating energy metabolism in obese humans.

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CHAPTER 2: SUBSARCOLEMMAL MITOCHONDRIA ISOLATED WITH THE PROTEOLYTIC ENZYME NAGARSE EXHIBIT GREATER PROTEIN SPECIFIC ACTIVITIES AND FUNCTIONAL COUPLING

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Abstract

Skeletal muscle mitochondria are arranged as a reticulum. Insight into the functional characteristics of such structure is achieved by viewing the network as consisting of "subsarcolemmal" (SS) and "inter-myofibrillar" (IMF) regions. During the decades, most, but not all, published studies have reported higher (sometimes over 2-fold) enzyme and enzyme-pathway protein-specific activities in IMF compared to SS mitochondria. We tested the hypothesis that non-mitochondrial protein contamination might account for much of the apparently lower specific activities of isolated SS mitochondria. Mouse gastrocnemii (n=6) were suspended in isolation medium, minced, and homogenized according to procedures typically used to isolate SS mitochondria. However, the supernatant fraction, collected after the first slow-speed (800g) centrifugation, was divided equally: one sample was exposed to nagarse (MITO+), while the other was not (MITO-). Nagarse treatment reduced total protein yield by 25%, while it increased protein-specific respiration rates (nmol O_2 min^{-1} mg^{-1}), by 38% under "resting" (state 4) and by 84% under maximal (state 3) conditions. Nagarse therefore increased the respiratory control ratio (state 3/state 4) by 30%. In addition, the ADP/O ratio was increased by 9% and the activity of citrate synthase (U/mg) was 49% higher.

Mass spectrometry analysis indicated that the MITO+ preparation contained less contamination from non-mitochondrial proteins. We conclude that nagarse treatment of SS mitochondria removes not only non-mitochondrial proteins but also the protein of damaged mitochondria, improves indices of functional integrity, and the resulting protein-specific activities.

Introduction

Mitochondrial oxidative phosphorylation fulfills two critical metabolic objectives: 1) synthesizing ATP at the rate demanded by cellular ATP-utilizing processes and 2) maintaining a robust cellular energy status (low cytosolic [ADP] and high ATP/ADP ratio). Almost 40 years ago Skulachev's laboratory provided evidence that mitochondria in mammalian striated muscle are structured as a continuous network or reticulum (Bakeeva, Chentsov YuS, & Skulachev, 1978). Recently, Glancy et al. (Glancy et al., 2015) provided compelling corroborative evidence for this model. Further, their report supports one of the fundamental hypotheses advanced by Skulachev over 40 years ago (Bakeeva et al., 1978), that the reticular arrangement provides the ability to rapidly transfer intracellular energy by propagating the proton-motive-force (Δp) from one region of the network to another (Amchenkova, Bakeeva, Chentsov, Skulachev, & Zorov, 1988; Bakeeva et al., 1978; Skulachev, 2001). According to this concept, the fraction of the network located near the plasma membrane ("subsarcolemmal" or SS mitochondria) contains protein stoichiometry particularly suited for Δp development, while the region deeper within the myocyte, the intermyofibrillar (IMF) fraction, is particularly tailored for transducing Δp into ATP synthesis and export. Reticular structure therefore facilitates meeting the demands for both rapid ATP turnover and the defense of cellular energetic

status. These older and more recent findings bracket decades of research supporting the mitochondrial reticulum concept. About the time Skulachev's paper demonstrated the mitochondrial reticulum in rat diaphragm (Bakeeva et al., 1978), Palmer et al. described procedures for independently isolating SS and IMF mitochondrial populations from rat heart (Palmer, Tandler, & Hoppel, 1977). Briefly, the tissue was minced, suspended in buffer, mechanically disrupted (liberating SS mitochondria) and centrifuged at slow speed, leaving SS mitochondria in the supernatant, while the IMF mitochondria were pelleted with the myofibrils. This first supernatant was then used to isolate SS mitochondria with high-speed spins. In the parallel IMF isolation procedure, the myofibrillar pellet was resuspended and incubated with the proteolytic enzyme nagarse to digest myofibrillar proteins and liberate the IMF mitochondria. Next, a second slow spin would yield a supernatant containing IMF mitochondria. The IMF supernatant was then centrifuged at high speed to pellet and wash the IMF mitochondria. These careful and detailed studies by Palmer et al. (Palmer et al., 1977; Palmer, Tandler, & Hoppel, 1985) provided convincing electron microscopic evidence that SS and IMF fractions were independently isolated, and their procedures became the generally accepted methodology upon which subsequent work was based. However, a curious pattern reported by Palmer et al., and many studies that followed (Bizeau, Willis, & Hazel, 1998; Cogswell, Stevens, & Hood, 1993; Palmer et al., 1977, 1985; Schwerzmann, Hoppeler, Kayar, & Weibel, 1989; Takahashi & Hood, 1993), was that essentially all activities of individual enzymes and/or oxidative enzyme pathways were uniformly higher in the IMF fraction compared to the SS, in some cases over 2-fold higher. This consistent finding raises obvious questions: If SS and IMF mitochondria are simply part of a continuous network, then

how could all protein-specific activities be higher in IMF? Another, related, question is: where and by what mechanism would the network transition from this SS (lower) to IMF (higher) protein-specific activity? One obvious alternative explanation is that these apparent differences simply reflect experimental artifact. SS mitochondria are isolated in the absence of nagarse exposure, while IMF isolation fundamentally depends on the nagarse incubation. Because nagarse treatment is the glaring difference between the two procedures, we tested the simple hypothesis that non-mitochondrial protein contamination accounts for the apparently lower specific activities of isolated SS mitochondria. Our data support this hypothesis and moreover advance the concept that nagarse treatment may also remove the protein within and perhaps also attached to damaged mitochondria that, left undigested, would otherwise diminish indices of mitochondrial structural and functional integrity.

Methods

Animal and muscle preparation

All procedures were in accordance with the guidelines regarding the care and use of animals by the Institutional Animal Care and Use Committee at Mayo Clinic. A total of six C57BL/6 J mice on standard chow diet and water ad libitum were used for all experiments. Mice ranged in age from 8 to 12 weeks. On the day of the experiment, the mice were euthanized by isoflourane inhalation and cervical dislocation. The left and right gastrocnemii were removed and immediately placed on an ice-cold petri dish, which was pre-rinsed with ice-cold modified Chappell-Perry Medium I (Solution I; mM): 100 KCl, 40 Tris–HCl, 10 Tris-Base, 5 $MgCl_2$, 1 EDTA, 1 ATP, pH 7.5. After removing the blood, fat and visible connective tissues, the gastrocnemii were placed into a pre-massed

beaker containing 2ml of ice-cold Solution I, reweighed, and the wet muscle mass was calculated. The mean gastrocnemii mass was 138.7±10.5 mg.

Isolation of mitochondria

Mitochondria were prepared according to the outline in Figure 2-1; all procedures were carried out on ice or at 4 °C and all centrifugations were 10 min in duration. Muscles were cleaned, weighed, minced with scissors in 9 volumes of ice-cold Solution I, and gently homogenized by hand using a ground glass-to-glass Potter-Elvehjem homogenizer (5 passes). The homogenate was centrifuged at 800g to obtain the supernatant (SN) containing mechanically released (SS) mitochondria. This SN was divided equally into two 1.5 ml homogenization tubes. Nagarse (bacterial proteinase Type XXIV, Sigma, P-8038) was prepared using Solution I (5mg g^{-1} ww) and added to one tube (SN+). An equal volume of Solution I containing no nagarse, was added to the other tube (SN-). After 7 min incubation, 1ml of Solution I was added to each tube (this terminated nagarse digestion in the SN+ tube). The SN+ and SN- samples were then centrifuged at 14,000g to obtain two mitochondrial pellets, one exposed to nagarse (MITO+) and the other not exposed (MITO-). After discarding the supernatant, each of the mitochondrial pellets were re-suspended in 0.5 ml of Solution II (mM): 100 KCl, 40 Tris–HCl, 10 Tris–Base, 5 $MgCl_2$, 1 EDTA, 0.2 ATP, and 1.5% BSA, pH 7.5. Following centrifugation at 7000g, the supernatants were discarded and the mitochondrial pellets were re-suspended in 0.5 ml of Solution III (identical to Solution II, but without BSA). After the final centrifugation at 4000g and removal of supernatant, the two final mitochondrial pellets were each re-suspended in identical volumes of mannitol-sucrose buffer containing (mM) 220 Mannitol, 70 Sucrose, 10 Tris–HCl, 1 EGTA, pH 7.40. The

volume of buffer used to resuspend each mitochondrial preparation was 1 µl per mg of original wet muscle. The protein content in the final mitochondrial preparations was determined by the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951). All protein concentrations reported below and in the Tables relate exclusively to the final mitochondrial preparations.

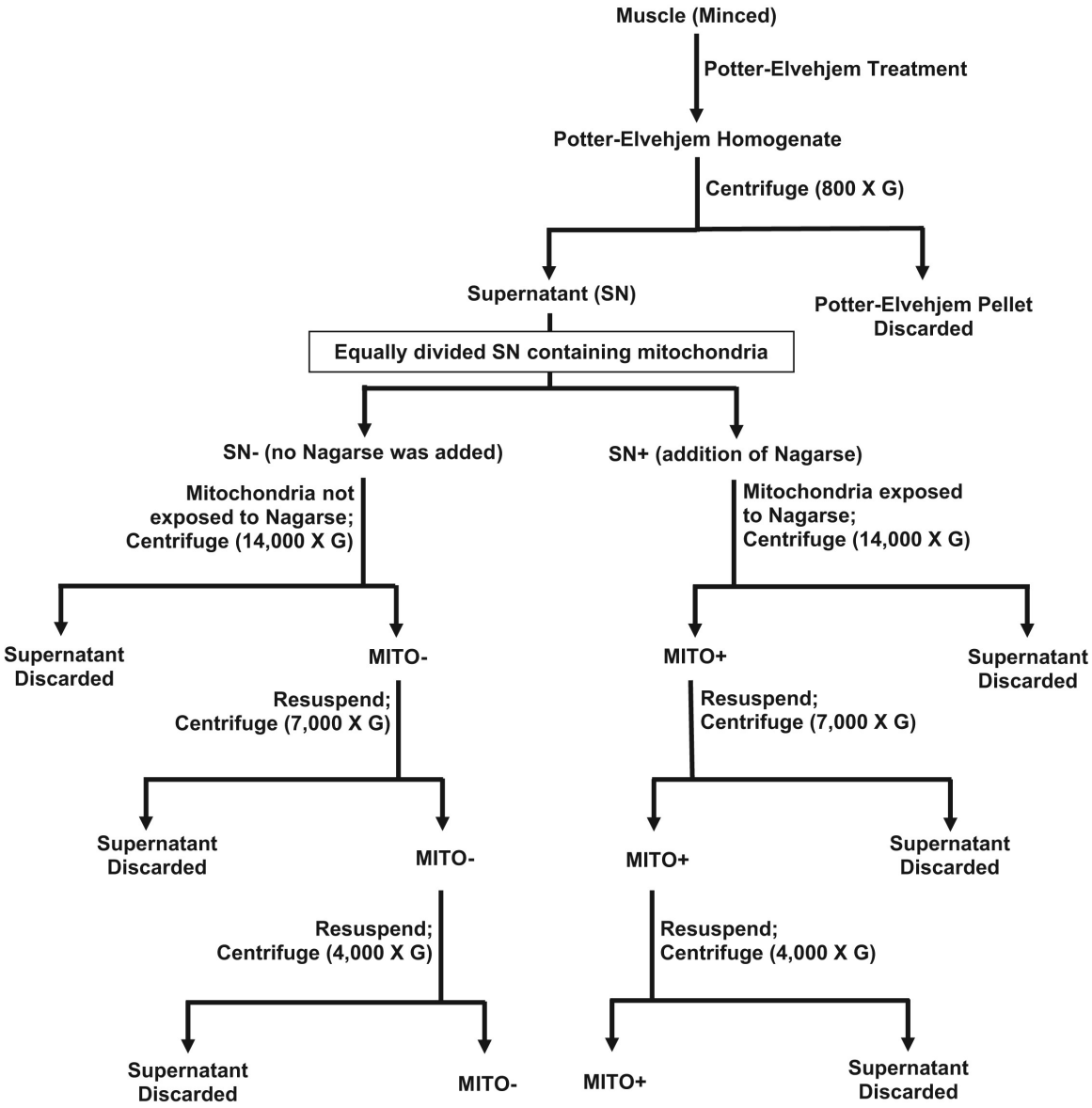

Figure 2-1. Isolation and treatment of the muscle mitochondria. A flow diagram is shown depicting the preparation of mechanically released mitochondrial sample that was subsequently either exposed or not to the enzyme nagarse. Details are given in the "Methods" (SN, supernatant).

Citrate synthase assay

Citrate synthase (CS) activity was determined spectrophotometrically at 37 °C by the method of Srere (Srere, 1969), as previously described (Lefort et al., 2010). In these assays aliquots of mitochondrial suspensions, stored at -80 °C, were assayed in buffer that included 0.05% Triton detergent to disperse the mitochondrial inner membrane and eliminate all enzyme latency.

Assays for mitochondrial O2 consumption and ATP production

Freshly isolated mitochondria were assayed for O_2 consumption rate (Jo) and ATP production rate (Jp). O_2 consumption was measured polarographically in a respiration chamber (Hansatech Instruments, Norfolk, UK) at 37 °C following general procedures we have previously described (Lefort et al., 2010). Mitochondrial respiration was fueled with the substrate combination pyruvate (1 mM)+malate (1 mM)+glutamate (10 mM) (PMG). Aliquots, typically 20 µl in volume, of mitochondrial suspension were added to 250 µl of respiration medium adapted from Wanders et al. (Wanders, Groen, Van Roermund, & Tager, 1984), which contained (in mM) 100 KCl, 50 MOPS, 10 K2PO4, 10 MgCl2, 1 EGTA, and 0.2% BSA, pH 7.00 (Lefort et al., 2010). Next, the PMG substrate combination was added and State 2 Jo was followed (respiration due primarily to proton leak). The addition of ADP to give a final concentration of 0.67 mM stimulated state 3, (maximal) Jo. Phosphorylation of this ADP resulted in state 4 Jo (Estabrook, 1967), and the respiratory control ratio (RCR) was calculated as state 3 Jo/state 4 Jo. The ADP/O ratio was determined as previously described (Estabrook, 1967). The State 3 (maximal) rate of ATP production was calculated as the product of

state 3 Jo times the ADP/O (taking the 2:1 molecular to atomic oxygen stoichiometry into account).

Protein separation by SDS-PAGE

Final MITO+ and MITO- suspensions were diluted 1:1 in 2 x Laemmli sample buffer before running on a SDS-PAGE gel. Laemmli sample buffer containing β-mercaptoethanol was prepared according to manufacturer's instructions (Bio-Rad, Hercules, CA). Samples were heated at 95 °C for 5 minutes and then loaded onto a pre-cast 10% SDS-PAGE gel (Bio-Rad, Hercules, CA). The gel was run at 60 V for 30 minutes, 110 V for 60 minutes and then 150 V for 10 minutes. Performing gel electrophoresis under these conditions allowed for adequate separation, visualization, and the ability to compare the MITO+ versus MITO- samples. Proteins were visualized using Coomassie blue. Gel image was captured using an ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Protein identification and quantification by mass spectrometry

To obtain an insight into the abundance of mitochondrial versus non-mitochondrial proteins contained in each of the MITO+ and MITO- preparations, aliquots from the samples analyzed on SDS-PAGE were also analyzed by mass spectrometry to identify and quantify proteins contained in each mitochondrial preparation. The mass spectrometry procedure was also used to identify and quantify the protein nagarse in the same preparations.

In-solution digest

A volume of isolated mitochondrial preparation, either treated or not treated with the enzyme nagarse, was added to a 9 times volume of dilution buffer [DB - 10%

acetonitrile (ACN) and 25mM Tris-HCl pH 8.5]. 8 µl trypsin (Sigma; St. Louis, MO) at 0.200 µg/ml was added to the protein sample and was allowed to incubate for 16 h at 37°C with gentle shaking, followed by addition of 50 µl 5% formic acid (FA) to halt the digestion. The resulting peptides were prepared for sample analysis similar to a previously published protocol (Kulak, Pichler, Paron, Nagaraj, & Mann, 2014). In brief, a stop-and-go extraction tip (StageTip) (Rappsilber, Mann, & Ishihama, 2007), was fitted with two C18 disk plugs using a customized tipping syringe (Rappsilber et al., 2007). The Stage Tip was activated with methanol, washed in 100 µl buffer B (0.1% FA, 80% ACN), and equilibrated in 100 µl buffer A (0.1% FA) twice. The peptides were then loaded onto the activated Stage Tip, washed twice in 100 µl buffer A, followed by elution in 50 µl Buffer B. The eluate was dried by vacuum centrifugation and stored at -80 °C prior to use. 6 µl of 0.1% FA (v/v) was added to re-suspend the dried samples, followed by sonication for 2 min. The sonicated samples were briefly centrifuged and 1 µl of sample was subsequently analyzed by mass spectrometry as described below.

Mass spectrometry

HPLC-ESI-MS/MSn was performed on a Thermo Electron Orbitrap Elite Velos Pro fitted with an EASY source (Thermo Electron, San Jose, CA). NanoLC was performed using a DIONEX/Thermo NCS-3500RS UltiMate 3000 with an EASY Spray column (Thermo Electron, 50 cm x 75-µm inner diameter, packed with PepMap RSLC C18 material, 2 µm); loading phase for 15 min; mobile phase, linear gradient of 1–37% ACN in 0.1% FA in 150 min, followed by a step to 95% ACN in 0.1% FA over 5 min, hold 10 min, and then a step to 1% ACN in 0.1% FA over 1 min and a final hold for 19 min (total run 200 min); Buffer A=0.1% FA in 100% H_2O; Buffer B=0.1% FA in 100%

ACN; flow rate, 300 nl/min. All solvents were mass spectrometry grade. A "top 15″ data-dependent MS/MS analysis was performed (acquisition of a full scan spectrum followed by collision-induced dissociation mass spectra of the 15 most abundant ions in the survey scan).

Database search

Tandem mass spectra were extracted by ProteoWizard msConvert, version 3 (Chambers et al., 2012) using the default settings. Charge state deconvolution and deisotoping were not performed. All MS/MS spectra were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the SwissProt_02_2015 database (16,706 entries, *mus musculus*) assuming the digestion enzyme trypsin and a maximum of 2 missed cleavages permitted. Mascot searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 PPM. Phosphorylation of serine, threonine, and tyrosine as well as oxidation of methionine was specified in Mascot as a variable modification while no fixe modifications were indicated.

Criteria for protein identification

Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Peptide Probabilities were assigned by the Peptide Prophet algorithm (Keller, Nesvizhskii, Kolker, & Aebersold, 2002) with Scaffold delta-mass correction as well as by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides.

Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller, Kolker, & Aebersold, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Quantification of mitochondrial and non-mitochondrial proteins

Progenesis software (Nonlinear Dynamics; Progenesis QI; Quayside, Newcastle Upon Tyne, UK) was used to quantify abundance of peptides associated with each protein. Quantification using non-conflicting peptides was selected to generate the protein report for the peptide ion abundance associated with each identified protein. Accession numbers were imported into SwissProt_02_2015 database and used to assign the subcellular location of the identified proteins (i.e. mitochondrial, non-mitochondrial). Peptide ion abundances for all mitochondrial and all non- mitochondrial proteins in each of the MITO+ and MITO- samples were added to calculate total mitochondrial and total non-mitochondrial protein abundances, respectively.

Quantification of the nagarse abundance

To quantify the abundance of nagarse present in the mitochondrial preparations treated/not treated with nagarse, we used the same approach described in the "Database search," section, but with the following exception: Mascot was set up to search the SwissProt_02_2015 database for *bacillus licheniformis*. We used the unique and stable identifier from the SwissProt_02_2015 database for nagarse, Q65LP7, to perform all queries. We also used the corresponding mnemonic identifier of the UniprotKB entry, Q65LP7_BACLD to perform all protein queries. The ion abundance for the peptides associated with the enzyme nagarse within each of the mitochondrial preparations were

compared with the total ion abundance for all the proteins within each of the mitochondrial preparations treated/not treated with nagarse from the Progenesis protein reports. The total ion abundances are in reference to alignments in Mascot search using SwissProt_02_2015 database for *mus musculus*. The ion abundances of the peptides associated with the enzyme nagarse are in reference to alignments in Mascot search using SwissProt_02_2015 database for *bacillus licheniformis*.

Statistical analyses

Differences between MITO+ and MITO- across variables of interest were compared using paired t-test. Data are reported as means ±SEM. Significance was set at $P < 0.05$.

Results

Protein content of final mitochondrial suspension

Isolating mitochondria with nagarse decreased the protein concentration of the final mitochondrial suspension by 30%. The MITO+ preparation was 2.12 ± 0.23 mg protein ml^{-1} compared to 2.80 ± 0.37mg.ml^{-1} in MITO-. When these protein concentrations (mg/ml) are multiplied by their respective suspension volumes (ml), the total protein yield (mg "mitochondrial" protein) of the isolation procedure is calculated. When this is done, the MITO+ protein yield was 0.290 ± 0.03 mg, compared to a MITO- value of 0.386 ± 0.05 mg (Table 2-1). Thus, nagarse exposure reduced, by 25%, the total protein isolated (Table 2-1).

Table 2-1. Total protein yield, enzyme specific activities, and indices of functional integrity of mitochondria isolated without and with nagarse.

	MITO−	MITO+	MITO+/MITO- (Fold Δ)	P Value
Total protein yield mg	0.386±0.05	0.290±0.03	0.75	0.02
Citrate synthase activity $nmol.min^{-1}.mg^{-1}$	827.4±65.4	1232.9±83.5	1.49	0.0001
State 3 J_O $nmol.min^{-1}.mg^{-1}$	77.6±8.6	142.6±16.8	1.84	0.003
State 4 J_O $nmol.min^{-1}.mg^{-1}$	22.5±2.4	31.0±3.5	1.38	0.01
State 3 J_P $nmol.min^{-1}.mg^{-1}$	384.9±36.9	771.4±83.4	2.00	0.002
RCR	3.6±0.4	4.6±0.4	1.30	0.002
ADP/O	2.5±0.1	2.7±0.1	1.09	0.02

Table 2-1. Values are means±SE; MITO+, mitochondria samples treated with nagarse; MITO−, mitochondria samples not treated with nagarse; total protein yield was calculated as the product of the protein concentration in the final mitochondrial suspension times the suspension volume; state 3 Jo, state 3 O_2 consumption rate; state 4 Jo, state 4 O_2 consumption rate; state 3 Jp, state 3 ATP production rate. RCR, respiratory control ratio, is State 3 (maximum) Jo divided by State 4 (resting) Jo; State 3 Jp is the product of State 3 Jo and the ADP/O ratio.

Citrate synthase activity

Citrate synthase, the first enzyme of the citric acid cycle, is a sturdy matrix enzyme often used to assess muscle mitochondrial content. Expressed per mg protein in the final mitochondrial suspension, citrate synthase activity (U mg^{-1}) was 49% higher when nagarse was included in the isolation procedure (Table 2-1). However, the total citrate synthase activity recovered in the final mitochondrial suspension, calculated by multiplying the Umg^{-1} by the total protein (mg) yield in the final suspension (see above), was not different in MITO+ (366.7 ± 53.0 nmol min^{-1}) compared to MITO- (329.3±54.1 nmol min^{-1}) (Table 2-2).

Table 2-2. Total yield of citrate synthase and respiratory activity of mitochondria isolated without and with nagarse.

	MITO−	MITO+	MITO+/MITO- (Fold Δ)	P Value
Citrate synthase activity $nmol.min^{-1}$	329.3±54.1	366.7±53.0	1.11	0.110
State 3 J_O $nmol.min^{-1}$	31.1±5.7	42.5±7.5	1.37	0.010
State 4 J_O $nmol.min^{-1}$	9.1±1.8	9.1±1.3	1.00	0.980
State 3 J_P $nmol.min^{-1}$	153.5±27.5	229.5±38.8	1.49	0.010

Table 2-2. Values are means±SE; MITO+, mitochondria samples treated with nagarse; MITO−, mitochondria samples not treated with nagarse; total yield of activity was calculated as the product of specific activity times the total protein yield (see mean values in Table 1); state 3 Jo, state 3 O_2 consumption rate; state 4 Jo, state 4 O_2 consumption rate; state 3 Jp, state 3 ATP production rate.

Respiratory rates and respiratory control ratio

Mitochondrial O_2 consumption (Jo) due to the combustion of Pyruvate + Malate + Glutamate (PMG Jo) requires the entire oxidative pathway of fully intact mitochondria, including several soluble matrix cofactors such as Coenzyme A and NAD+. Saturating ADP was added to stimulate the maximum O_2 consumption rate (state 3 Jo). When mitochondria phosphorylate all of the added ADP to ATP, they transition to "resting" (state 4) respiration. Table 2-1 reports that nagarse treatment increased both state 3 Jo (by 84%) and state 4 Jo (by 38%), when these values are expressed per mg protein (nmol O_2 min^{-1} mg^{-1}). Thus, nagarse treatment especially increased State 3 Jo, while it increased State 4 more modestly. As a result, nagarse increased the respiratory control ratio (RCR=State 3 Jo/State 4 Jo) by 30% (Table 2-1). The total yield of State 3 Jo activity recovered in the final mitochondrial suspension was calculated as above for citrate synthase, by multiplying the respiration rates per mg by the total protein (mg) yield in the final suspension. Unlike citrate synthase, which was not statistically different, total state 3 Jo was 37% higher in MITO+(42.5±7.5 nmol min^{-1}) compared to MITO-(31.1±5.7 nmol min^{-1}) (Table 2-2). The total yield of State 4 Jo activity was essentially identical in MITO- and MITO+ (Table 2-2).

ADP/O coupling and maximum ATP production

Nagarse exposure modestly improved, by 9%, the ADP/O ratio, 2.7±0.1 vs. 2.5±0.1, in MITO+ vs. MITO-, respectively (Table 2-1). The product of State 3 Jo times the ADP/O yields the State 3 ATP production rate (State 3 Jp). Because both factors were elevated by nagarse treatment, State 3 Jp was dramatically (100%) higher in MITO+ compared to MITO-, 771.4±83.4 vs. 384.9±36.9 nmol ATP min^{-1} mg^{-1} respectively

(Table 2-1). Again, multiplying these protein-specific State 3 Jp values by the total protein (mg) yield indicated that nagarse exposure increased the total yield of State 3 Jp by 49%: 229.5±38.8 nmol ATP/min in MITO+ vs. 153.5±27.5 nmol ATP/min in MITO- (Table 2-2).

Protein detection by SDS-PAGE and identification and quantification by mass spectrometry

Consistent with the Lowry protein determinations, nagarse treatment decreased the visually apparent protein detected using SDS-PAGE in representative MITO+ vs. MITO- preparations (Figure 2-2A). Mass spectrometry analysis of all samples also showed less protein, including mitochondrial protein, content in the MITO+ suspensions (Figure 2-2B). In MITO+ preparations approximately 83% of the total protein content was identified as mitochondrial proteins. In contrast, in MITO- preparations mitochondrial proteins could account for only approximately 73% of total protein. The ratio of identified MITO proteins versus non-MITO proteins was higher in MITO+ samples compared to MITO- (Figure 2-2C). Nagarse (apr; subtilisin Carlsberg; EC:3.4.21.62; UniProtKB accession number, Q65LP7) was essentially absent in the final suspensions of mitochondria subjected to nagarse treatment. In MITO+ preparations the tryptic peptide abundance of nagarse represented only 0.008% of the total peptide abundance. In MITO+ this value was 0.0002%.

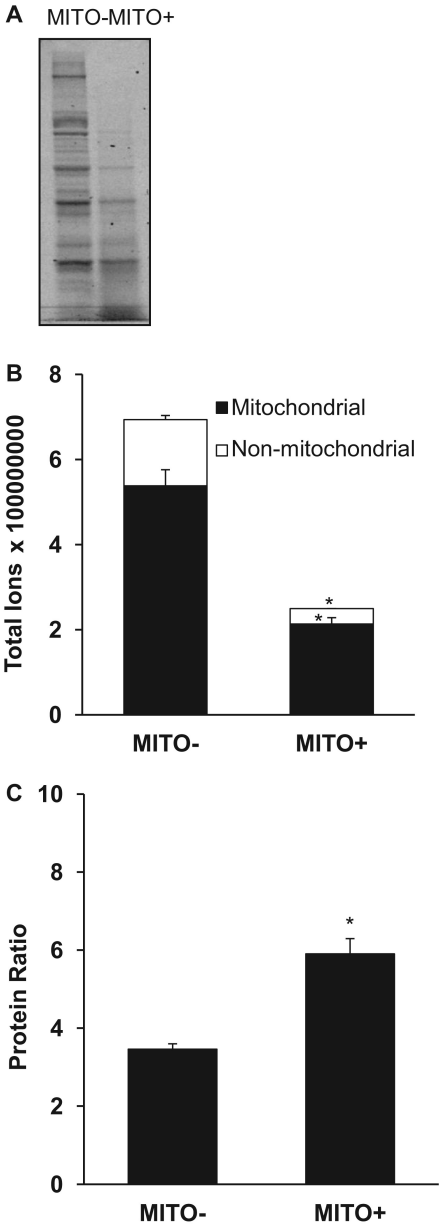

Figure 2-2. Protein abundance in mitochondrial suspensions isolated without and with nagarse. Protein abundance detected by SDS-PAGE/Coomassie Blue (A), HPLC-ESI-MS/MS quantitative proteomics analysis to determine peptide ion abundance of mitochondrial and non-mitochondrial proteins in MITO+ and MITO− samples (B), and protein ratio of mitochondrial-to-non-mitochondrial protein in the same samples (C). Values are means±SE; MITO+, mitochondria samples treated with nagarse; MITO−, mitochondria samples not treated with nagarse; *P<0.05 between MITO+ and MITO-.

Discussion

The major findings reported here are that nagarse exposure substantially reduced (by 25%) the protein yield in the final mitochondrial suspension, and proteomic analysis indicated that the elimination of non-mitochondrial protein accounted for much of this reduction. By removing non-mitochondrial protein, nagarse treatment increased mitochondrial enzyme and enzyme pathway protein-specific activities. Expressed per mg protein, nagarse treatment increased citrate synthase activity by 49% and State 3 Jo even more dramatically, by 84%. Moreover, nagarse also modestly increased the ADP/O ratio, so that, per mg protein, State 3 Jp improved by 100%. The data generally indicate that nagarse treatment effectively removes contaminating non-mitochondrial proteins and substantially improves indices of mitochondrial catalytic potential and energetic coupling.

The total isolated CS activity was not significantly affected by nagarse treatment, while nagarse increased the total yield of State 3 Jo by 37%, and State 3 Jp by 49%. The spectrophotometric assay of citrate synthase activity versus the polarographic assay of mitochondrial oxidative phosphorylation fueled by PMG, evaluate two vastly different parameters of mitochondrial structure and function. Citrate synthase is a high activity matrix enzyme with well-known stability (D'Souza & Srere, 1983), and is routinely measured in either whole tissue homogenates or mitochondrial suspensions in the presence of a detergent such as Triton X-100, as it was in the present study. In marked contrast, the oxidation of the substrate combination PMG, whether the mitochondria are "resting" or stimulated by saturating ADP, requires, at the least, the entire integrated oxidative pathway and, in particular, robust inner membrane structural integrity. It can be

argued that the different out-comes of the CS and Jo assays suggest that nagarse treatment somehow modified the first, slow speed, supernatant in away that protected mitochondrial units (vesicles) from damage during the subsequent high speed centrifugation and resuspension steps of the isolation procedure. This interpretation is developed below.

Whether mitochondria are partially damaged, have been briefly opened and then resealed, or are fully intact, citrate synthase activity may well remain unaffected. Thus, it can be envisioned that in the absence of nagarse treatment some mitochondria, both fully intact and partially damaged, in the first supernatant were subsequently further damaged during the course of the isolation procedure, but they nevertheless carried citrate synthase activity into the final suspension. Nagarse treatment, on the other hand, would be expected to proteolytically remove the activity of exposed citrate synthase in damaged mitochondria. The data of Table 2-2 indicate that these intact mitochondria in the final MITO+ suspension carry roughly similar citrate synthase as the MITO-. The net effect is that the total yield of citrate synthase activity in the final suspension is not significantly different. However, in MITO+ more of this citrate synthase activity reside in fully intact mitochondria, which are capable of coupled oxidative phosphorylation. The total yield of State 3 Jo was 37% higher and State 3 Jp activity was 49% higher in MITO+ final suspensions. In contrast, the total yield of State 4 Jo was essentially identical in the two preparations. Nagarse in the first supernatant may have somehow protected against mitochondrial damage during subsequent centrifugation and resuspension steps, perhaps by digesting non-mitochondrial proteins in some type of linkage (Boncompagni & Rossi, 2009; de Brito & Scorrano, 2008) to mitochondria. In the absence of nagarse treatment,

during the high- speed centrifugations and pellet resuspensions these linkages somehow increased the likelihood of structural insult to mitochondrial vesicles. The damage was not severe enough to liberate citrate synthase from its matrix binding (D'Souza & Srere, 1983), but was sufficient to preclude the development of the very high driving forces required for oxidative phosphorylation. Damaged organelles in MITO- preparations are consistent with the lower RCR and ADP/O values observed in MITO-. It therefore appears that nagarse promoted the release of mitochondria from non-mitochondrial protein, which was digested and resulted in less protein yield and less damage to mitochondria during isolation. Mass spectrometry data further support this interpretation. MITO- preparations had much greater abundance of non-mitochondrial proteins. In nagarse treated preparations, both mitochondrial and non-mitochondrial protein yields were reduced (Figure 2-2B). This suggests that sufficiently damaged mitochondria in the first supernatant were exposed to nagarse entry/attack, digested and did not make it into the final suspension. The mitochondria within a muscle cell are interconnected as a reticulum (network) (Amchenkova et al., 1988; Bach et al., 2003; Bakeeva et al., 1978; Glancy et al., 2015; Kirkwood, Munn, & Brooks, 1986). Mechanical homogenization, however gentle, of muscle tissue therefore must destroy this reticular structure, because electron micrographs of isolated mitochondria, for example those of Palmer et al. (Palmer et al., 1977), clearly show the isolated organelles as individual vesicles. During homogenization, as these vesicles form, some membrane damage and leakage of essential matrix cofactors (NAD, CoA, adenylates, etc.) into the isolation medium would be expected (Bremer, Wojtczak, & Skrede, 1972). Nevertheless, when these procedures are performed carefully by experienced hands, and particularly when nagarse is included in

the isolation procedure, the resulting vesicles are capable of nearly matching the maximum O_2 consumption rates (U F Rasmussen & Rasmussen, 2000; Ulla F. Rasmussen, Vielwerth, & Rasmussen, 2004) and ATP free energy (Hitchins, Cieslar, & Dobson, 2001; Jeneson, Westerhoff, Brown, Van Echteld, & Berger, 1995; Kemp, Meyerspeer, & Moser, 2007) measured in intact muscle using Fick O_2 mass balance and ^{31}P-MRS, respectively. Moreover, the isolated mitochondria also control respiration and ATP production over the same range of energy phosphate levels observed in vivo (Glancy, Willis, Chess, & Balaban, 2013; Jeneson, Westerhoff, Brown, Van Echteld, & Berger, 1995; Lefort et al., 2010; Messer, Jackman, & Willis, 2004). Thus, mitochondria isolated using mechanical homogenization and exposure to protease can be nearly as functional as mitochondria in vivo.

Conclusions

Nagarse treatment of mitochondria removes non-mitochondrial proteins as well as proteins of damaged mitochondria, and improves indices of functional integrity and resulting protein-specific activities.

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CHAPTER 3: PLASMA AMINO ACIDS STIMULATE UNCOUPLED RESPIRATION OF MUSCLE SUBSARCOLEMMAL MITOCHONDRIA IN LEAN BUT NOT OBESE HUMANS

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Abstract

Obesity is associated with mitochondrial dysfunction in skeletal muscle. Skeletal muscle mitochondria are arranged as a reticulum; mitochondria in the periphery of the cell, directly beneath the sarcolemma, subsarcolemmal (SS) and mitochondria located in the interior of the cell proximal to myofibrils, intermyofibrillar (IMF). Obese individuals have reduced ATP production associated with early fatigability during activity and more sedentary lifestyle. SS mitochondria being more adaptable than IMF mitochondria, may be prone to metabolic changes in obesity, but better able to respond to treatments for obesity, such as increased protein intake/amino acids (AA). Increasing the plasma amino acid (AA) concentrations stimulates mitochondrial ATP production in lean individuals. Therefore, the objective of this study was to determine whether four-hour infusion of AA improves skeletal muscle mitochondria ATP production equally in healthy lean and obese adults; in other words we sought to determine whether acute elevation in plasma AAs enhances muscle mitochondrial respiration and ATP production in skeletal muscle mitochondria of obese adults. Using standard polarography techniques, we measured maximal oxygen consumption rates to assess SS and IMF mitochondria function during

saline (i.e., control) and AA infusions. Eligible participants were healthy lean (BMI, < 25 kg/m^2; age, 37 ± 3 yrs; n = 10) and obese (BMI > 30 kg/m^2; age 35 ± 3 yrs; n = 11) subjects. The intervention in this study was a single trial of saline infusion followed by a four-hour AA infusion. SS and IMF mitochondria were isolated from muscle biopsies collected at the end of the saline and AA infusions. The main outcomes to this study revealed that mitochondrial ADP-stimulated respiration and ATP production rates increased following a four-hour AA infusion in SS mitochondria of lean ($P < 0.05$), but not obese, subjects. Furthermore, we show that four-hour AA infusion increased the uncoupled (i.e. non ADP-stimulated) respiration of SS mitochondria in the lean subjects only ($P <0.05$). AA infusion had no effect in any of these parameters on IMF mitochondria in either lean or obese subjects ($P > 0.05$). From these finding we conclude that increasing plasma amino acids concentrations enhances the capacity for respiration and ATP production of muscle SS mitochondria, but not IMF mitochondria, in lean individuals in parallel with increase in uncoupled respiration. However, neither of these parameters increases in muscle SS mitochondria or IMF mitochondria in obese individuals. In summary, amino acid infusion results in increased maximal oxygen consumption and uncoupled respiration in subsarcolemmal, but not intermyofibrillar mitochondria in lean, but not obese subjects.

Introduction

Obesity is an underlying cause for diabetes, heart disease, and cancer. The financial burden on the healthcare system to treat obesity continues to rise as access to food increases in parallel with the adoption of more sedentary lifestyle. Mitochondria from skeletal muscle exist in a reticulum (Bakeeva, Chentsov YuS, & Skulachev, 1978;

Glancy et al., 2015; Kirkwood, Munn, & Brooks, 1986; Ogata & Yamasaki, 1997) and according to some evidence, obese individuals have reduced skeletal muscle mitochondrial function as indicated by lower citrate synthase activity (Machado et al., 2012), reduced capacity for mitochondrial electron transport (Kelley, He, Menshikova, & Ritov, 2002), or ATP production (Abdul-Ghani et al., 2009). However, such differences in mitochondrial function are not evident when lean and obese subjects are matched for physical activity levels (Karakelides et al., 2010). Reduced physical activity in obese individuals (Cooper, Page, Fox, & Misson, 2000) may impair muscle mitochondrial function (Safdar et al., 2010) and exacerbate the metabolic consequences of obesity. Regardless of the mechanism(s) involved, diminished skeletal muscle mitochondrial function in obesity has causally been linked to insulin resistance (previously reviewed (Montgomery & Turner, 2014)). Skeletal muscle mitochondria are unique with respect to their arrangement within myocytes: mitochondria near the periphery of the cell are termed subsarcolemmal (SS) mitochondria and those near the myofibrils are termed intermyofibrillar (IMF) mitochondria. These two mitochondrial subpopulations are biochemically distinct (Palmer & Hoppelt, 1985). The previously reported differences in mitochondrial ATP production observed in muscle from obese could be specific to the population of skeletal muscle mitochondria studied. Furthermore the SS mitochondria has been shown to be more responsive to metabolic changes, compared to IMF mitochondria (Bizeau, Willis, & Hazel, 1998; Chomentowski, Coen, Radiková, Goodpaster, & Toledo, 2011; Cogswell, Stevens, & Hood, 1993; Crescenzo et al., 2006; Koves et al., 2005). Consequently, metabolic changes associated with obesity may particularly affect the SS mitochondria. Muscle mitochondria have been previously studied relative to their

subcellular location, and with regards to their content/size (Samjoo et al., 2013) or substrate metabolism (Hoshino, Yoshida, Kitaoka, Hatta, & Bonen, 2013; Stefanyk et al., 2011). To our knowledge there is limited evidence evaluating respiratory capacity separately in SS and IMF mitochondria when comparing lean and obese humans. In relevant evidence, Ritov et al (Ritov et al., 2005) have shown that electron transport chain activity is reduced in Type 2 Diabetes patients as well as obese adults in both SS and IMF muscle mitochondria, and in parallel with greater reduction in SS mitochondrial content. On the other hand, SS mitochondria show more pronounced improvements in function in response to interventions, such as exercise, than IMF mitochondria (Menshikova et al., 2006). Therefore, studying SS and IMF mitochondria independently allows for a more detailed exploration of the mechanisms that can lead to alterations in energy metabolism in the muscle of obese individuals. Improving mitochondrial metabolism has been proposed as a novel approach to treating obesity and insulin resistance (Montgomery & Turner, 2014).

Practical treatments for obesity include nutritional and exercise interventions. Lifestyle interventions to treat obesity such as nutrition, specifically the increased intake of protein/amino acids have been shown to effect skeletal muscle mitochondrial function (Guillet et al., 2009; Tatpati et al., 2010). Increased availability of amino acids has been shown to promote protein synthesis in skeletal muscle at the total (Bohé, Low, Wolfe, & Rennie, 2003; Guillet et al., 2009; Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe, 2005, 2006; Volpi, Kobayashi, Sheffield-Moore, Mittendorfer, & Wolfe, 2003) as well as mitochondrial protein levels (Bohé et al., 2003; Guillet et al., 2009; Stump, Short,

Bigelow, Schimke, & Nair, 2003; Tatpati et al., 2010); resulting in higher skeletal muscle mitochondrial ATP production (Stump et al., 2003; Tatpati et al., 2010).

Plasma amino acids (AAs), in addition to their well-known effects on stimulating mitochondrial protein synthesis (Katsanos et al., 2006), improve mitochondrial ATP production of SS mitochondria of young healthy lean subjects (Tatpati et al., 2010). Generally, SS mitochondria appear more responsive than IMF mitochondria to physiological perturbations induced by muscle use (or disuse) (Hood, 2001). To our knowledge, no studies have evaluated the effects of increasing the plasma AAs on the function of IMF mitochondria in humans, and, more importantly, how responses in SS and IMF mitochondria may differ between lean and obese humans. Moreover, the metabolism of certain AAs, such as the branched-chain AAs (BCAAs), is impaired in skeletal muscle in obesity (Lerin et al., 2016), which could differentially impact the effects of plasma AA availability on mitochondrial function. Therefore, we hypothesized: 1) the AA would stimulate ATP production in skeletal muscle SS mitochondria only and 2) the stimulatory effect of AA would be lower in obese compared with lean adults. Measurements of citrate synthase activity, hormones and substrates were used to elucidate the underlying mechanisms responsible for improving capacity of ATP production in skeletal muscle mitochondria. These findings may be translated into interventions to improve energy metabolism in skeletal muscle from obese individuals. Therefore, determining interventions and the mechanisms, which improve capacity of ATP production in skeletal muscle from obese individuals is expected to have the following benefits: 1) decrease fatigability, 2) increase levels of activity, and 3) help improve overall skeletal muscle energy metabolism in obese individuals.

Our objective was to compare the effects of increased plasma AA concentrations on SS and IMF mitochondrial function, specifically as it relates to oxygen consumption and ATP production in lean and obese individuals. To this end, we hypothesized that elevated plasma AA concentrations will stimulate oxygen consumption and ATP production in skeletal muscle SS mitochondria only, and that this effect will be observed in lean but not obese subjects.

Methods

Subjects

Ten healthy sedentary lean (male = 4, females = 6; age = 37±3.1 years; BMI = 22.0±0.7 kg/m2; Body fat = 23.8±2.6%) and eleven healthy sedentary obese (male = 6, females = 5; age = 35±3.1 years; BMI = 34.5±1.1 kg/m2; Body fat = 33.7±2.2%) individuals participated in this study. The Institutional Review Board at Mayo Clinic approved the studies and all experimental procedures were performed in the Clinical Studies Infusion Unit (CSIU) at Mayo Clinic in Scottsdale, Arizona. The purpose, design, and the risks associated with this study were explained to each subject before obtaining written consent. Participants were determined healthy based on screening procedures that involved medical history, routine physical examination, and standard laboratory tests, which included blood metabolic panel, urinalysis, and a 2-hour oral glucose tolerance test (OGTT). Body fat was determined using bioelectrical impedance analysis (BIA 310e, Biodynamics Corporation, Seattle, WA). Maximal oxygen uptake (VO_2max) was measured (Ultima™ Series, MGC Diagnostics Corporation, Saint Paul, MN) on a separate day following the screening by using an incremental (30 Watts/min) cycle ergometer (Lode Corival, Lode B.V., Groningen, Netherlands) test to exhaustion. Subject

characteristics are shown in Table 3-1. Participants returned to the CSIU on a separate day from their screening visit for the main experiment, which included intravenous infusion of saline followed by infusion of AAs and collection of blood and muscle samples.

Table 3-1 Subject anthropometric and metabolic characteristics.

Characteristic	Lean	Obese	P Value
Gender (M/F)	4/6	6/5	
Age, years	37 ± 3	35 ± 3	0.5970
Body Weight, kg	62 ± 3	103 ± 4	< 0.0001
BMI, kg/m^2	22 ± 1	34 ± 1	< 0.0001
Body fat (%)	24 ± 3	34 ± 2	0.0097
FFM, kg	43 ± 3	68 ± 2	< 0.0001
VO_2max, ml/kg FFM/min	35 ± 7	34 ± 7	0.9000
Matsuda Index	9.2 ± 0.9	4.5 ± 1.2	0.0076
2-hr OGTT glucose, mmol/l	4.9 ± 0.1	5.4 ± 0.2	0.0076
$HbA_{1c,}$ (%)	5.3 ± 0.1	5.6 ± 0.1	0.0517

Table 3-1. Data are presented as mean ± SEM; BMI, body mass index; FFM, fat free mass; VO_2max, maximal oxygen uptake; Matsuda index, insulin sensitivity index calculated from the plasma glucose and insulin responses during an oral glucose tolerance test (OGTT).

Experimental Design

Volunteers arrived at the CSIU at approximately 0630 h following an overnight fast. All subjects were instructed to refrain from any form of exercise for the 3-days prior to the study. An intravenous line was inserted into an antecubital vein for infusions, while a second intravenous line was placed in a dorsal hand vein to collect arterialized blood samples (i.e., heated-hand technique) (Sonnenberg & Keller, 1982). After that, a saline infusion was started (time 0) and continued for 300 min. At 300 min, an amino acid mixture infusion (15% Clinisol; Baxter Healthcare Corporation, Deerfield, IL) was started with a priming dose, 82 mg kg/FFM, and then maintained at 240 mg kg/FFM/hr for 240 min (i.e., 540 min from the beginning of the experiments). Specifically, there were two periods in the experimental protocol; one describes the response of skeletal muscle mitochondrial function at the end of the saline infusion and the second describes skeletal muscle mitochondrial function at the end of amino acids infusion period. Blood samples were collected at 60 minutes and 300 minutes after the start of the saline infusion, and at 30 minutes and 240 minutes after the initiation of the AA mixture infusion. Muscle samples were collected at the end of the saline and AA infusion periods to evaluate skeletal muscle mitochondria function.

A Bergström biopsy needled was used to collect percutaneous muscle biopsies (~100 mg) of the *vastus lateralis* under local anesthesia (lidocain, 2%). After removing the blood, fat and visible connective tissues, the muscle biopsy was placed into a pre-massed beaker containing 2 ml of ice-cold Solution I (modified Chappell-Perry Medium I (Solution I; mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 $MgCl_2$, 1 EDTA, 1 ATP, pH 7.5), reweighed, and the wet muscle mass was calculated. The mean *vastus lateralis*

masses, collected at the end of the saline and AA infusion periods, from lean and obese individuals were 105.0±8.9 vs. 115.6±6.9 and 106.8±12.3 vs. 114.7±6.3 (mg), respectively. There were no significant differences in the masses of muscle biopsies collected in either the lean or obese groups following any of the study periods as determined by Unpaired t test; data represents mean muscle mass ± SEM; $P > 0.05$.

Isolation of skeletal muscle mitochondria

All procedures were carried out on ice or at 4°C and all centrifugations were 10 min in duration. Muscles were minced with scissors in 9 volumes of ice-cold Solution I (mM; 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 MgCl2, 1 EDTA, 1 ATP, pH 7.5.), and gently homogenized by hand using a ground glass-to-glass Potter-Elvehjem homogenizer (5 passes). The homogenate was centrifuged at 800 x g to obtain the supernatant (SN 1) containing mechanically released SS mitochondria (Figure 3-1). The pellet from the initial 800 x g centrifugation, Potter-Elvehjem Pellet (PEP), containing IMF mitochondria, was subjected to incubation with Nagarse (Type XXIV, Sigma, P-8038). Nagarse was prepared using Solution I and added (5 mg·g-1 WW) to the PEP to liberate IMF mitochondria. After a 7-min incubation, 1 ml of Solution I was added to the digested PEP, to stop the enzymatic digestion. The resuspended PEP was then centrifuged at 800 x g to separate the non-mitochondrial proteins and membranes from IMF mitochondria. This supernatant (SN 2) containing IMF mitochondria was transferred to a new tube. The SN 1 containing SS mitochondria and the separate SN 2 containing IMF mitochondria were centrifuged at 14,000 x g to obtain two mitochondrial pellets, SS mitochondria and IMF mitochondria. After discarding the supernatant, each of the mitochondrial pellets were resuspended in 0.5 ml of Solution II (mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5

$MgCl_2$, 1 EDTA, 0.2 ATP, at pH 7.5. Following centrifugation at 7000 x g, the supernatants were discarded and the mitochondrial pellets were resuspended a third time in 0.5 ml of Solution II. After the final centrifugation at 4000 x g and removal of the supernatant, the two final mitochondrial pellets (i.e. SS mitochondria and IMF mitochondria) were each resuspended in identical volumes of mannitol-sucrose buffer containing (mM) 220 Mannitol, 70 Sucrose, 10 Tris-HCl, 1 EGTA, pH 7.40. The protein content in the final mitochondrial preparations was determined by the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951). Citrate synthase (CS) activity in the same preparations was determined spectrophotometrically at 37°C using procedures we have previously described in chapter 2 (Kras et al., 2016).

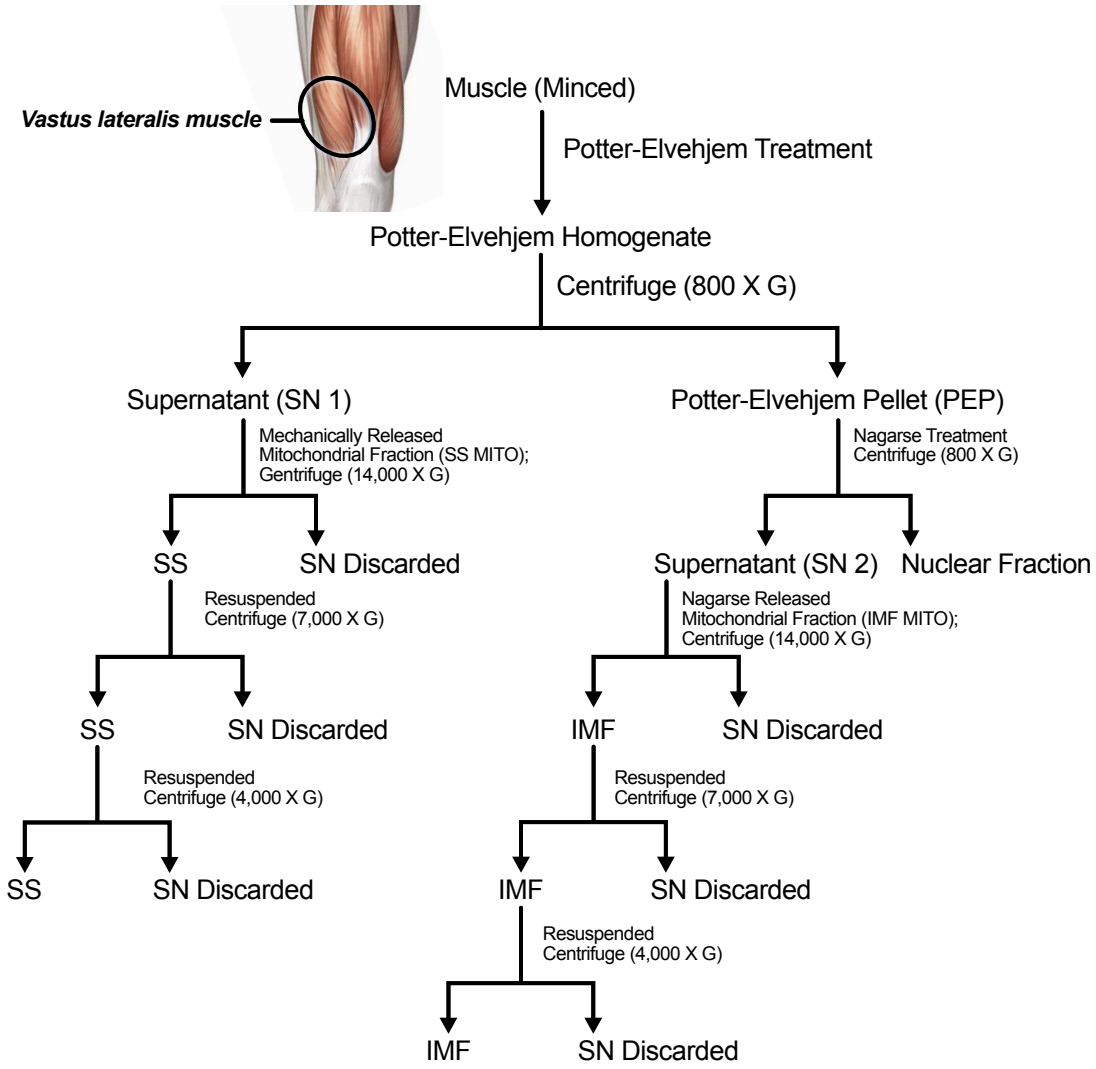

Figure 3-1. A schematic workflow for isolating skeletal muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from lean and obese individuals; SN – supernatant.

Assays for mitochondrial O_2 consumption and ATP production

Freshly isolated mitochondria were assayed for O_2 consumption rate (Jo) and ATP production rate (Jp). O2 consumption was measured polarographically in a respiration chamber (Hansatech Instruments, Norfolk, UK) at 37°C following general procedures we have previously described (Kras et al., 2016) and described in chapter 2. Complex-I-mediated and complex-II-mediated mitochondrial respiration were assessed with saturating amounts of either Malate (1mM) +Pyruvate (1mM) +Glutamate (10mM) (MPG) or Succinate (10mM) (SUCC), respectively. Aliquots of mitochondrial suspension were added to 0.230 ml of respiration media, adapted from Wanders, (Wanders, Groen, Van Roermund, & Tager, 1984) containing (in mM) 100 KCl, 50 MOPS, 100 Glucose, 10 K_2PO_4, 10 $MgCl_2$, 1 EGTA, and 0.2% BSA, pH 7.00. The MPG or SUCC substrates were added to the respiration chamber, and state 2 Jo was followed (respiration primarily due to proton leak). The addition of ADP to give a final concentration of 0.67 mM stimulated state 3, (maximal) Jo. Phosphorylation of this ADP resulted in state 4 Jo (Estabrook, 1967), and the respiratory control ratio (RCR) was calculated as state 3 Jo/state 4 Jo. The ADP/O ratio was determined as previously described (Estabrook, 1967). The State 3 (maximal) rate of ATP production was calculated as previously described (Kras et al., 2016).

Determination of muscle mitochondrial DNA content

Total DNA was isolated from whole muscle homogenates stored at -80°C, using TRI-Reagent® (T9424) (Sigma-Aldrich; Molecular Research Center), and by following the manufacturer's instructions. DNA was quantified using the NanoDropTM 1000 Spectrophotometer Thermo Fisher Scientific (Wilmington, DE, USA). Concentration was

determined by the absorbance at 260 nm, and purity was assessed using the 260:280 ratio. PCR for the detection of mitochondrial NADH-ubiquinone oxidoreductase chain 2 (MTND2) and nuclear beta-actin (ACTB) (Vogel et al., 2008) was performed in duplicate using the Applied Biosystems ABI 7900HT sequence detection instrument and software. TaqMan® Fast Universal PCR master mix reagents and the Assay-On-DemandTM gene expression primer pair and probes (Thermo Fisher Scientific) for MTND2 (Hs02596874_g1) and ACTB (Hs03023880_g1), were added to 20 ng DNA and 1 ng of DNA, respectively. All samples were run in duplicate for each gene. A DNA standard curve was performed to optimize amplification. Reactions were run with the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s, and 60°C for 60 s. The threshold cycle number (Ct) was calculated using SDS software v.2.3 (Applied Biosystems) and an automatic setting of the baseline. These values were used for the calculation of the mitochondrial DNA copy numbers (mtDNA) expressed as $2\Delta Ct$, and where $\Delta Ct = Ct_{ACTB} - Ct_{MTND2}$, and as described previously (Ritov et al., 2005; Szuhai et al., 2001).

Mitochondrial respiration experiments of isolated rodent mitochondria treated with amino acids

All procedures were in accordance with the guidelines regarding the care and use of animals by the Institutional Animal Care and Use Committee at Mayo Clinic. Three C57BL/6J mice, 10-12 weeks old, were used for the experiments. Mice were euthanized by isoflourane inhalation and cervical dislocation. The left and right gastrocnemii were removed and immediately placed on an ice-cold petri dish, which was pre-rinsed with ice-cold Solution I (composition described above in “Isolation of skeletal muscle

mitochondria"). Mitochondrial isolation and measurement of mitochondrial respiration followed the procedures we describe for the human tissue. Only subsarcolemmal mitochondria were isolated for this set of experiments. After the isolation, the mitochondrial fraction was divided equally into two separate tubes. Mitochondrial protein content in each tube was determined by Lowry as previously described (Lowry et al., 1951) and was not different between isolation preparations from either SS mitochondrial fraction. One of the fractions was treated with the amino acids found in the amino acid solution infused in the human experiments, while the other was not (i.e., control). For the fraction treated with the amino acids, 40 μg of isolated mitochondrial protein was incubated with the amounts of amino acids found in 47 μl of the infused amino acid solution (15% Clinisol; Baxter Healthcare Corporation, Deerfield, IL). These amino acid amounts were based on theoretical calculations, and in a way that approximate exposure of muscle mitochondria to amino acids at concentrations measured in plasma during the amino acid solution infusion. Protein yield ($mg.g^{-1}$) in either of the final isolation preparations of SS mitochondria not treated with and treated with amino acids was not different; 6.3 ± 0.7 versus 5.49 ± 0.4, (n=3). A workflow diagram is presented in Figure 3-2.

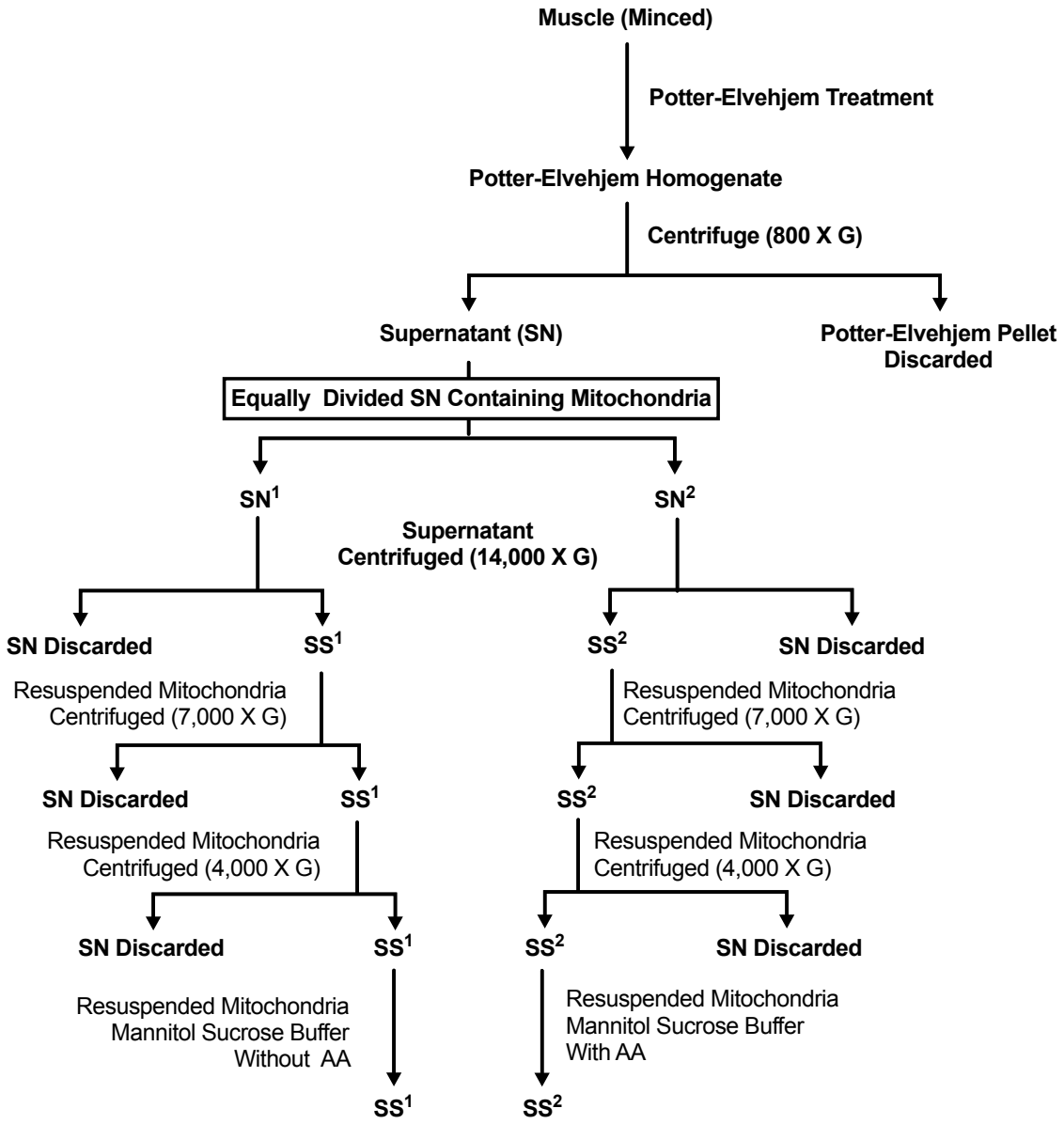

Figure 3-2. Isolation and amino acids treatment in isolated mouse muscle mitochondria. A flow diagram depicting the preparation of mechanically released subsarcolemmal (SS) mitochondria either exposed (SS^2) or not exposed (SS^1) to a 15% mixture of amino acids (AA). Details are given in the methods section of this chapter titled "*Mitochondrial respiration experiments of isolated rodent mitochondria treated with amino acids*"; (SN, Supernatant).

Mitochondrial fractions remained on ice until mitochondrial respiration measurements were performed at approximately 90 minutes following the treatment of the isolated mitochondria with the amino acids (preliminary experiments showed robust differences between control and amino acid-treated mitochondria after treatment of mitochondria with amino acids for at least 90 minutes). Mitochondrial respiration measurements were performed in triplicate (and in a random order between control and amino acid-treated mitochondria) from mitochondria isolated from the three mice, and by following the procedures we described in the methods section of above, "Assays for mitochondrial O_2 consumption and ATP production".

Plasma hormone, glucose and amino acid concentrations

Arterialized blood samples were analyzed for concentrations of plasma insulin and C-peptide using commercially available kits (ALPCO Diagnostics, Salem, NH) and glucose using an automated glucose analyzer (STAT 2300; Yellow Springs Instruments). Concentrations of the plasma AAs were measured using high-performance liquid chromatography (HPLC). In brief, plasma samples for the determination of AA concentrations were treated with an equal volume of 10% trichloracetic acid, followed by the derivatization of the samples and standards with o-phthalaldehyde (Carroll et al., 2005; Liu, 2000). Derivatized samples and standards were injected onto an Agilent 1100 Series HPLC system (G1312A and G1329A, Agilent Technologies, Santa Clara, CA, USA), and separation of AAs was achieved using a Shimadzu C18, 50mm x 4.6mm column (Shimadzu Scientific Instruments, Columbia, MD, USA). Peaks were monitored at 230nm excitation/450nm emission (Agilent Technologies, G1321A). The

concentration of individual AAs was determined by comparison with a standard curve developed from known AA concentrations of each AA.

Statistical analysis

Data are presented as means ± SEM. Area under the curve (AUC) values for variables of interest were calculated using the trapezoidal rule. Two-way with repeated measures ANOVA was used to test for the main effects of AAs and obesity, as well as their interaction on the variables of interest. Bonferroni correction was performed for multiple comparison tests. Non-paired two-tailed t tests were used for single measurements between lean and obese groups. Statistical significance was set at $P < 0.05$. Analyses were performed using the GraphPad Prism7 statistical software (GraphPad Software, San Diego, CA).

Results

Amino acid concentrations

Individual plasma AA (Aspartate, Glutamine, Asparagine, Serine, Glutamine, Threonine, Arginine, Tyrosine, Methionine, Valine, Phenylalanine, Isoleucine, Leucine) concentrations increased 2- to 6-fold in response to the AA mixture infusion ($P < 0.05$) in both lean and obese subjects (Table 3-2). The sum of the individual plasma AA concentrations, BCAA concentrations, and essential AA (EAA) concentrations, were all increased ($P < 0.001$) during the AA infusion when compared to the saline infusion in both lean and obese subjects (Figure 3-3).

Table 3-2. Plasma amino acids concentrations during saline and following amino acids infusion periods between lean and obese subjects.

	Lean		Obese	
Amino Acid	Saline Infusion	AA Infusion	Saline Infusion	AA Infusion
Aspartate	5±0	21±3[a]	12±1	31±3[a]
Glutamate	54±7	133±20[c]	148±7	172±24[b]
Asparagine	28±3	53±7[c]	48±3	43±2[d]
Serine	128±14	209±21[c]	196±5	183±18[c]
Glutamine	389±51	595±68[c]	786±46	525±39[c]
Threonine	179±24	334±41[b]	300±24	362±43[a]
Arginine	92±10	271±26[a]	138±3	263±29[a]
Tyrosine	55±5	85±9[d]	132±3	108±13[a]
Methionine	60±11	208±32[c]	114±12	299±42[c]
Valine	236±29	609±56[a]	474±21	718±69[b]
Phenylalanine	60±7	209±23[b]	110±4	297±29[a]
Isoleucine	73±15	221±19[b]	148±7	265±25[a]
Leucine	132±25	355±27[a]	244±9	387±37[a]

Table 3-2. Plasma amino acids concentations ($\mu mol.L^{-1}$) measured during saline and following amino acid infusion periods between lean and obese (n=10, lean) (n=11, obese). Data presented as mean ± SEM. Two-way with repeated measures ANOVA was used to analyze the data. Bonferroni correction was performed for multiple comparison tests ($^{a}P < 0.0001$, $^{b}P < 0.001$, $^{c}P < 0.01$, $^{d}P < 0.05$ versus saline infusion).

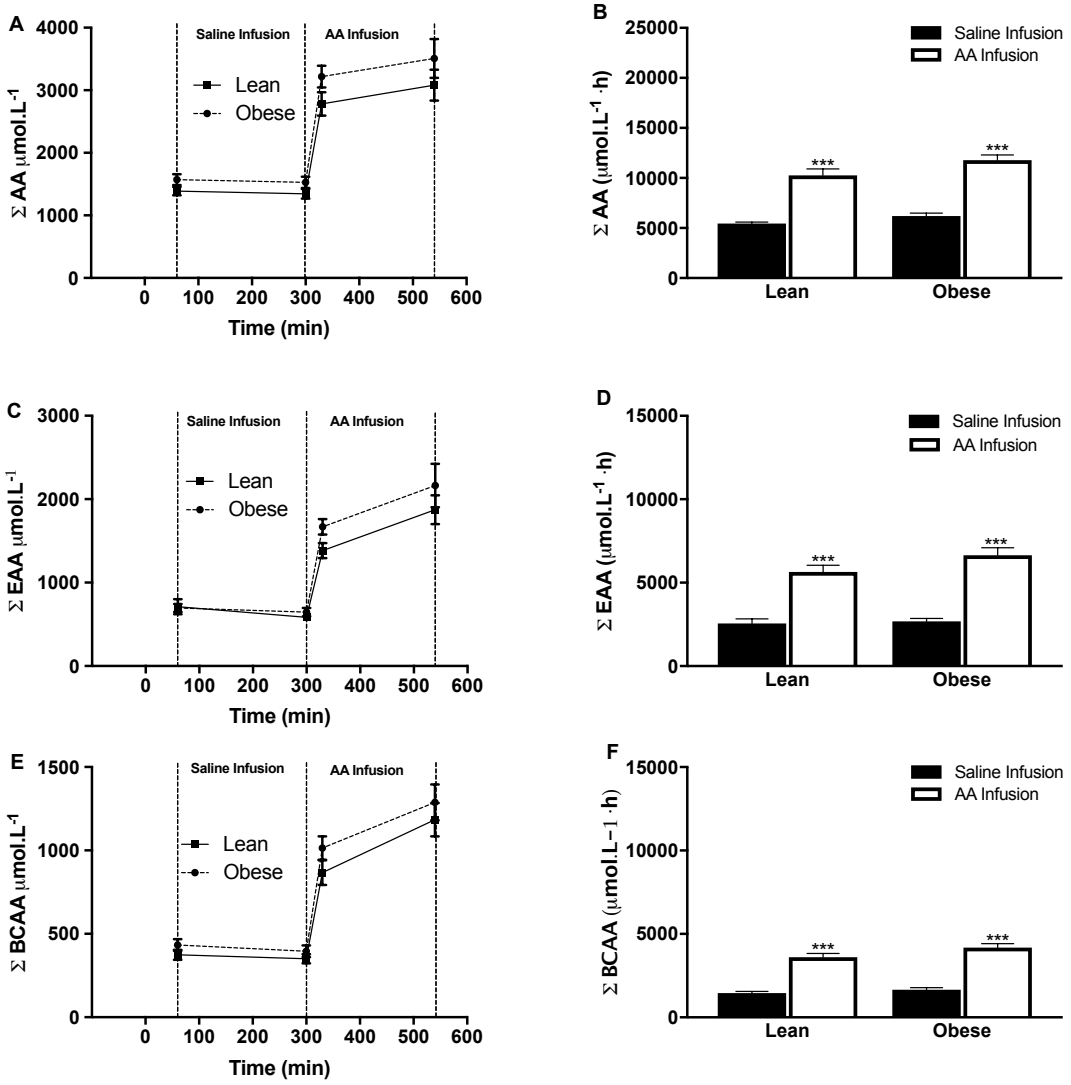

Figure 3-3. Sum of total amino acid (AA), essential amino acid (EAA), and branched-chain amino acid (BCAA) concentrations ($\mu mol.L^{-1}$) at the initiation of the experiments (t =60), at the end of the saline infusion period (t=300), 30 min after the start of the amino acid infusion (t=330), and at the end of the amino acid infusion period (t=540); A) all detected plasma amino acids (AA), C) essential amino acids (EAA), and E) branched-chain amino acids (BCAA) measured in plasma during saline or following amino acids infusion periods. Corresponding area under the curve (AUC) values ($\mu mol.L^{-1} \cdot h$) describing the B) AA , D) EAA, and F) BCAA responses during the saline and AA infusion periods. Data is presented as mean ± SEM. Two-way with repeated measures ANOVA was used to analyze the data. Bonferroni correction was performed for multiple comparison tests (***$P < 0.001$, versus saline infusion).

Hormones and glucose concentrations

Insulin

Plasma insulin responses during the saline and AA infusions are shown in Figure 3-4A. ANOVA revealed significant main effects for obesity ($P < 0.001$) and AAs ($P < 0.001$), as well as their interaction ($P < 0.01$) on the calculated plasma insulin AUC values. Multiple comparison tests indicated that plasma insulin AUC was greater in response to AA infusion when compared to that during the saline infusion within the obese group only (Figure 3-4B).

C-peptide

Plasma C-peptide responses during the saline and AA infusions are shown in Figure 3-4C. ANOVA revealed significant main effects for obesity ($P < 0.05$) and AAs ($P < 0.001$), as well as their interaction ($P < 0.05$) on the calculated C-peptide AUC values. Multiple comparisons tests showed that C-peptide AUC values were greater within both lean and obese groups during the AA infusion when compared to the saline infusion (Figure 3-4D).

Glucose

Plasma glucose responses during the saline and AA infusions are shown in Figure 3-4E. Plasma glucose AUC increased significantly during AA infusion when compared to that during saline infusion within the obese group only (Figure 3-4F).

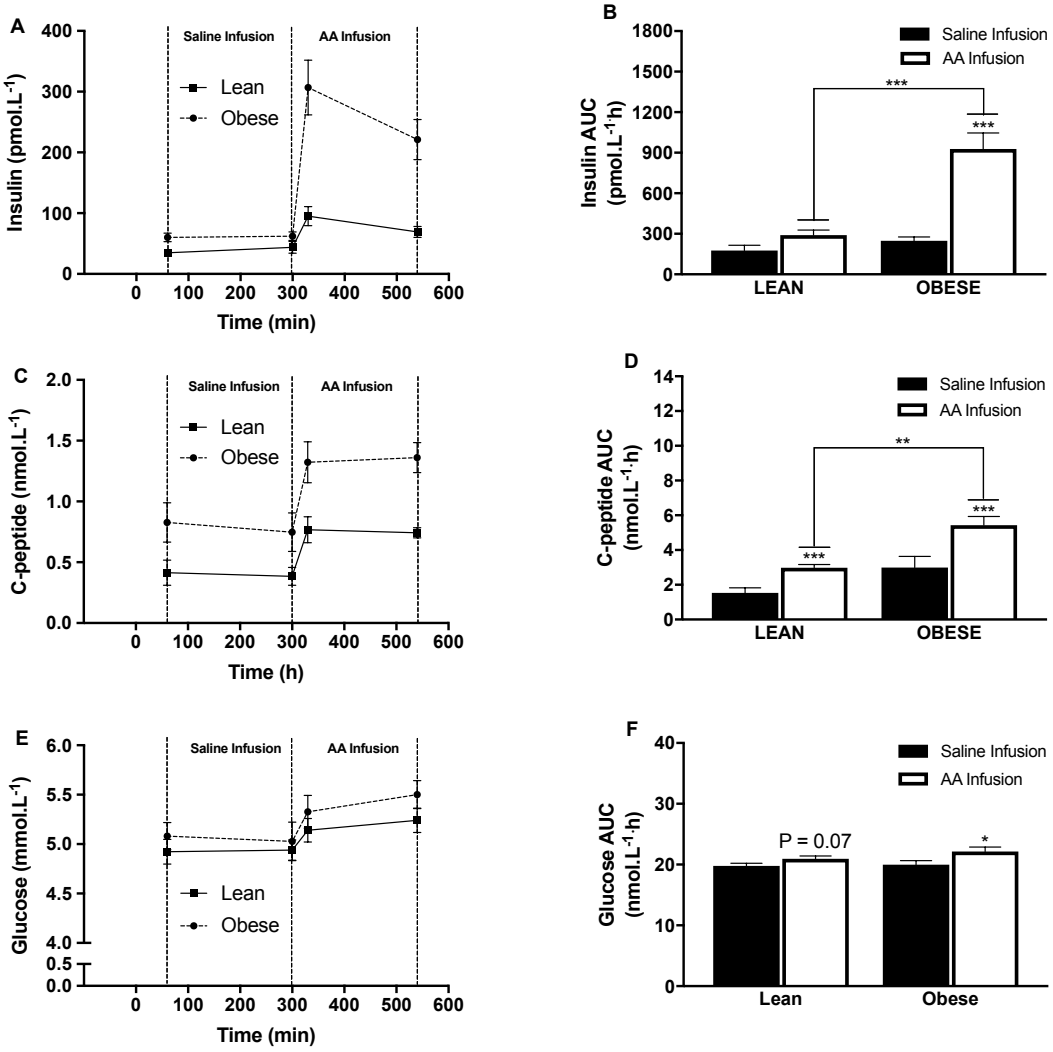

Figure 3-4. Hormone and glucose concentrations. Measurements were performed at the initiation of the experiments (t =60), at the end of the saline infusion period (t=300), 30 minutes after the start of the AA infusion (t=330), and at the end of the AA infusion period (t=540) for either A) insulin (pmol.L^{-1}), C) C-peptide (nmol.L^{-1}), or E) glucose (mmol.L^{-1}). The corresponding area under the curve (AUC) values for either B) insulin (pmol.L^{-1}·h) or D) C-peptide (nmol.L^{-1}·h) describing the hormone and F) glucose responses during the saline and AA infusion periods. Data is presented as mean ± SEM. Two-way with repeated measures ANOVA was used to analyze the data. Bonferroni correction was performed for multiple comparison tests (***P < 0.001, **P < 0.01, *P < 0.05 versus saline infusion, unless otherwise shown).

Mitochondrial protein and citrate synthase activity

No significant main effects or interaction were found for protein concentration in either the SS or IMF mitochondrial fractions ($P > 0.05$; Table 3-3). Similarly, no significant main effects or interaction were found for citrate synthase activity, a marker of mitochondrial content (Larsen et al., 2012), or citrate synthase specific activity in either the SS or IMF mitochondrial fractions ($P > 0.05$; Table 3-3). Furthermore, no differences were found for mtDNA content between lean and obese subjects (Figure 3-5). These findings for comparable muscle mitochondria content between lean and obese are in line with those from other investigations with subjects of similar age (Fisher-Wellman et al., 2014; Karakelides et al., 2010; Samjoo et al., 2013).

Table 3-3. Protein and citrate synthase activity yields as well as citrate synthase specific activity in the subsarcolemmal and intermyofibrillar mitochondrial fractions during saline (i.e., control) and amino acid infusions.

	Lean		Obese	
	Saline Infusion	AA Infusion	Saline Infusion	AA Infusion
Protein (mg·g wet muscle^{-1})				
Subsarcolemmal	3.1 ± 0.2	3.1 ± 0.2	3.2 ± 0.4	2.8 ± 0.4
Intermyofibrillar	2.1 ± 0.4	2.0 ± 0.3	2.2 ± 0.3	2.4 ± 0.4
CS activity (μmol·min^{-1}·g^{-1} wet muscle)				
Subsarcolemmal	2.2 ± 0.2	2.1 ± 0.3	2.2 ± 0.3	1.8 ± 0.3
Intermyofibrillar	1.3 ± 0.2	1.5 ± 0.3	1.5 ± 0.2	1.6 ± 0.2
CS specific activity (μmol·min-1·mg^{-1})				
Subsarcolemmal	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
Intermyofibrillar	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

Table 3-3. Protein yield and enzyme activities determined in subsarcolemmal and intermyofibrillar mitochondria during saline and following amino acids infusion periods between lean and obese. No significant effects were detected in any of the measurements for protein yield (mg·g wet muscle^{-1}), total citrate synthase activity ((μmol·min^{-1}·g^{-1} wet muscle), or citrate synthase specific activity (μmol·min-1·mg^{-1}), during saline or following amino acids infusion periods in either group. Data is presented as mean ± SEM; $P > 0.05$; CS, citrate synthase. Two-way with repeated measures ANOVA was used to analyze the data.

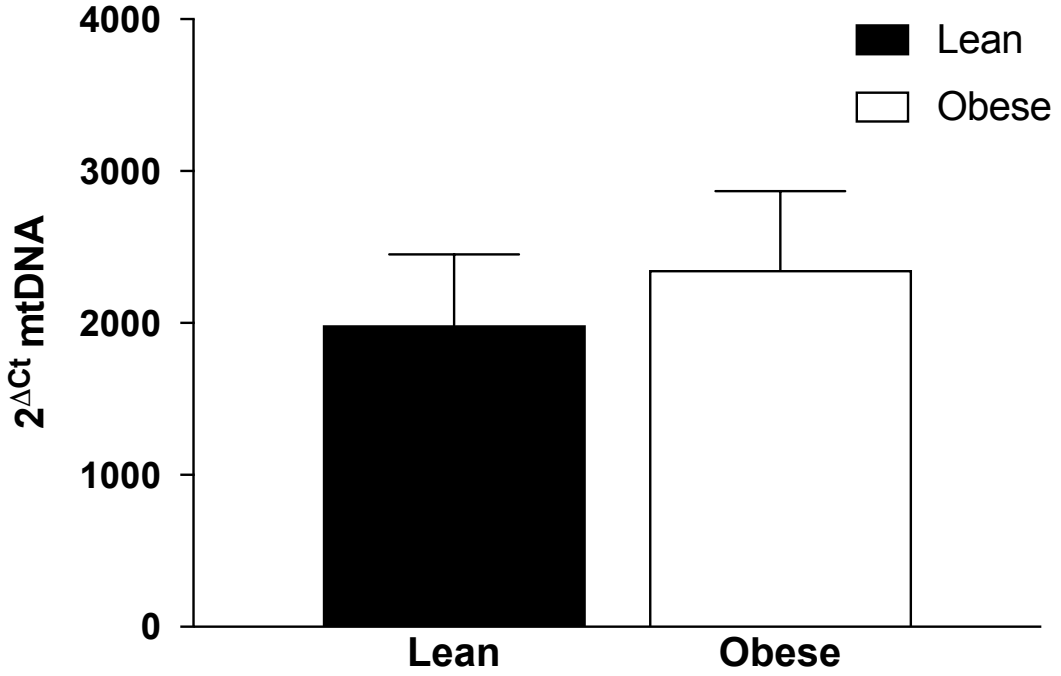

Figure 3-5. Baseline mitochondrial DNA (mtDNA) copy numbers between the lean and obese subjects using primers and probes directed to mitochondrial NADH-ubiquinone oxidoreductase chain 2 (MT-ND2) and normalized to nuclear beta-actin (ACTB). Data presented as mean ± SEM.

Maximal (state 3) J_o and ATP production

We detected no significant main effects for either obesity or AAs on state 3 J_O in SS mitochondria, and when using either malate+pyruvate+glutamate (MPG) or succinate (SUCC) substrates ($P > 0.05$). However, there was significant interaction between factors ($P < 0.01$). Multiple comparison tests revealed increase in state 3 J_O in the lean, but not obese, subjects in response to the AA infusion (Figure 3-6A and C). Similar to the finding in state 3 J_O in SS mitochondria, state 3 J_P increased in the SS mitochondria using MPG, and trending towards significance using SUCC ($P=0.066$), in lean, but not obese, subjects (Figure 3-6E and G). Also, the findings were the same when state 3 J_O and J_P were expressed relative to muscle wet weight instead of mitochondrial protein (Figure 3-7). No significant differences were detected in state 3 J_O and J_P for the IMF mitochondria within or between groups ($P > 0.05$; Figure 3-6, Figure 3-7; right panels).

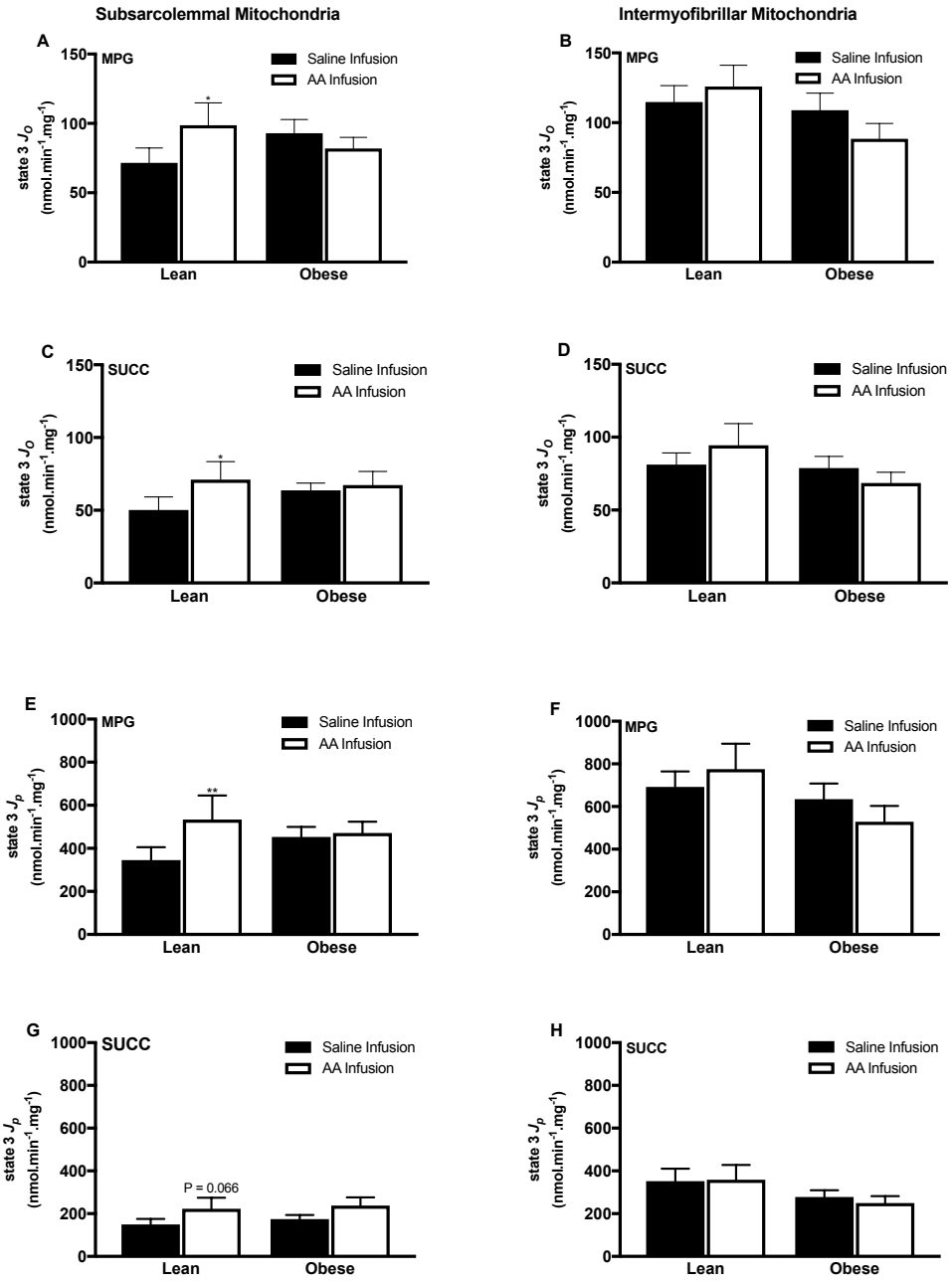

Figure 3-6. State 3 O_2 consumption (State 3 J_O) and ATP production (State 3 J_P) per milligram (mg) of isolated protein. State 3 J_O and J_P of subsarcolemmal (left side) and intermyofibrillar (right side) mitochondria were measured at the end of the saline (Saline Infusion) and AA (AA Infusion) infusion periods. State 3 J_O and J_P were measured in the presence of Malate (1 mM) + Pyruvate (1 mM) + Glutamate (10 mM) (MPG) or Succinate (10 mM) (SUCC) as substrates, and maximal rates for J_O and J_P were stimulated by addition of ADP (0.67 mM). Data is presented as mean ± SEM. Two-way with repeated measures ANOVA was used to analyze the data. Bonferroni correction was performed for multiple comparison tests (**$P < 0.01$, *$P < 0.05$ versus saline infusion).

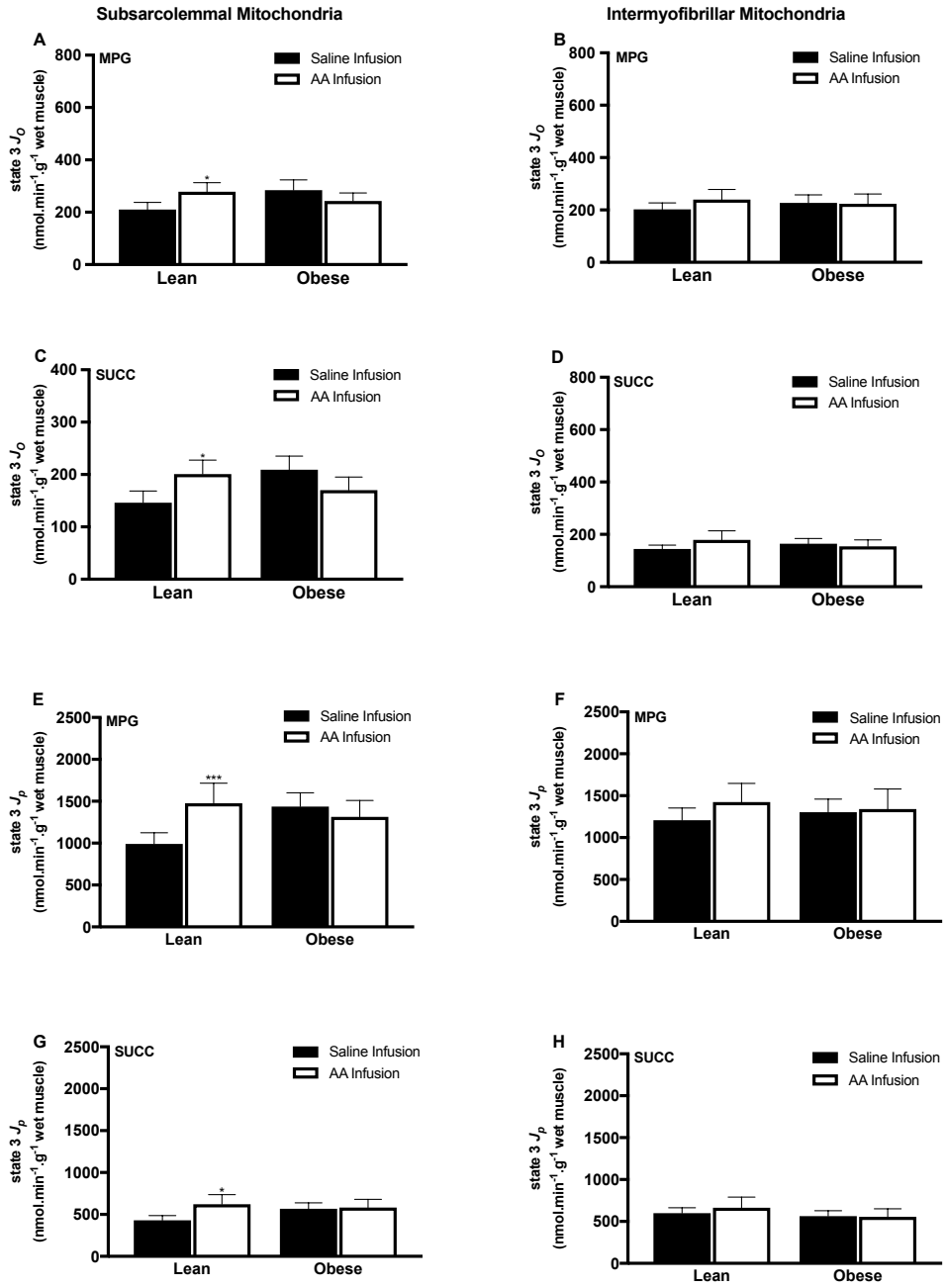

Figure 3-7. State 3 O_2 consumption (State 3 J_O) and ATP production (State 3 J_P) per gram (g) of wet muscle. State 3 J_O and J_P of subsarcolemmal (left side) and intermyofibrillar (right side) mitochondria were measured at the end of the saline (Saline Infusion) and AA (AA Infusion) infusion periods. State 3 J_O and J_P were measured in the presence of Malate (1 mM) + Pyruvate (1 mM) + Glutamate (10 mM) (MPG) or Succinate (10 mM) (SUCC) as substrates, and maximal rates for J_O and J_P were stimulated by addition of ADP (0.67 mM). Data is presented as mean ± SEM. Two-way with repeated measures ANOVA was used to analyze the data. Bonferroni correction was performed for multiple comparison tests (**$P < 0.01$, *$P < 0.05$ versus saline infusion).

"Resting" (states 2 and 4) J_O

State 2 J_O of SS mitochondria did not differ between lean and obese prior to the AA infusion, but it increased in the lean subjects only in response to the AA infusion (Figure 3-8A). State 4 J_O of SS mitochondria did not differ between lean and obese prior to the AA infusion, but it increased in the lean subjects only in response to the AA infusion (Figure 3-8E and G). No differences were detected for either state 2 J_O or state 4 J_O in the IMF mitochondria either within or between groups ($P > 0.05$; Figure 3-8; right panel).

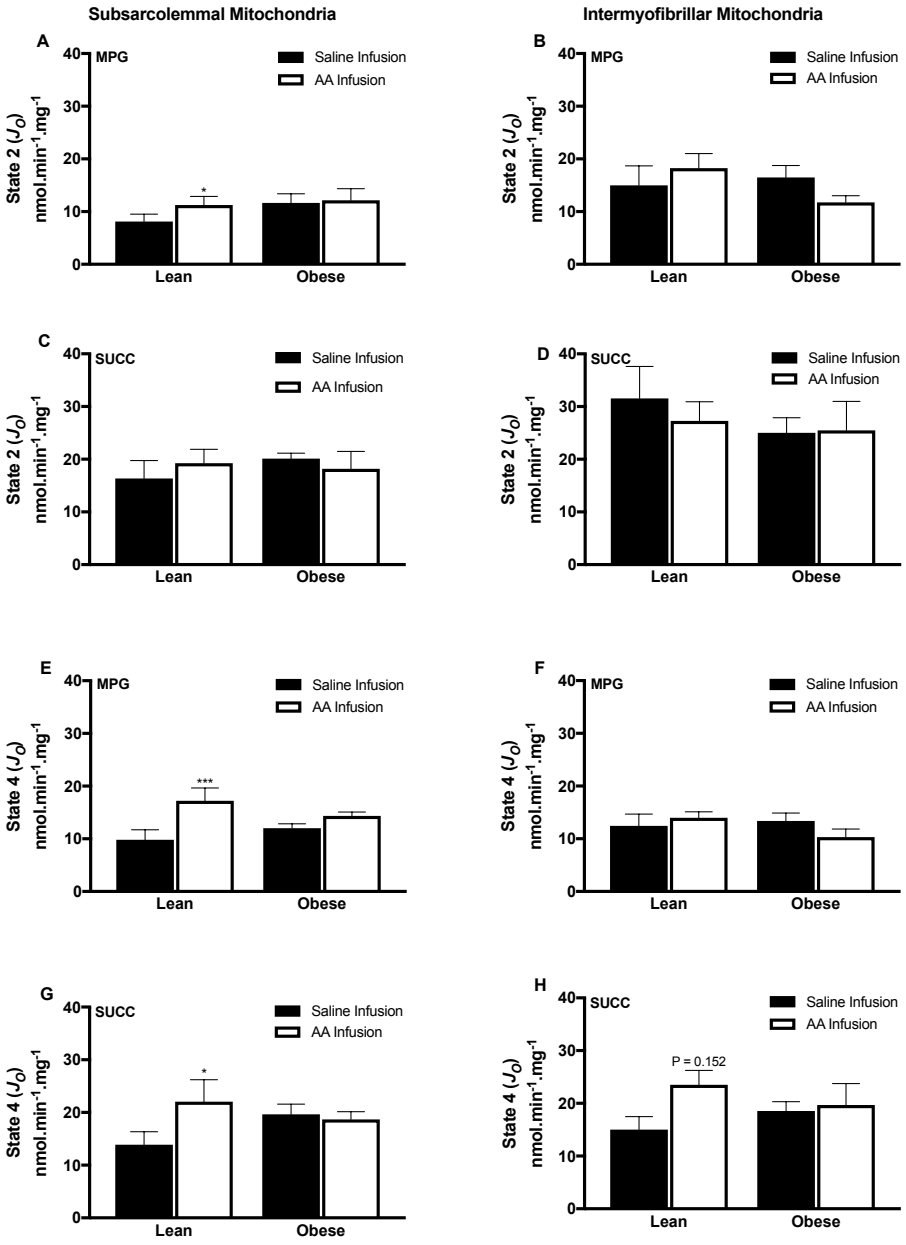

Figure 3-8. State 2 (State 2 J_O) and state 4 (State 4 J_O) O_2 consumption. State 2 J_O and state 4 J_O of subsarcolemmal (left side) and intermyofibrillar (right side) mitochondria were measured at the end of the saline (Saline Infusion) and AA (AA Infusion) infusion periods. State 2 J_O was measured in the presence of Malate (1 mM) + Pyruvate (1 mM) + Glutamate (10 mM) (MPG) or Succinate (10 mM) (SUCC) as substrates. State 4 J_O was measured following state 3 J_O after depletion of ADP in the medium. Data is presented as mean ± SEM. Two-way with repeated measures ANOVA was used to analyze the data. Bonferroni correction was performed for multiple comparison tests (***$P < 0.001$, *$P < 0.05$ versus saline infusion).

Respiratory control ratio (RCR) and ratio of phosphorylation of ADP to Oxygen (ADP/O)

ANOVA showed significant main effect of AAs on the calculated respiratory control ratio (RCR) (i.e., state 3 J_O/state 4 J_O) in SS mitochondria with MPG ($P < 0.01$). Multiple comparison tests indicated an ~ 30% decrease in RCR in response to the AA infusion in the lean subjects only (Figure 3-9A). No significant differences were detected for RCR in the IMF mitochondria within or between groups ($P > 0.05$) (Figure 3-9; right panels). ADP/O data are presented in Figure 3-9E thru H. ANOVA showed a significant main effect of AAs on ADP/O in SS mitochondria with MPG (Figure 3-9E) ($P < 0.01$). Multiple comparison tests indicated significant increase in ADP/O in response to AA in the obese subjects only (Figure 3-9E). No significant differences were detected for ADP/O in the IMF mitochondria within or between groups (Figure 3-9; right panel) ($P > 0.05$).

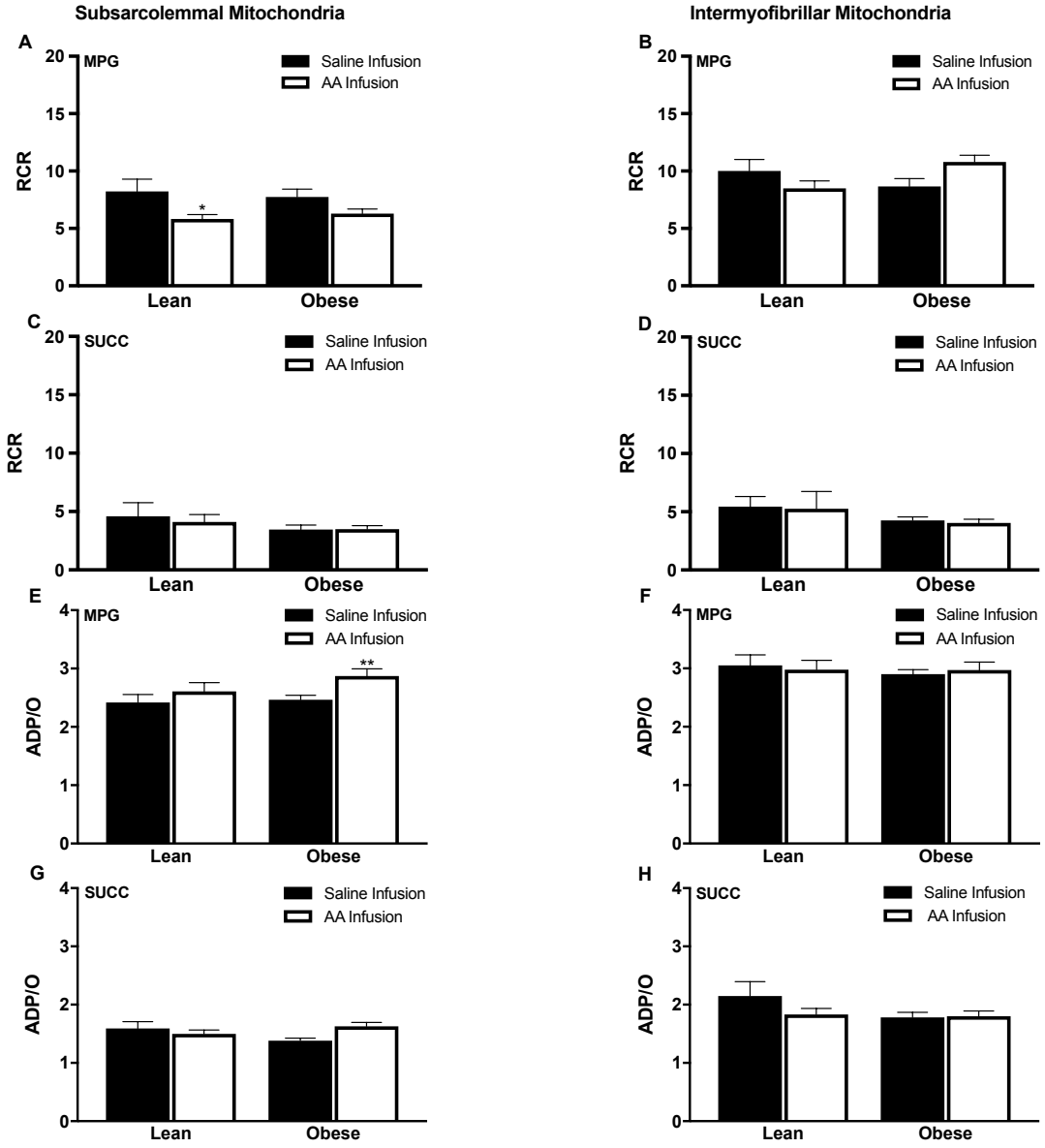

Figure 3-9. Respiratory control (RCR); (state 3 J_O/state 4 J_O) and ADP/O ratios. RCR and ADP/O were measured in the presence of Malate (1 mM) + Pyruvate (1 mM) + Glutamate (10 mM) (MPG) or Succinate (10 mM) (SUCC) as substrates; Data presented as mean ± SEM. Two-way with repeated measures ANOVA was used to analyze the data. Bonferroni correction was performed for multiple comparison tests (**P < 0.01, *P < 0.05 versus saline infusion).

Maximal (state 3) J_o, ATP production (J_P) from isolated subsarcolemmal mitochondria from rodent gastrocnemius treated with amino acids

Representative oxygraph traces are shown in Figure 3-10A and B. The state 3 J_O of SS mitochondria not treated with 15% mixture of amino acids (-AA) was lower compared to the state 3 J_O of SS mitochondria treated with amino acids (+AA) when using either malate+pyruvate+glutamate (MPG) (Figure 3-10C and D). As expected, the ATP production rate was also higher in SS mitochondria treated with amino acids when compared to SS mitochondria not treated with amino acids (Figure 3-10D.) Our results show that treating isolated mitochondria from mouse gastrocnemius, with a 15% mixture of AA, increased rates of ADP stimulated oxygen consumption (state 3 J_O) as well as improved the rate of ATP production (state 3 J_P) (Figure 3-10). It is worth noting; treating isolated mitochondria with AA for 30 minutes on ice resulted in no significant changes in maximum ADP stimulated state 3 Jo. However, changes in state 3 Jo were observed after a time exceeding 90 minutes of 15% amino acid incubation.

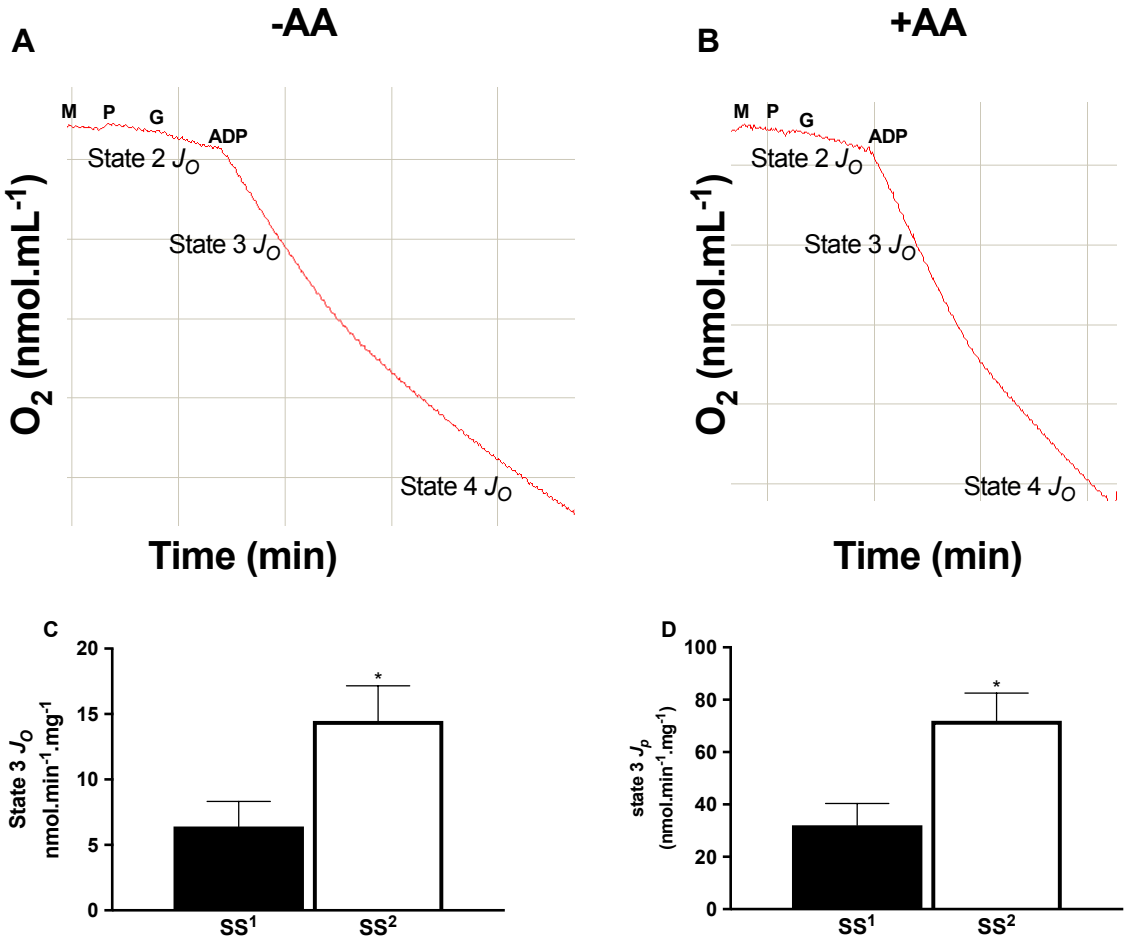

Figure 3-10. Representative oxygraph traces of mitochondrial respiration of isolated rodent mitochondria A) not treated (-AA) or B) treated (+AA) with amino acids obtained using an oxygraph respirometer (Hansatech Instruments, Norfolk, UK). Malate (M; 1 mM) + pyruvate (P; 1 mM) + glutamate (G; 10 mM) were added and State 2 J_O, State 2 O_2 consumption, was followed in both –AA and +AA SS mitochondria. State 3 J_O was initiated with the addition of ADP (0.67 mM), and the maximal state 3 J_O rate was measured in the – AA and + AA. Following the phosphorylation of available ADP, -AA and +AA SS mitochondria returned to "resting state" and the state 4 J_O, State 4 O_2 consumption, was followed for ADP/O and respiratory control ratio determinations. Average maximal state 3 Jo and state 3 Jp measurements associated with mitochondrial respiration in isolated SS mitochondria from mouse gastrocnemius (n=3) either C) not treated or D) treated with amino acids; SS mitochondria not treated with amino acids (SS^1); SS mitochondria treated with amino acids (SS^2); Data is presented as mean ± SEM. *P < 0.05).

"Resting" (states 2 and 4) J_O, RCR and ADP/O in isolated SS mitochondria from mouse treated with amino acids

Both the state 2 J_O and state 4 J_O in isolated SS mitochondria from mouse gastrocnemius increased in response to the AA treatment (Figure 3-11A and B). However there were no significant differences in the ADP/O or RCR in response to the AA treatment in isolated mitochondria from mouse (Figure 3-11C and D).

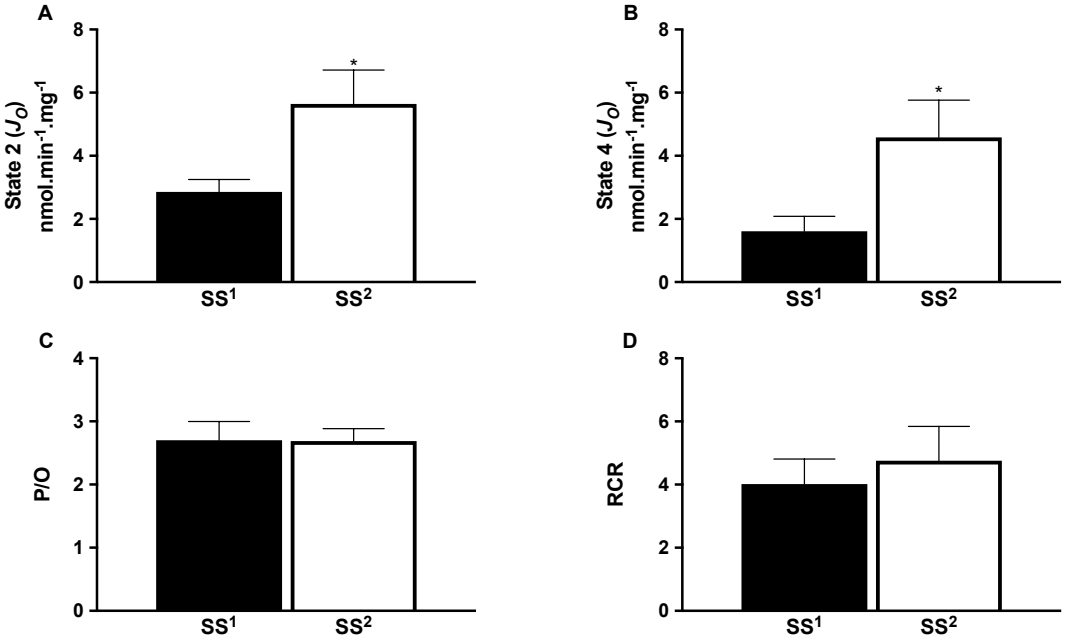

Figure 3-11. The state 2 Jo and State 4 Jo and functional indices of isolated SS mitochondria from mouse in response to amino acid treatment. A) State 2 J_O and B) state 4 J_O of subsarcolemmal (SS) mitochondria either not treated (SS^1) or treated (SS^2) with amino acids. State 2 J_O was measured in the presence of Malate (1 mM) + Pyruvate (1 mM) + Glutamate (10 mM) (MPG) as substrates. State 4 J_O was measured following state 3 J_O after depletion of ADP (0.67 mM) in the medium. RCR and ADP/O of subsarcolemmal (SS) mitochondria either not treated (SS^1) or treated (SS^2) with amino acids were measured in the presence of MPG. The Data is presented as mean ± SEM; (*$P < 0.05$).

Discussion

The current study examined the effects of elevated plasma AA concentration on skeletal muscle mitochondrial function in sedentary lean and obese adults. The main finding shows elevated plasma AA concentrations increase capacity of ATP production of SS mitochondria from muscle of lean participants but not obese subjects. Moreover, only the lean subjects showed increase in uncoupled respiration in response to elevation of plasma amino acids.

Current evidence suggest that elevation of plasma amino acids enhances the capacity of subsarcolemmal fraction of muscle mitochondria to produce ATP (Tatpati et al., 2010), and our results add further support to this evidence. Furthermore, whereas previous findings were limited to ATP production (Tatpati et al., 2010), we have measured mitochondrial respiration to more fully characterize mitochondrial function in skeletal muscle. In this regard, increased capacity for ATP production in the presence of elevated plasma amino acids in SS mitochondria in the lean subjects is supported by an increased state 3 Jo in this mitochondrial subpopulation.

Based on the mitochondrial substrates used, our results show that greater mitochondrial oxygen consumption in the SS mitochondria of the lean subjects when plasma amino acids increased was mediated by increased catalytic potential of both complex I (malate+pyruvate+glutamate as substrates) and complex II (succinate as substrate) of the mitochondrial electron transport chain. However, about 85% of muscle mitochondria are located in the interior of the cell proximal to the myofibrils, intermyofibrillar mitochondria (Hood, 2001). Because maximal mitochondrial oxygen consumption (and ATP production) did not improve in the IMF mitochondria under the

same conditions, our findings reveal a role of plasma amino acids in regulating specifically the SS compartment of the muscle mitochondrial reticulum. In this regard, we show that SS mitochondria in lean subjects are more responsive than IMF mitochondria in modifying their catalytic potential in response to increased plasma amino acids concentrations.

Reduced responsiveness of muscle SS mitochondria in the obese subjects to the elevated plasma AAs could have possibly resulted from reduced transport of plasma AAs into the muscle, secondary to reduced muscle AA transporters in the obese subjects. Although whether such a mechanism is implicated in the present findings remains to be determined, current evidence suggests that the content of skeletal muscle AA transporters does not differ between lean and obese subjects at basal or during elevated plasma AAs (Beals et al., 2016). On the other hand, lack of stimulation of mitochondrial maximal oxygen consumption by the plasma AAs in the obese subjects may be, at least in part, due to a trend for an already elevated respiratory capacity in these subjects at basal. Specifically, state 3 J_O in SS mitochondria of the obese subjects during saline infusion was 94% of the AA-stimulated state 3 J_O in SS mitochondria of the lean subjects. Our results, therefore, during saline infusion do not show mitochondrial dysfunction previously reported in obese subjects (Abdul-Ghani et al., 2009; Kelley et al., 2002), but are in agreement with other findings showing no differences in muscle mitochondrial function with obesity (Fisher-Wellman et al., 2014; Karakelides et al., 2010; Samjoo et al., 2013). In line with our findings in humans, basal state mitochondrial enzyme activities in rodents were 20-50% higher in the presence of increased muscle lipid availability (Turner et al., 2009), which is a typical observation in obese humans as well

(Malenfant et al., 2001). Current evidence suggests that mitochondria complexes in skeletal muscle in obese humans are exposed to relative greater amounts of redox cofactors (i.e., NADH) (Lefort et al., 2010). Therefore, it is possible that increased availability of mitochondrial substrate in the basal state in the muscle of the obese subjects provides a constant stimulus that increases the catalytic potential of mitochondrial electron transport chain complexes, thus minimizing the stimulation of these complexes by the increased plasma AA concentrations.

In addition to the utilization of protons to generate ATP via the ATP synthase complex, mitochondria also demonstrate proton leak (i.e., uncoupled respiration) across the mitochondrial inner membrane, running in parallel with the ATP synthesis. In this regard, measures of state 4 J_O in isolated mitochondria indicate mainly proton leak (Nicholls, 1977). Previous reports have documented increased proton leak in response to fasting (Mollica et al., 2006) and acute exercise (Madsen, Ertbjerg, & Pedersen, 1996). In the present studies, state 4 J_O increased (~ 75% with MPG) in response to the AA infusion in the SS mitochondria of the lean subjects only. Because state 4 J_O did not change in IMF mitochondria, our results show that SS mitochondria have greater flexibility than IMF mitochondria to modify uncoupled respiration in response to acute increase in plasma AAs. It is noted that state 2 J_O, which also reflects proton leak in the presence of reducing substrate(s) but absence of ADP (Makrecka-Kuka, Krumschnabel, & Gnaiger, 2015), and, therefore, is not affected by recycled ADP present during the state 4 J_O measurements, was also significantly higher in response to the increase in plasma AAs. To our knowledge, this study provides the first evidence showing that increased

plasma AA concentrations enhance uncoupled respiration in human skeletal muscle mitochondria.

Our finding related to state 4 J_O show that acute elevation of plasma AAs induces "metabolic inefficiency" in the SS mitochondria of lean subjects. From a physiological perspective, this suggests increased capacity for fuel/energy utilization in SS mitochondria in the muscle of these subjects. It has been argued that such a response may have beneficial effects because it can limit the generation of reactive oxygen species due to excess nutrients (Liesa & Shirihai, 2013), and also prevent muscle nutrient overload observed in pathophysiological circumstances (Iossa et al., 2004). In line with our finding for increased uncoupled respiration at the mitochondrial level, AA infusion acutely increases whole body oxygen consumption and energy expenditure (Weissman et al., 1983). Therefore, failure to increase uncoupled respiration in obese individuals in response to increased plasma AA concentrations (i.e., during the postprandial period) may impact the ability of these individuals to increase their overall energy expenditure and regulate body weight.

Because insulin alone stimulates mitochondrial ATP production (Stump et al., 2003), enhanced mitochondrial function may be mediated by increased plasma insulin concentrations during the AA infusion. However, plasma insulin concentrations were not significantly higher when mitochondrial respiratory capacity increased during AA infusion compared to saline infusion in the lean subjects. Furthermore, insulin decreases proton leak (Nisr & Affourtit, 2014), which is opposite from what we observed in the lean subjects after AA infusion in the present study. Increased proton leak in the present study, therefore, would be the direct result of the increase in plasma AA concentrations,

and in the same manner that fatty acids alone increase mitochondrial uncoupling/proton leak (Tonkonogi, Krook, Walsh, & Sahlin, 2000). Using rodent muscle we found that treating isolated mitochondria with AAs increased mitochondrial respiration (Figure 3-10). These findings, together with evidence for improved mitochondrial function in cell lines treated with the amino acid leucine (Sun & Zemel, 2009), indicate a possibly independent role of plasma AAs in enhancing mitochondrial function. In this regard, protein posttranslational modifications appear as a likely mechanism involved in the observed effects of plasma AAs on mitochondria. Future studies are warranted to examine the effects of AAs in modifying directly the activity of individual mitochondrial proteins.

A limitation of the present study is that complex II activity was evaluated without the presence of rotenone. This results in the formation of oxaloacetate that inhibits succinate dehydrogenase, and when compared to the experimental condition where succinate measurements are performed in the presence of rotenone (Gnaiger, 2014). However, we still found an increase in respiratory capacity (i.e., state 3 J_O) under our experimental conditions in the lean but not obese subjects. It is possible that, as recent evidence indicates, robust respiration can be measured without rotenone at least up to a certain respiratory state (Bai, Fink, Yu, & Sivitz, 2016). Regardless, our overall findings, and when considering that the same effect of elevated plasma AAs on mitochondrial respiration was observed for Complex I activity, show stimulation of mitochondria function by plasma AA in lean but not obese humans.

In conclusion, our results describe specificity of plasma AAs in stimulating respiration of SS (but not IMF) mitochondria, and specifically enhancing uncoupled

respiration (i.e., state 4 J_O) while sustaining increased capacity for ATP production (i.e., state 3 J_O and J_P). However, none of these effects of plasma AAs were observed in obese individuals. Reduced response of uncoupled respiration to increased plasma AA concentrations may play a role in attenuating overall energy expenditure in obese individuals.

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CHAPTER 4: PROTEOME DIFFERENCES IN SKELETAL MUSCLE MITOCHONDRIA BETWEEN LEAN AND OBESE HUMANS

Abstract

Skeletal muscle is largely responsible for regulating whole-body energy metabolism. Mitochondrial dysfunction and dysregulation of energy metabolism is implicated in the etiology of obesity. We sought to investigate differences in the proteome of skeletal muscle mitochondria between lean and obese individuals. We hypothesized that obesity would result in a reduction in the abundance, as well as redistribution of mitochondrial proteins, within the mitochondrial reticulum, composed of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. We isolated SS and IMF mitochondrial fractions from *vastus lateralis* muscle biopsies using standard differential centrifugation techniques from sedentary lean (n=16; 9M/7F) and obese (n=17; 9M/8F) subjects (age: 33±3 vs. 31±2 years, mean±SE, $P > 0.05$; BMI: 23±1 vs. 34±1 kg/m^2, $P < 0.05$; Matsuda insulin-sensitivity index: 10.1±1.5 vs. 4.2±0.8, $P < 0.05$). Our study identified 674 and 550 mitochondrial specific proteins with at least two peptides in SS mitochondria and IMF mitochondria, respectively. The total abundance of normalized spectra in SS and IMF mitochondria was 2% and 9% lower, respectively, in muscle of obese subjects. We found 73 and 41 mitochondrial proteins from SS mitochondria and IMF mitochondria, respectively, exhibiting differential expression between the lean and obese subjects. Pathway analysis of the differentially expressed mitochondria proteins in SS and IMF mitochondria of obese by Kyoto encyclopedia of genes and genomes (KEGG) and REACTOME software revealed significant enrichment of the following

mitochondrial biological pathways ($q < 0.01$): citric acid cycle (TCA), electron transport chain (ETC), amino acid metabolism, and pyruvate metabolism. Additionally, the differentially expressed mitochondria proteins in IMF mitochondria obese subjects were associated with significant enrichment in mitochondrial biological pathways ($P < 0.01$) specific to IMF mitochondria from obese subjects including, metabolism of lipids, electron transport, and adenosine triphosphate (ATP) synthesis. Finally, our study shows the abundance of proteins associated with mitochondrial proton-motive force generation through NADH: ubiquinone oxidoreductase (complex I), in the cell periphery (i.e. SS mitochondria), is not different between lean and obese. However, the level of proteins assigned to ATP synthase (complex V), proteins that use mitochondrial proton-motive force for the phosphorylation of adenosine diphosphate (ADP), in the cell interior (i.e. IMF mitochondria), is lower in obese subjects. Overall our findings suggest obesity is associated with protein composition changes in SS and IMF mitochondria associated with key biological processes responsible for regulating energy metabolism in skeletal muscle.

Introduction

Obesity is the underlying cause for diabetes, heart disease, cancer and impacts skeletal muscle mitochondrial function (K. Hojlund et al., 2010; Kim, Hickner, Cortright, Dohm, & Houmard, 2000; Toledo, 2014). Skeletal muscle mitochondria are unique with respect to their arrangement within myocytes, forming a continuous network or reticulum. Specifically, subsarcolemmal (SS) mitochondria are located near the periphery, while intermyofibrillar (IMF) are located near the myofibrils (Bakeeva, Chentsov YuS, & Skulachev, 1978; Glancy et al., 2015; Kayar, Hoppeler, Mermod, & Weibel, 1988; Kirkwood, Munn, & Brooks, 1986; T. Ogata & Yamasaki, 1997; Patel,

Glancy, & Balaban, 2016). Obesity results from an energy surplus. The excess energy is speculated to reside in the accumulation of lipid droplets within skeletal muscle (Gray, Tanner, Pories, MacDonald, & Houmard, 2003; Morino et al., 2005; V. B. Schrauwen-Hinderling et al., 2007; Vera B Schrauwen-Hinderling, Hesselink, Schrauwen, & Kooi, 2006; Toledo et al., 2008) and is associated with insulin resistance and impaired energy metabolism (Amati et al., 2011). Energy metabolism imbalance in muscle may in part be due to alterations within the mitochondria reticulum, in turn affecting the ability of myocytes to regulate the intracellular energy metabolism within skeletal muscle.

Recent evidence suggests the mitochondrial reticulum in skeletal muscle provides the pathway to transfer intracellular energy rapidly throughout the mitochondria network (Amchenkova, Bakeeva, Chentsov, Skulachev, & Zorov, 1988; Glancy et al., 2015; Patel et al., 2016). In other words, protein stoichiometry of SS mitochondria near the plasma membrane may play a critical role in the development of the proton-motive force, while the protein stoichiometry of the IMF mitochondria promotes the utilization of the proton-motive force. Impaired mitochondria oxidative phosphorylation (OXPHOS) has been previously suggested to be due to abnormal phosphorylation at specific sites of enzymes involved in the catalysis of ATP production (K. Hojlund et al., 2010). In addition, studies have shown reduced content and functional capacities of muscle mitochondria (Kurt Hojlund et al., 2008; Kelley, He, Menshikova, & Ritov, 2002; Mogensen et al., 2007; Ritov et al., 2005) in obesity. The extent to which obesity alters the stoichiometry of proteins in SS mitochondria and IMF mitochondria in obesity requires further investigation.

Preserving tightly-coupled bioenergetics functions in mitochondria isolated from skeletal muscle is possible (Cogswell, Stevens, & Hood, 1993; D. a. Hood, 1996; Kras et al., 2016; Palmer, Tandler, & Hoppel, 1977, 1985). However, the compartmentalized nature of mitochondria in skeletal muscle, SS mitochondria region and IMF mitochondria region, requires specialized fractionation techniques that make possible independent isolation of SS and IMF mitochondria. Studying SS and IMF mitochondria independently from muscle of lean and obese individuals may reveal differences in skeletal muscle mitochondria content with respect to location within the myocyte (i.e. SS or IMF mitochondria) along with differences in protein composition related to the capacity for energy distribution through out the myocyte at rest. Revealing the proteome of SS and IMF mitochondria between lean and obese individuals may provide insight into the following observations with respect to the proteins and pathways altered by obesity, specifically: 1) lower abundance of electron transport chain enzymes (Lefort et al., 2010); 2) reduced ATP production (Abdul-Ghani et al., 2009; Jheng et al., 2012; Karakelides et al., 2010; Minet & Gaster, 2010); 3) reduced amino acid catabolism (Katsanos & Mandarino, 2011; Koves et al., 2008; Newgard et al., 2009); 4) reduced lipid oxidation (Boyle, Zheng, Anderson, Neufer, & Houmard, 2012; Kim et al., 2000); and 5) insulin resistance (De Filippis et al., 2008; Evans, Maddux, & Goldfine, 2005; Karakelides et al., 2010).

Previous studies have focused on the global picture of changes in protein abundance in skeletal muscle in obesity by way of whole muscle tissue homogenates which are further separated by two-dimensional electrophoresis (Giebelstein et al., 2012; Théron et al., 2014). These approaches are laborious and costly. Other approaches,

involve fractionation of cellular components, such as mitochondrial proteins (Ferreira et al., 2010; K. Hojlund et al., 2010; Lefort et al., 2010; Ma et al., 2017). These studies are mostly based on two dimensional gel electrophoresis approach, with exception of Ma et al. (Ma et al., 2017), who used a Percoll gradient to purify the crude mitochondrial enriched pellet. They also used an "on-pellet-digestion" step. Similar to the approach we use in the current study. This approach has the advantage of being cost-effective, while simultaneously providing high-resolution in identification of peptides (León, Schwämmle, Jensen, & Sprenger, 2013; Rappsilber, Mann, & Ishihama, 2007). Moreover, in our hands this approach has permitted, for the first time, the investigation of the proteomes of sub populations of mitochondria within skeletal muscle from humans, linking differential expressions of proteins with pathways associated with regulating energy metabolism.

SS mitochondria seems particularly adapted to maintaining the energy charge needed for membrane-related processes, including signal transduction, ion exchange, substrate transport, and substrate activation (Cogswell et al., 1993; D. A. Hood, 2001). SS mitochondria and IMF mitochondria exhibit different bioenergetics properties and previous reports of impaired mitochondrial metabolism observed in muscle from obese could be specific to this population of mitochondria. SS mitochondria are likely more affected by obesity due to the ability of SS mitochondria to respond to metabolic changes compared to IMF mitochondria (Bizeau, Willis, & Hazel, 1998; D. a. Hood, 1996; Koves et al., 2005; Palmer et al., 1977, 1985; Ritov et al., 2005). Specifically, proteins of SS mitochondria are likely more affected by obesity compared to proteins of IMF mitochondria. To this point, the SS-to-IMF mitochondrial protein abundance ratios in

obese individuals as determined in the current study may account for the metabolic differences observed in obesity compared to healthy lean individuals.

The objective of this study was to perform label-free quantitative proteomics to identify proteins in isolated SS and IMF mitochondria fractions from muscle of obese and lean subjects. One of the major and novel findings from this study showed alterations in mitochondria protein composition in SS and IMF mitochondria during obesity. Specifically, the total abundance of mitochondria identified proteins in SS and IMF mitochondria was reduced in obese individuals relative to lean controls. We further showed that the proteins responsible for the reduced total abundance of mitochondrial proteins in SS and IMF mitochondria were primarily involved with the regulation of energy metabolism in muscle; electron transport and formation of ATP by chemiosmotic coupling. Our hypothesis, mitochondrial proteins known for using the proton-motive force would be reduced in IMF mitochondria from obese individuals, was supported by evidence of a reduced abundance of proteins assigned to electron transport and the enzyme complex ATP synthase. We further show evidence to support previous findings that skeletal muscle mitochondria from obese subjects have higher catalytic potential by our evidence of higher abundance of proteins involved in citric acid cycle. Overall, our data from this study shows that skeletal muscle mitochondria protein composition involved in key biological processes responsible for regulating energy metabolism in skeletal muscle is altered during obesity.

Subjects and Methods

Subjects

Sixteen healthy sedentary lean and seventeen apparently healthy sedentary obese individuals participated in this study. Subject characteristics are reported in Table 1. The Institutional Review Board at Mayo Clinic approved the studies and all experimental procedures were performed in the Clinical Studies Infusion Unit (CSIU) at Mayo Clinic in Scottsdale, Arizona. Each participant was considered healthy based on medical history, routine physical examination, screening laboratory tests, metabolic panel, bioelectrical impedance analysis, and a two-hour oral glucose tolerance test (OGTT). The purpose, design, and the risks associated with this study were explained to each subject before obtaining written consent. Following a screening visit, all participants returned to the CSIU on a separate day for the main experiment that included collection of muscle biopsy.

Experiment Design

Participants arrived at the CSIU at approximately 0630 h following an overnight fast. All subjects were instructed to refrain from any form of exercise for the 3-days prior to the study. A *Bergström* biopsy needled was used to collect percutaneous muscle biopsies (~100 mg) of the *vastus lateralis under local anesthesia (lidocain, 2%).* After removing blood, fat and visible connective tissues, the muscle biopsy was placed into a pre-massed beaker containing 2 ml of ice-cold Solution I (modified Chappell-Perry Medium I (Solution I; mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 $MgCl_2$, 1 EDTA, 1 ATP, pH 7.5), reweighed, and the wet muscle mass was calculated. Average *vastus lateralis* muscle samples from lean and obese individuals collected were 107±9 mg (lean) vs. 122±12 mg (obese), respectively.

Methods

Isolation of mitochondria

Isolated mitochondria from skeletal muscle of lean and obese individuals were prepared according to the outline in Figure 4-1; all procedures were carried out on ice or at 4°C and all centrifugations were 10 min in duration. Muscle biopsies were cleaned, weighed, minced with scissors in 9 volumes of ice-cold Solution I, and gently homogenized by hand using a ground glass-to-glass Potter-Elvehjem homogenizer (5 passes). The homogenate was centrifuged at 800 x g to obtain the supernatant (SN 1) containing mechanically released subsarcolemmal mitochondria (SS mitochondria). The pellet from the initial 800 x g centrifugation, Potter-Elvehjem Pellet (PEP), containing intermyofibrillar mitochondria (IMF mitochondria), was subjected to 7-minute incubation with Nagarse, a bacterial enzyme protease. Nagarse (bacterial proteinase Type XXIV, Sigma, P-8038) was prepared using Solution I (5 $mg.g^{-1}$ WW) and added to the PEP to liberate IMF mitochondria. After 7 min incubation, 1 ml of Solution I was added to the digested PEP, to slow enzymatic digestion. The resuspended PEP was then centrifuged at 800 x g to separate the non-mitochondrial proteins and membranes from IMF mitochondria. The supernatant SN 2 containing IMF mitochondria was transferred to a new tube. The SN 1 containing SS mitochondria and the separate SN 2 containing IMF mitochondria were centrifuged at 14,000 x g to obtain two mitochondrial pellets, SS mitochondria and IMF mitochondria. After discarding the supernatant, each of the mitochondrial pellets were resuspended in 0.5 ml of Solution II (mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 $MgCl_2$, 1 EDTA, 0.2 ATP, at pH 7.5. Following centrifugation at 7000 x g, the supernatants were discarded and the mitochondrial pellets were

resuspended a third time in 0.5 ml of Solution II. After the final centrifugation at 4000 x g and removal of supernatant, the two final mitochondrial pellets (i.e. SS mitochondria and IMF mitochondria) were each resuspended in identical volumes of mannitol-sucrose buffer containing (mM) 220 Mannitol, 70 Sucrose, 10 Tris-HCl, 1 EGTA, pH 7.40.

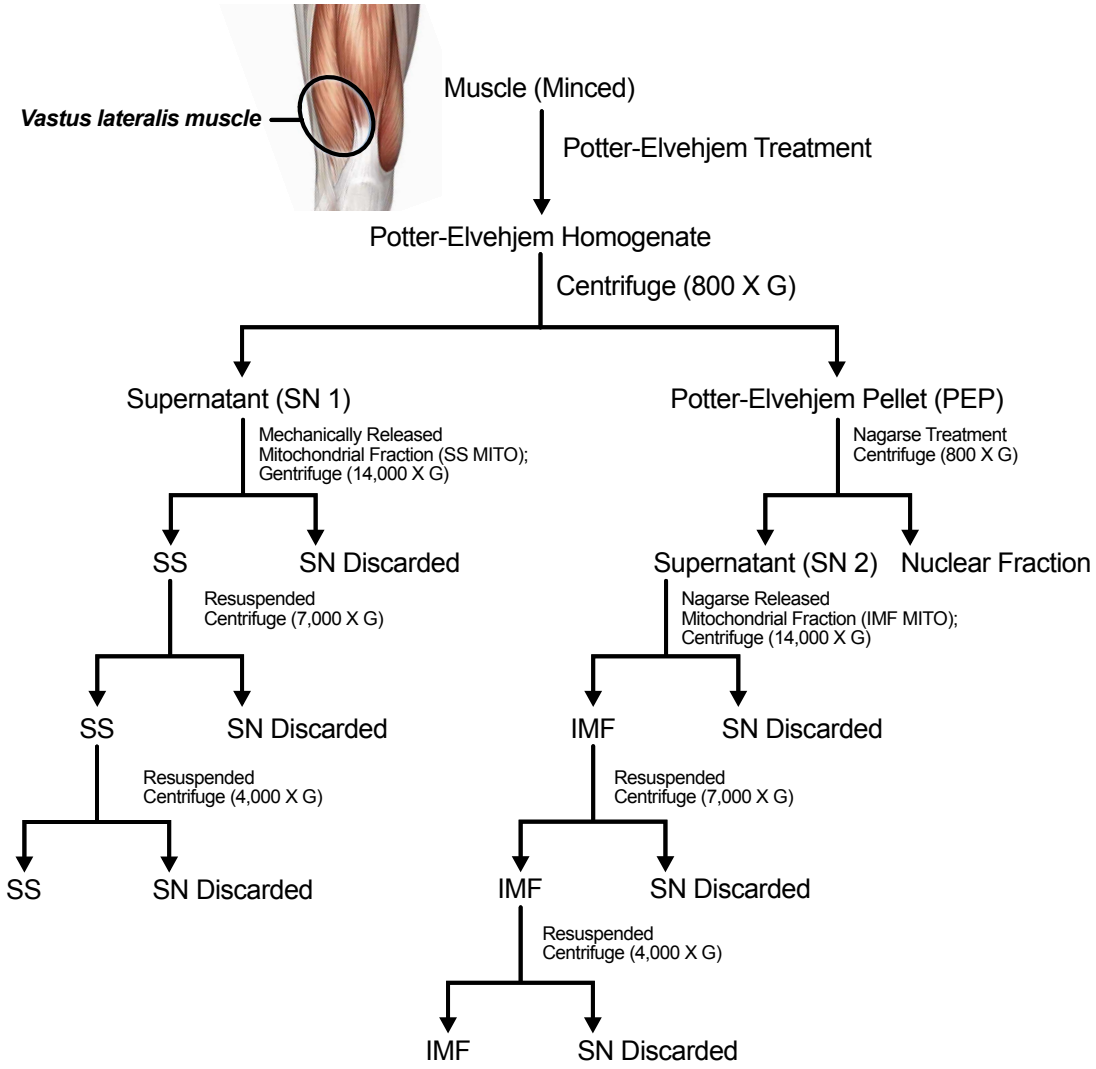

Figure 4-1. Isolation of skeletal muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from lean and obese individuals; SN - supernatant.

Citrate synthase activity

All citrate synthase assays carried out at 37º Celsius. Mitochondrial suspension was diluted 1:10 in mannitol sucrose buffer. This sample was then added to a cuvette containing (in mM) 0.025 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.125 acetyl-CoA, and 0.05% Triton detergent in a final volume of 0.5 mL. The addition of 0.25 mM oxaloacetate initiated substrate-dependent activity and absorbance was followed at 412 nm for three minutes to account for background activity. Activity was calculated using the millimolar extinction coefficient of 13.6 for the appearance of the mercaptide ion; formed when CoA is liberated during the citrate synthase reaction, reacting with DTNB. The degree of homogenization and the yield of mitochondria were estimated from citrate synthase activity assays carried out in aliquots of fractions from whole muscle homogenate described in Figure 4-1. Total citrate synthase activity was determined in the whole muscle homogenate ($\mu mol.min^{-1}$). Total citrate synthase activity ($\mu mol.min^{-1}$) was determined in 800xg (SN1) the Potter-Elvehjem pellet (PEP), the 800xg (SN2) and each of the final SS mitochondrial and IMF mitochondrial fractions. Fractions of total activity in SS mitochondria and IMF mitochondria fractions versus the total citrate synthase recovered from either SN1, SN2 represent the mitochondrial yield (Fractional Yield %). The protein content in the final mitochondrial preparations was determined by the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951).

In-solution digest of mitochondrial proteins

Skeletal muscle mitochondria proteomics analyses were performed using an in-solution digest protocol as previously described (Kras et al., 2016). In brief; A volume of isolated SS mitochondria or IMF mitochondria preparation, was added to a 9 times

volume of dilution buffer [DB; 10% acetonitrile (ACN) and 25mM Tris–HCl pH 8.5]. 8 µl trypsin (Sigma; St. Louis, MO) at 0.200 µg/ml was added to the protein sample and was allowed to incubate for 16 h at 37 °C with gentle shaking, followed by addition of 50 µl 5% formic acid (FA) to halt the digestion. The resulting peptides were prepared for sample analysis similar to a previously published protocol (Kulak, Pichler, Paron, Nagaraj, & Mann, 2014). In brief, a stop-and-go extraction tip (StageTip) (Rappsilber et al., 2007), was fitted with two C18 disk plugs using a customized tipping syringe (Rappsilber et al., 2007). The Stage Tip was activated with methanol, washed in 100 µl buffer B (0.1% FA, 80% ACN), and equilibrated in 100 µl buffer A (0.1% FA) twice. The peptides were then loaded onto the activated Stage Tip, washed twice in 100 µl buffer A, followed by elution in 50 µl Buffer B. The eluate was dried by vacuum centrifugation and stored at -80 °C prior to use. 6 µl of 0.1% FA (v/v) was added to re-suspend the dried samples, followed by sonication for 2 min. The sonicated samples were briefly centrifuged and 1 µl of sample was subsequently analyzed by mass spectrometry as described below.

Mass spectrometry

HPLC-ESI-MS/MS was performed on a Thermo Electron Orbi-trap Elite Velos Pro fitted with an EASY source (Thermo Electron, San Jose, CA). NanoLC was performed using a DIONEX/Thermo NCS-3500RS UltiMate 3000 with an EASY Spray column (Thermo Electron, 50 cm x 75-mm inner diameter, packed with PepMap RSLC C18 material, 2 µm); loading phase for 15 min; mobile phase, linear gradient of 1–37% ACN in 0.1% FA in 150 min, followed by a step to 95% ACN in 0.1% FA over 5 min, hold 10 min, and then a step to 1% ACN in 0.1% FA over 1 min and a final hold for 19

min (total run 200 min); Buffer A = 0.1% FA in 100% H_2O; Buffer B = 0.1% FA in 100% ACN; flow rate, 300 nl/min. All solvents were mass spectrometry grade. A "top 15″ data-dependent MS/MS analysis was performed (acquisition of a full scan spectrum followed by collision-induced dissociation mass spectra of the 15 most abundant ions in the survey scan).

Database Searching

Tandem mass spectra were extracted by msconvert version 3 using default settings. Charge state deconvolution and deisotoping were not performed. The fragment mass spectra were then searched against the human SwissProt_2017_01 database, using Mascot (Matrix Science, London, United Kingdom; version 2.4) and X! Tandem. Cross-correlation of Mascot search results with X! Tandem was accomplished with Scaffold (version Scaffold_4.6.1; Proteome Software, Portland, OR). Probability assessment of peptide assignments and protein identifications were made through the use of Scaffold. Only peptides with > 95%probability were considered. The assumption was set for the digestion enzyme strict trypsin. X! Tandem was set up to search the 2017_Jan_human database (SwissProt_2017_01, 313776 entries) also assuming strict trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, oxidation of methionine and carbamidomethyl of cysteine were specified in X! Tandem as variable modifications. Oxidation of methionine and carbamidomethyl of cysteine were specified in Mascot as variable modifications.

Criteria for Protein Identification

Scaffold (version Scaffold_4.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein Prophet algorithm assigned all protein probabilities (Nesvizhskii, Keller, Kolker, & Aebersold, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Area-proportional Venn diagrams were drawn by using free software available from BioinfoRX; (http://apps.bioinforx.com/).

Protein Quantitation for Comparative Proteomics

Samples used in the comparative proteomics experiments presented in this work were normalized total spectral count (TSC). Proteins identified as mitochondrial by gene ontology (GO:0005739) were used for final analysis. Briefly, Gene ontology (GO) describes function with respect to three aspects: molecular function (molecular-level activities performed by gene products), cellular component (the locations relative to cellular structures in which a gene product performs a function), and biological process (the larger processes, or 'biological programs' accomplished by multiple molecular activities) (Gene et al., 2011; Gene & Consortium, 2017). Missing TSC values across each of the identified proteins were replaced by the lowest reported TSC within each of the groups (i.e. lean or obese) (Lazar, Gatto, Ferro, Bruley, & Burger, 2016). TSC were used to determine the

normalized spectral abundance factors (NSAF) for protein abundance across samples as previously described (Hwang et al., 2010; Lefort et al., 2009); TSC were normalized to the length of respective identified protein. The sum of the normalized TSC (spectral abundance factor; SAF) was applied to SAF within the sample to generate the normalized spectral abundance factor (NSAF). We observed confounding clustering of samples run on different days. Therefore samples were batch mean centered (Gregori et al., 2012) to correct for this observed batch effect. In brief, each of the determined NSAF values was shifted by the mean of the identified protein within each group, to center the NSAF values to the mean. Means for the identified proteins, adjusted to zero, were shifted to the median NSAF values. A few proteins had negative values and they were corrected by shifting the whole row to a minimum value of 0 spectral count by subtracting the minimum NSAF across all samples, which resulted in samples corrected for variances observed due to batch effects. The batch corrected NSAF values were then used for the final analysis and discovery was determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli; FDR < 0.05 (Benjamini, Krieger, & Yekutieli, 2006).

There were a large number of proteins assigned in at least one of the 33 subjects. Therefore only proteins detected in ≥ 17 of the 33 subjects were used for statistical comparisons (K. Hojlund et al., 2010; Hwang et al., 2010; Xie et al., 2016). Gene Ontology (GO) annotation was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (http://david.abcc.ncifcrf.gov) (Huang, Sherman, & Lempicki, 2009). Protein identifications assigned to mitochondria by GO:0005739, detected in >50% of subjects, were used in the downstream

final analysis to determine differentially expressed proteins in SS mitochondria and IMF mitochondria between lean and obese subjects. Figure 4-2 and Figure 4-3 detail the workflow to determine differences in the proteome of SS mitochondria and IMF mitochondria, respectively, between lean and obese individuals. Normalized spectral abundance factors were mean scaled to 1 for lean when analyzing abundance of normalized spectra from manually curated sets of proteins between lean and obese subjects.

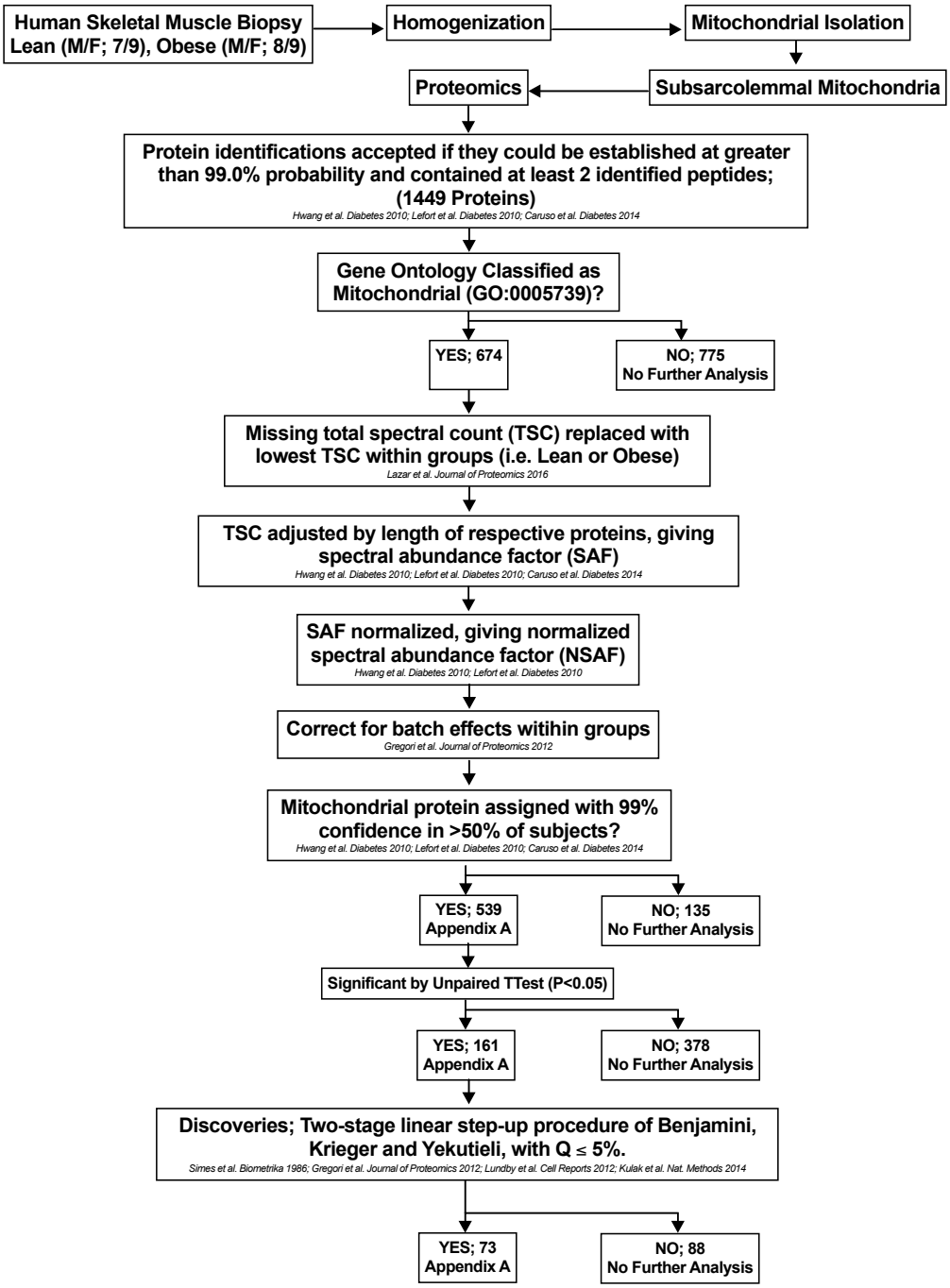

Figure 4-2. Workflow showing the steps for protein quantitation and comparative proteomics in skeletal muscle subsarcolemmal (SS) mitochondria. Appendix A provides mitochondria protein identifications as indicated in the workflow.

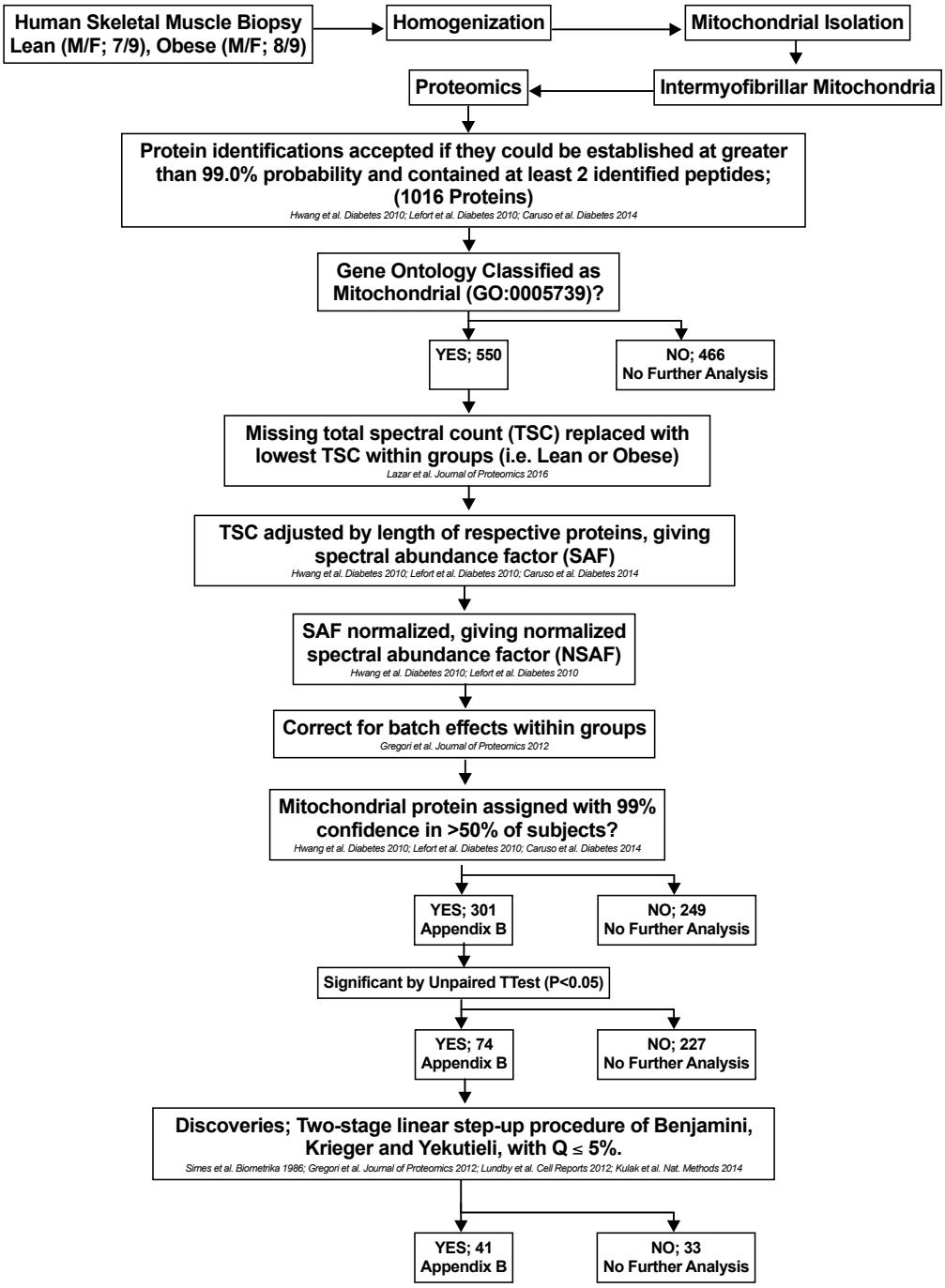

Figure 4-3. Workflow showing the steps for protein quantitation and comparative proteomics in skeletal muscle intermyofibrillar (IMF) mitochondria. Appendix B provides mitochondria protein identifications as indicated in the workflow schematic.

Principal component, heatmap analysis, and functional annotations for mitochondria protein identifications in SS and IMF mitochondria

Heatmaps, hierarchical clustering, and Principal Component Analysis (PCA) was performed using ClustVis (http://biit.cs.ut.ee/clustvis/) (Metsalu & Vilo, 2015). The STRING 10.5 database (Search Tool for the Retrieval of Interacting Genes/Proteins) was used to determine functional associations between differentially expressed proteins between lean and obese. Pathway analysis was also performed using STRING version 10.5 database (http://string-db.org), STRING analysis options were based on "evidence" mode, disconnected nodes were hidden, we did not add or remove any protein partners (Szklarczyk et al., 2015). More comprehensive pathway enrichment analysis was performed using Kyoto Encyclopedia of Genes and Genome (KEGG) (H. Ogata et al., 1999) and REACTOME software (Fabregat et al., 2016; Milacic et al., 2012).

Statistical Analysis

Data are presented as means ± SEM. Unpaired; parametric two-tailed t tests were used to determine which identified proteins were differentially expressed between lean and obese groups ($P < 0.05$). Significant differentially expressed proteins (DEP) between lean and obese were then further scrutinized and discovery determination was based on a false discovery rate (FDR) ($p < 0.10$) (GraphPad Prism7); Statistical significance was set at $P \leq 0.05$. Analyses performed using statistical software (GraphPad Prism7 Software, San Diego, CA).

Results

Physical and metabolic characteristics for study participants with (n = 17; BMI > 30 kg/m^2) and without (n=16) obesity are shown in Table 4-1. The obese subjects had significantly higher BMI, fat mass, fasting plasma insulin, and HbA_{1c}, compared to lean controls. However, systolic and diastolic blood pressures, fasting blood glucose levels, plasma non-esterified fatty acids (NEFA), as well as total plasma cholesterol were comparable to the lean subjects, indicative of a relatively healthy group of obese individuals with similar age and gender distribution.

Table 4-1. Characteristics of the study participants (n=33) classified as either lean or obese.

	Lean	Obese	pValue
n (F/M)	16(7/9)	17(8/9)	
Age (Years)	33.0±2.5	31.5±2.5	0.667
Weight (kg)	68.5±2.6	98.5±3.5	<0.001
Height (cm)	170.9±1.9	170.6±2.4	0.924
BMI (kg.m-2)	23.3±0.6	34.2±0.8	<0.001
FFM (kg)	49.8±3.1	64.8±2.8	<0.01
FM (kg)	10.8±1.0	21.7±1.0	<0.001
Body fat mass (%)	22.5±2.2	34.2±1.6	<0.001
VO2max ($ml.min^{-1}$)	2086±164	2286±134	0.350
VO2max ($ml.kgFFM\text{-}1.min^{-1}$)	30.4±1.9	23.6±1.4	<0.01
Waist circumference (cm)	80.1±1.9	106.7±3.2	<0.001
Hip circumference (cm)	98.1±1.8	118.1±2.6	<0.001
Waist-to-hip ratio	0.82±0.01	0.91±.03	<0.01
Systolic blood pressure (mmHg)	127±6	121±3	0.461
Diastolic blood pressure (mmHg)	73±2	76.2±2	0.368
Fasting blood glucose ($mg.dL^{-1}$)	87.4±1.6	91.4±3.5	0.310
Fasting plasma insulin ($\mu IU.ml^{-1}$)	5.0±0.8	11.1±1.2	<0.001
Matsuda-ISI	10.1±1.5	4.2±0.8	<0.01
HOMA-IR	1.1±0.2	2.6±0.3	<0.01
HbA1C (%)	5.2±0.1	5.5±0.1	<0.05
Plasma triglycerides ($mg.dL^{-1}$)	86.6±13.7	133.1±20.5	0.069
Plasma NEFA ($mmol.L^{-1}$)	0.34±0.05	0.39±0.04	0.347
Total plasma cholesterol ($mg.dL^{-1}$)	170.9±9.4	177.3±6.5	0.579
Plasma HDL-Cholesterol ($mg.dL^{-1}$)	61.3±3.9	46.5±2.9	<0.01
Plasma LDL-Cholesterol ($mg.dL^{-1}$)	92.3±7.8	104.3±6.3	0.242
TSH ($mIU.L^{-1}$)	2.1±0.4	2.4±0.3	0.509

Table 4-1 Baseline subject characteristics values are presented as mean ± SEM; BMI, body mass index, FFM, fat free mass, Matsuda Index, indices of sensitivity to insulin, Homeostatic model assessment, assess β-cell function and insulin resistance (IR), HbA_{1c}, fraction of glycated hemoglobin, TSH, thyroid stimulating hormone. Significance determined by unpaired t test; $P < 0.05$.

Proteome analysis of skeletal muscle mitochondria

A total of 1,449 proteins were assigned in ≥ 1 of the 33 subjects from the subsarcolemmal mitochondrial fractions, while a total of 1,016 proteins were assigned in ≥ 1 of the 33 subjects from the intermyofibrillar mitochondrial fractions. 674 identified proteins were assigned to mitochondria by gene ontology (GO) in SS mitochondria and 550 identified proteins were assigned to mitochondria by GO in IMF mitochondria. Among the 674 SS and 501 IMF mitochondria protein identifications, 539 and 301 were detected in ≥ 17 subjects in SS and IMF mitochondria, respectively (Appendix A, Appendix B). Area-proportional Venn diagrams show numbers of protein identifications and overlap of data sets from either SS or IMF mitochondria (Figure 4-4).

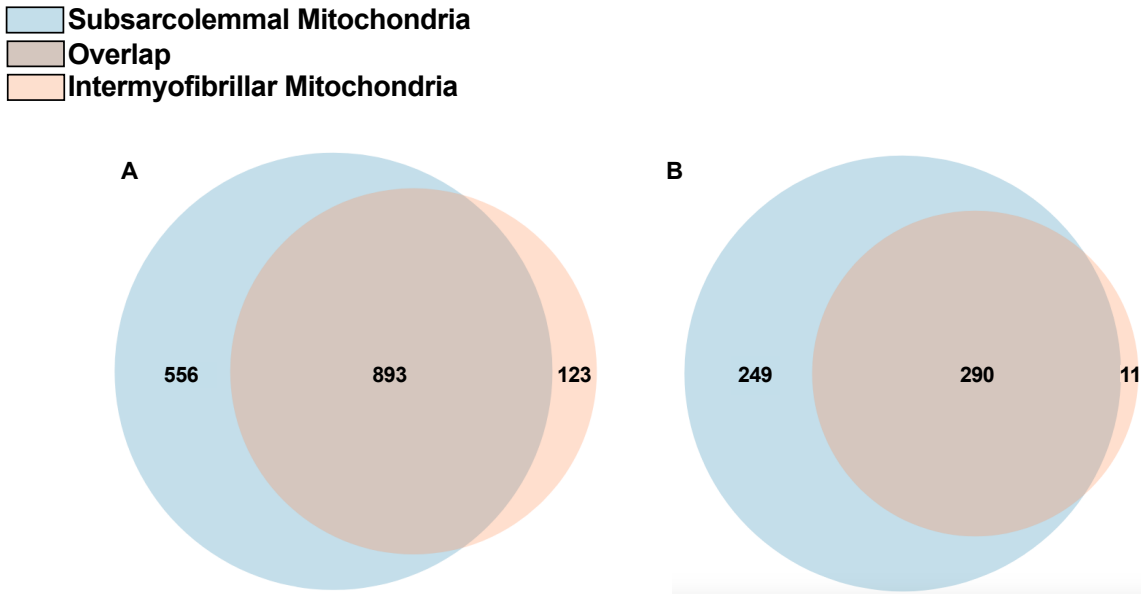

Figure 4-4. Area proportional Venn diagrams showing protein identifications, overlapping identifications in subsarcolemmal or intermyofibrillar mitochondria from lean and obese (n≥17) revealed by proteomics; A) 556 proteins from subsarcolemmal mitochondrial fraction detected in ≥1 subjects, 893 proteins overlap and detected in both subsarcolemmal and intermyofibrillar mitochondrial fractions in ≥1 subjects, and 123 intermyofibrillar mitochondria proteins detected in ≥1 subjects; B) 249 proteins assigned to mitochondria by gene ontology (GO: 0005739), in subsarcolemmal mitochondria detected in >50% of all subjects, 290 proteins assigned to mitochondria that overlap both subsarcolemmal mitochondria and intermyofibrillar mitochondria in >50% of all subjects, and 11 proteins assigned to mitochondria from intermyofibrillar mitochondria detected in >50% of all subjects.

Total normalized spectral abundance in either SS or IMF mitochondria between lean and obese

Comparing the total abundance of normalized spectra from either the 539 or 301 mitochondria protein identifications in either SS or IMF mitochondria between the lean and obese groups revealed the following: total abundance of normalized spectra (∑NSAF) from 539 mitochondria proteins in SS mitochondria was reduced by 2 % in obese subjects compared to lean controls (Figure 4-5A); (493.1 ± 1.9, (n=17) vs. 500.7 ± 1.7, (n=16)); P = 0.0046); the ∑NSAF from 301 mitochondria proteins in IMF mitochondria was reduced by 9% in obese subjects compared to lean controls (Figure 4-5B); (548.5 ± 10.58, (n=17) vs. 604.2 ± 6.385, (n=16)); P = 0.0001.

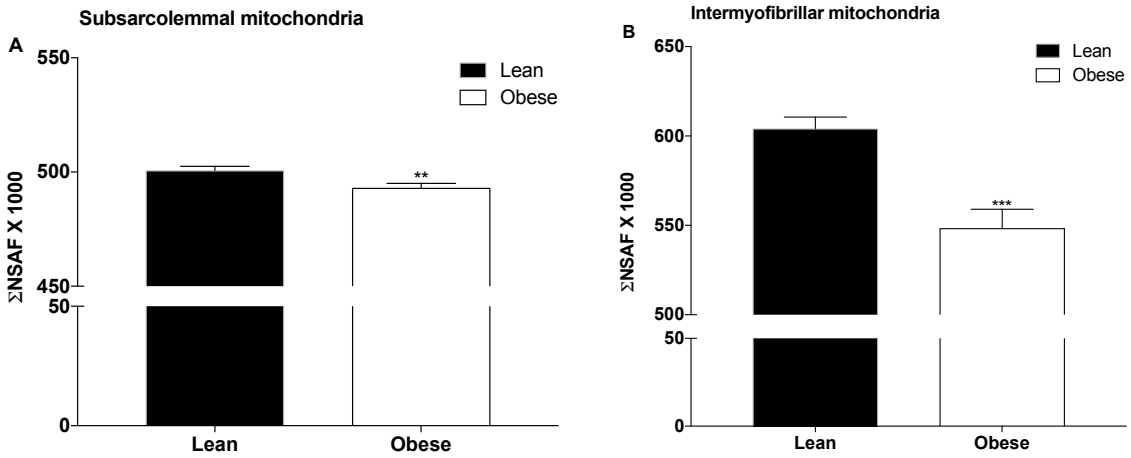

Figure 4-5. Total abundance of normalized spectral abundance factors (∑NSAF) from 539 and 301 mitochondrial protein identifications measured in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, respectively in lean and obese subjects. The ∑NSAF of 539 mitochondria protein identifications in A) SS mitochondria was 2% lower in obese compared to lean controls while the ∑NSAF from 301 mitochondria protein identifications in B) IMF mitochondria was 9 % lower in the obese group, compared to lean controls; ∑NSAF = sum of determined normalized spectral abundance factor (NSAF) for each of the 539 and 301 mitochondria protein identifications in SS and IMF mitochondria respectively, shown in Figure 4-4B; Data presented are ∑NSAF X 1000 ± SEM; Statistical significance determined by parametric Unpaired t test; (**, $P < 0.01$, ***, $P < 0.001$).

Total abundance of normalized spectra in SS and IMF mitochondria between lean and obese involved with regulation of energy metabolism

To determine which sets of proteins might be responsible for the significantly reduced abundance of normalized spectra in SS and IMF mitochondria of obese, we investigated manually curated protein sets assigned to key biological processes involved with regulation of energy metabolism in skeletal muscle. One well-established key biological process in mitochondria associated with energy metabolism is oxidation-reduction process (GO:0055114). Therefore, we sought to investigate differences in total abundance of normalized spectra assigned to the key biological process of oxidation-reduction, which includes proteins involved in the citric acid cycle (TCA), the respiratory electron transport chain and ATP synthase. We hypothesized that SS and IMF mitochondria from obese would have a lower total abundance of normalized spectra (ΣNSAF) assigned to TCA, ETC and ATP synthase compared to lean controls, based on the results in Figure 4-5 showing a reduced total abundance of normalized spectra in SS and IMF mitochondria from obese. However, the ΣNSAF assigned to TCA, ETC and ATP synthase was not different in SS mitochondria between groups (Figure 4-6A). Furthermore, the ΣNSAF assigned to TCA and succinate-coenzyme Q reductase (complex II) were significantly higher in IMF mitochondria of obese compared to lean controls, while the ΣNSAF for NADH: ubiquinone oxidoreductase (complex I), cytochrome c – oxidoreductase (complex III), and ATP synthase (complex V) were all significantly reduced in IMF mitochondria of obese (Figure 4-6B).

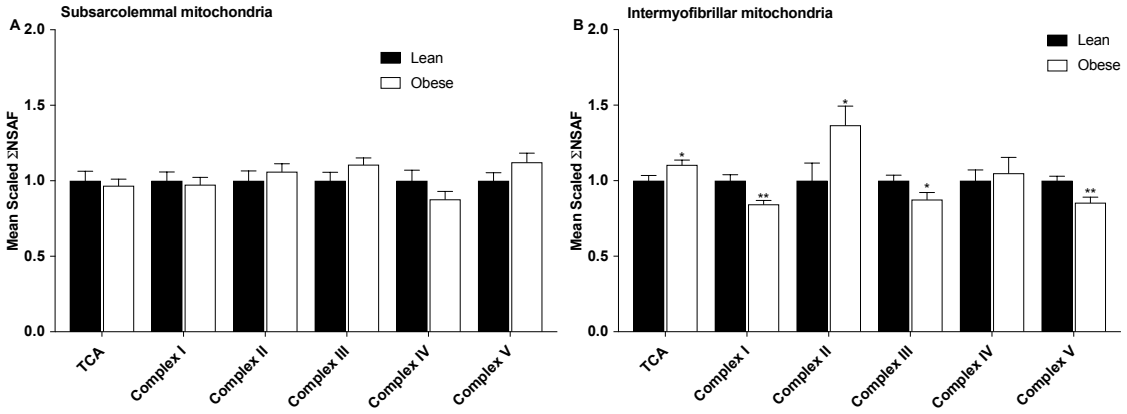

Figure 4-6. Mean scaled total abundance of normalized spectra (ΣNSAF) for manually curated protein sets assigned to citric acid cycle (TCA), electron transport chain complexes I - IV (ETC), and ATP synthase (complex V) from skeletal muscle mitochondria between lean and obese individuals; mean scaled ΣNSAF for TCA, ETC and complex V from A) SS and B) IMF mitochondria between lean and obese; ΣNSAF = sum of normalized spectral abundance factors (NSAF) for each of the mitochondria protein identifications associated with complex I – V, and TCA. Mean scaled ΣNSAF adjusted to 1 for the lean. Data is presented as mean scaled ΣNSAF ± SEM; Statistical significance determined using Unpaired, parametric t test within each pathway; (*, $P < 0.05$; **, $P < 0.01$)

Ratios of normalized spectra assigned to citric acid cycle versus electron transport and ATP synthase between lean and obese

With evidence showing an increased total abundance of normalized spectra assigned to TCA along with an overall reduced abundance of normalized spectra assigned to electron transport chain and ATP synthase in IMF mitochondria of obese (Figure 4-6B), we wanted to investigate the relative abundance of mitochondria protein identifications assigned to TCA versus ETC plus ATP synthase in both SS and IMF mitochondria between lean and obese subjects. As expected there was no difference in the ratio for total abundance of normalized spectra assigned to TCA versus ETC plus ATP synthase in SS mitochondria between groups (Figure 4-7A). However there was a significantly higher ratio for total abundance of normalized spectra assigned to TCA versus ETC plus ATP synthase in IMF mitochondria between groups (Figure 4-7B) ($P < 0.05$).

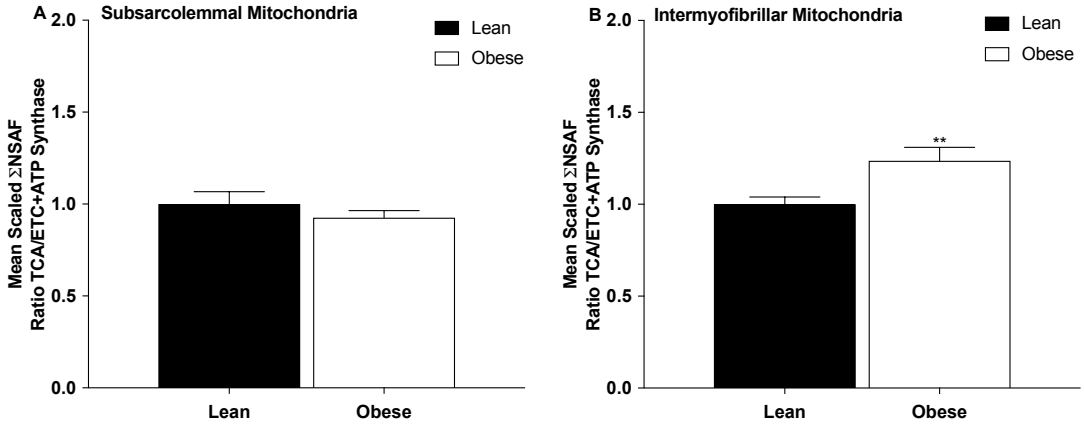

Figure 4-7. Mean scaled total abundance of normalized spectra (∑NSAF) ratios for manually curated protein sets assigned to citric acid cycle (TCA) versus the electron transport chain complexes I - IV (ETC), and ATP synthase (complex V) from skeletal muscle mitochondria between lean and obese individuals; mean scaled ∑NSAF ratio for TCA versus ETC + complex V from A) SS and B) IMF mitochondria between lean and obese; ∑NSAF = sum of normalized spectral abundance factors (NSAF) for each of the mitochondria protein identifications associated with manually curated protein sets of interests, within each subject ($n \geq 30$) sampled. Data is presented as mean scaled ∑NSAF ratio ± SEM; Statistical significance determined using Unpaired, parametric t test; (**, $P < 0.01$).

A total of 73 and 41 differentially expressed protein identifications in SS and IMF mitochondria between lean and obese individuals

Among 539 and 301protein identifications in SS and IMF mitochondria, respectively, 73 and 41 were significantly different between lean and obese subjects as determined by Unpaired parametric t test ($P < 0.05$) and passing false discovery rate (FDR) criteria ($P < 0.1$) with a FDR cutoff $P \leq 0.05$. Following identification of the 73 and 41 differentially expressed proteins (DEP) in SS and IMF mitochondria, respectively, between lean and obese, REACTOME analyses were performed on these proteins with respect to the direction of their expression to reveal significant pathway assignments. Specifically, Uniprot identifications of the DEPs in SS and IMF mitochondria were mapped to pathways and over-representation analysis was performed using REACTOME software. REACTOME pathways were over-represented (enriched) in the submitted protein identifications.

Among the 73 DEPs in SS mitochondria from obese, 28 and 45 were up- and down-regulated, respectively (Appendix A). Significantly enriched pathways (i.e. pathways that were over-represented in the differentially expressed proteins in SS mitochondria) are reported as the –log transformed false discovery rate p value (i.e. –log (p Value)). The most significant pathways enriched in DEP in SS mitochondria of obese subjects relative to lean controls are shown in Figure 4-8A, and included: the citric acid cycle (TCA) and respiratory electron transport ($P < 0.0001$); respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins ($P < 0.0001$); mitochondrial protein import ($P < 0.0001$); mitochondrial translation ($P < 0.0001$); complex I biogenesis ($P < 0.0001$); and metabolism of amino acids and derivatives ($P < 0.001$).

REACTOME analysis in submitted DEP in SS mitochondria of obese subjects is presented in Appendix C.

Among the 41 DEPs in IMF mitochondria from obese, 13 and 28 were up and down-regulated, respectively (APPENDIX B). The most significant pathways that were over-represented in DEP in IMF mitochondria of obese subjects relative to lean controls are shown in Figure 4-8B including: the citric acid (TCA) cycle and respiratory electron transport ($P < 0.0001$); formation of ATP by chemiosmotic coupling ($P < 0.0001$); complex I biogenesis ($P < 0.0001$); mitochondrial protein import ($P < 0.0001$); and Fatty acid metabolism ($P < 0.001$). REACTOME analysis in submitted DEP in IMF mitochondria of obese subjects is presented in Appendix D.

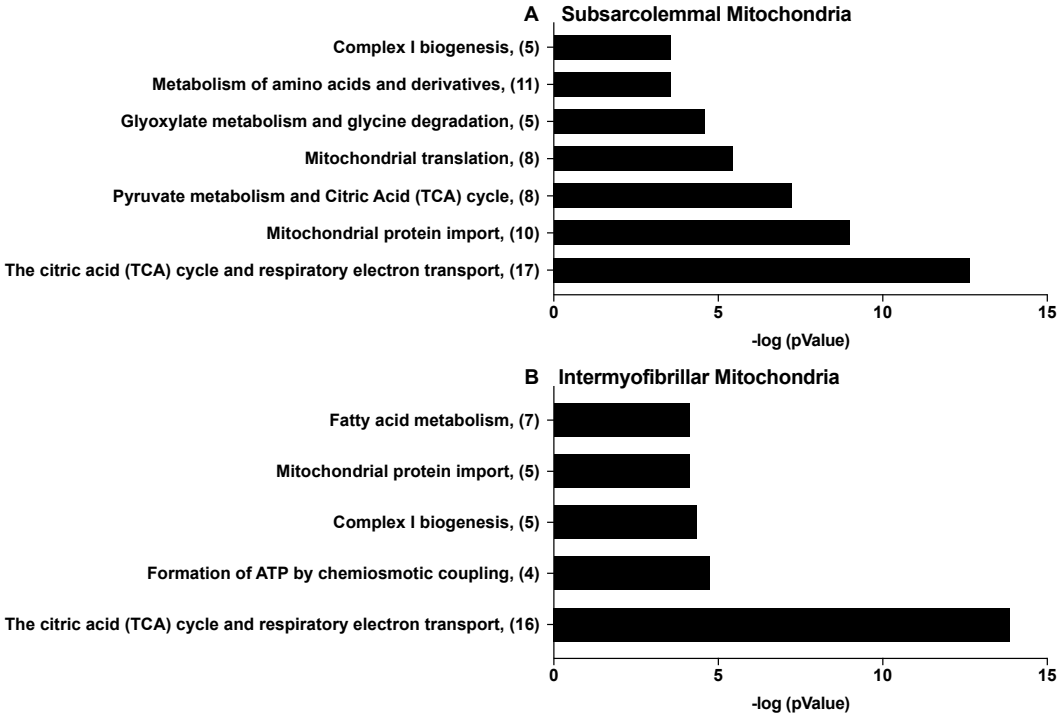

Figure 4-8. Pathways associated with the differentially expressed proteins in SS and IMF mitochondria between lean and obese individuals. Significant pathways of differentially expressed proteins (DEPs) in A) SS and B) IMF mitochondria between lean and obese individuals based on the REACTOME database analysis. The total numbers of differentially expressed proteins for a given pathway are given in parentheses. Tables showing REACTOME analysis in submitted DEPs in SS and IMF mitochondria between lean and obese individuals appear in Appendices C and D, respectively.

Proteins involved in mitochondrial energy metabolism are differentially expressed in SS and IMF mitochondria between lean and obese individuals

Based on REACTOME pathway analysis, the majority of the differentially expressed proteins in SS and IMF mitochondria between lean and obese were associated with the citric acid cycle and electron transport (Figure 4-8A and B). The analysis of the subsets of differentially expressed proteins in SS and IMF mitochondria of obese, assigned to pathways based on REACTOME in Figure 4-8, revealed the that in total there were 12 and 29 differentially expressed mitochondria proteins that were up- and down-regulated in SS mitochondria, respectively, in obese relative to lean controls. Meanwhile there were 12 and 16 differentially expressed mitochondria proteins that were up- and down-regulated in IMF mitochondria, respectively, in obese compared to lean controls (Figure 4-9).

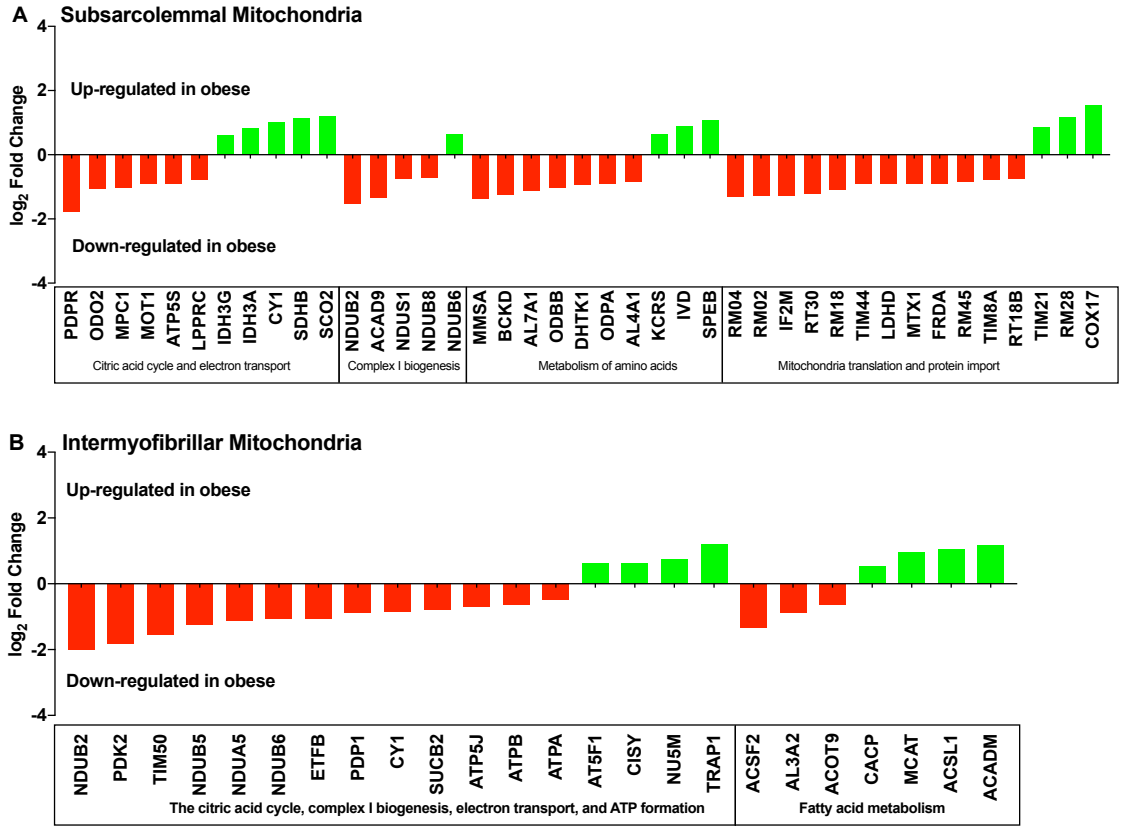

Figure 4-9. A subset of differentially expressed proteins located in SS and IMF mitochondria participating in pathways associated with mitochondria energy metabolism. The log_2 fold change of differentially expressed proteins (DEPs) in A) SS and B) IMF mitochondria of obese relative to lean controls. The green and red bars represent up and down-regulated proteins, respectively, in obese relative to lean controls. Functional annotations assigned to protein groups were REACTOME based. REACTOME analyses of DEPs are located in Appendices C and D.

Protein-protein interactions of differentially expressed proteins in SS and IMF mitochondria of obese individuals

Using a more comprehensive protein-protein interaction network database, STRING, we detected 56 protein-protein interaction networks, based on key biological processes by gene ontology, in SS mitochondria enriched with ≥ 5 of the 73 significantly differentially expressed proteins ($P < 0.05$) including: respiratory electron transport chain (ETC) ($P < 0.0001$); cellular amino acid catabolic process ($P < 0.0001$); oxidative phosphorylation (OXPHOS) ($P < 0.0001$); mitochondrial translational initiation ($P < 0.0001$); mitochondrial electron transport, NADH to ubiquinone ($P < 0.0001$); mitochondrial translation ($P < 0.0001$); mitochondrial ATP synthesis coupled electron transport ($P < 0.0001$); and tricarboxylic acid cycle (TCA) ($P < 0.0001$) (Appendix E).

Among the 41 DEPs in IMF mitochondria in obese, using STRING, we detected 24 protein-protein interaction networks, also based on key biological processes by gene ontology, in IMF mitochondria enriched with ≥ 5 differentially expressed assigned proteins ($P < 0.05$) including: respiratory electron transport chain ($P < 0.0001$); mitochondrial electron transport, NADH to ubiquinone ($P < 0.0001$); mitochondrial ATP synthesis coupled electron transport ($P < 0.0001$); and oxidative phosphorylation ($P < 0.0001$) (Appendix F).

Differentially expressed proteins in SS mitochondria and IMF mitochondria from obese individuals organize into distinguishable clusters

Principal component analysis (PCA) was performed on the expression levels for the 73 DEPs in SS mitochondria of obese (up-regulated ≥ 1.43 or down-regulated ≤ 0.68; $P \leq 0.05$); (Appendix A) and 41 DEPs in IMF mitochondria of obese (up-regulated ≥ 1.46 or down-regulated ≤ 0.71; $P \leq 0.05$); (Appendix B). Separate principal component analyses (PCA) of the 73 and 41 differentially expressed proteins (DEP), indicated above, in either SS or IMF mitochondria, revealed that difference in relative fold changes in DEP from obese relative to lean, were sufficient to differentiate each of the groups from the others; (Figure 4-10A) (Figure 4-11A). We compared the 73 DEP in SS mitochondria as well as the 41 DEP in IMF mitochondria from obese and results are presented as heatmaps (Figure 4-10B) and (Figure 4-11B). Rows of heatmaps were centered and unit variance scaling was applied. Both rows and columns were clustered using correlation distance and average linkage. Hierarchical clustering analysis of DEP in either SS or IMF mitochondria organized into distinguishable clusters; up (Green bar) and down-regulated (Red bar) DEP in SS and IMF mitochondria of obese relative to lean controls.

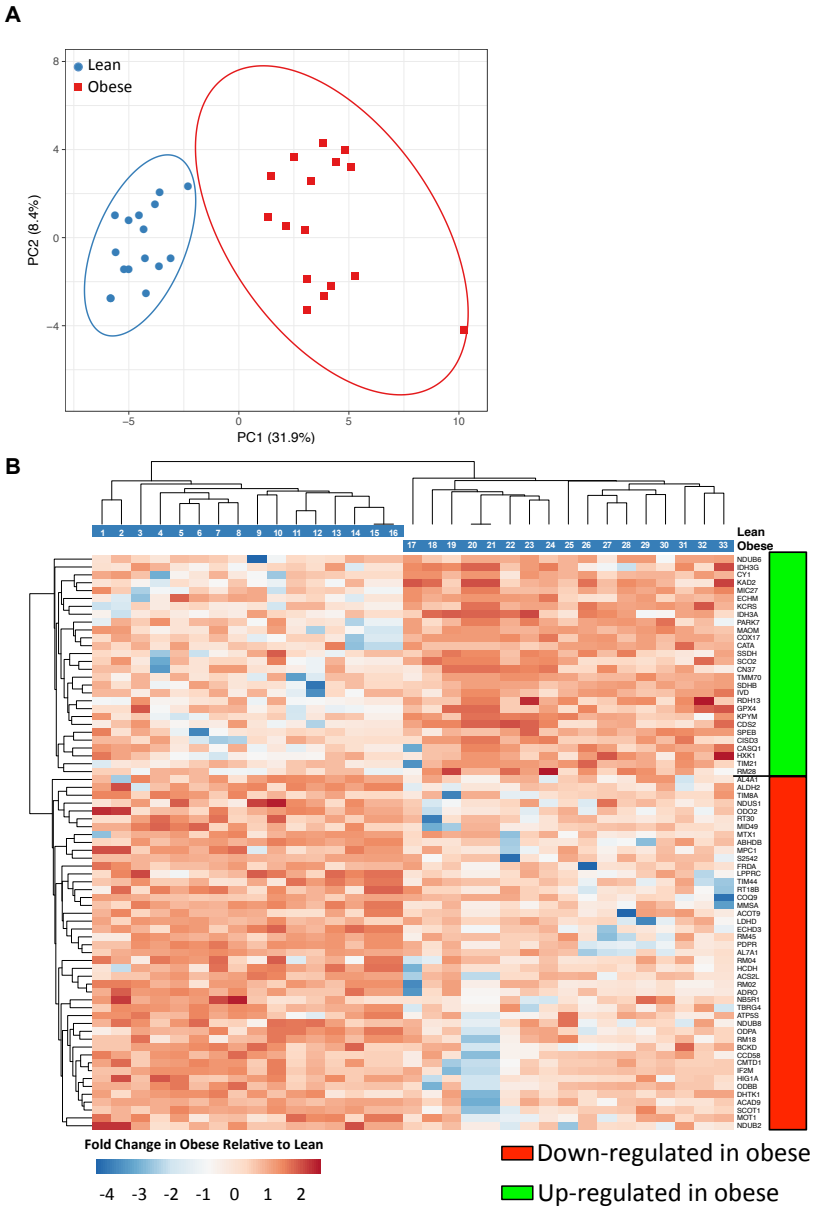

Figure 4-10. Principal component analysis and heatmaps for differentially expressed mitochondrial protein identifications in human skeletal muscle subsarcolemmal mitochondria. A) Principal component analysis (PCA) and B) heatmap of 73 differentially expressed proteins in subsarcolemmal mitochondria; up-regulated (Green) and down-regulated (Red) differentially expressed proteins in subsarcolemmal mitochondria of obese relative to lean. Columns represent each subject in the study. Columns 1 - 16 = lean controls; columns 17 – 33 = obese subjects.

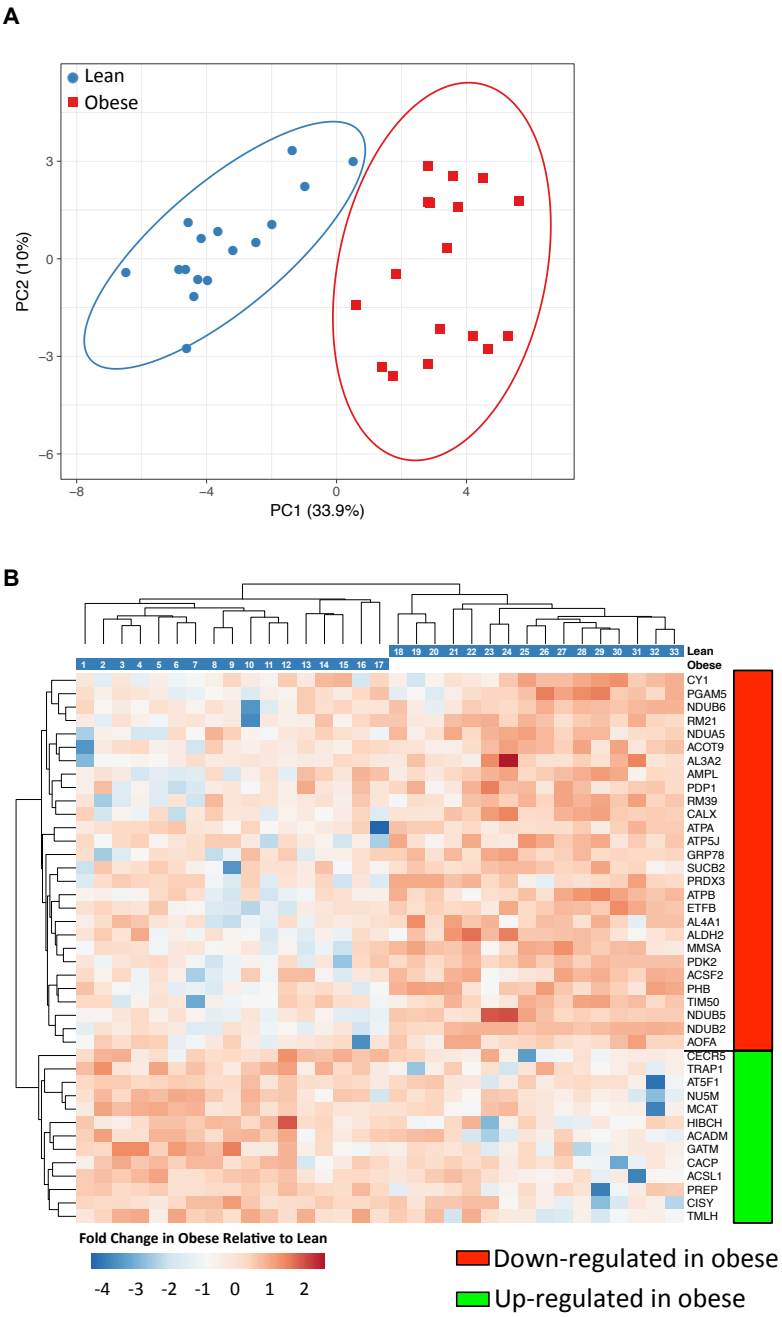

Figure 4-11. Principal component analysis and heatmaps for differentially expressed mitochondrial protein identifications in human skeletal muscle intermyofibrillar mitochondria. A) Principal component analysis (PCA) and B) heatmap of 41 differentially expressed proteins in intermyofibrillar mitochondria; up-regulated (Green) and down-regulated (Red) differentially expressed proteins in subsarcolemmal mitochondria of obese relative to lean. Columns represent each subject in the study. Columns 1 - 17 = obese subjects; columns 18 – 33 = lean controls.

Comparing SS-to-IMF protein abundance ratios from overlapping protein identifications in SS and IMF mitochondria between lean and obese individuals

Based on the findings from this studying showing differences in SS and IMF mitochondria of obese, we wanted to investigate if there might be differences in SS-to-IMF protein abundance between lean and obese individuals. Therefore, using the 290 overlapping mitochondria protein identifications in SS and IMF mitochondria we determined an SS-to-IMF protein abundance ratio for all 290 mitochondria proteins. We found there was no difference in the average normalized spectral abundance for SS-to-IMF protein abundance ratios between groups as determined by Unpaired parametric t test (Figure 4-12A). Additionally, using 290 overlapping mitochondria protein identifications to compare SS-to-IMF protein abundance ratios for manually curated protein sets assigned to citric acid cycle, electron transport, and ATP synthase showed no differences between groups in (Figure 4-12B). However, 6 proteins had SS-to-IMF protein abundance ratios that significantly differed between lean and obese subjects (Figure 4-13). The SS-to-IMF protein abundance ratios of obese relative to lean were expressed as $\log_2$ fold change and grouped according to biological processes (GO) (Figure 4-13). Among the 6 proteins with significantly different SS-to-IMF protein abundance ratios in obese, 4 and 2 were up- and down-regulated respectively; NU5M, NDUB8, KCRS, PHB, CY1, KAD2. Upon further analysis by REACTOME, the top significantly enriched pathways included the citric acid (TCA) cycle and respiratory electron transport; ($P < 0.01$) (Table 4-2).

Results from the current study revealed more than a 300% increase in the detected abundance of adenylate kinase 2, (KAD2), a highly abundant skeletal muscle mitochondrial associated isoform, in SS mitochondria of obese individuals ($P = 0.0018$)

(Appendix A). Also, relevant to the current study was the reorganization and distribution of KAD2 within the mitochondrial reticulum between lean and obese individuals. The current study revealed a higher abundance of KAD2 in subsarcolemmal mitochondria from obese individuals, consequently resulting in the higher SS-to-IMF protein abundance ratio in obese relative to lean controls (Figure 4-13). On the contrary, the detected abundance of KAD2 from the intermyofibrillar mitochondrial fractions from muscle of obese individuals was nearly 22% lower; although statistical significance was not achieved ($q = 0.108$). Similar to KAD2, creatine kinase, mitochondrial 2 (Sarcomeric), isoform (KCRS), was 56% higher in SS mitochondria of obese, which resulted in a higher SS-to-IMF protein abundance ratio in obese; suggests the possibility that these skeletal muscle proteins are differentially distributed within the mitochondrial reticulum during various physiological conditions, such as obesity (Figure 4-13).

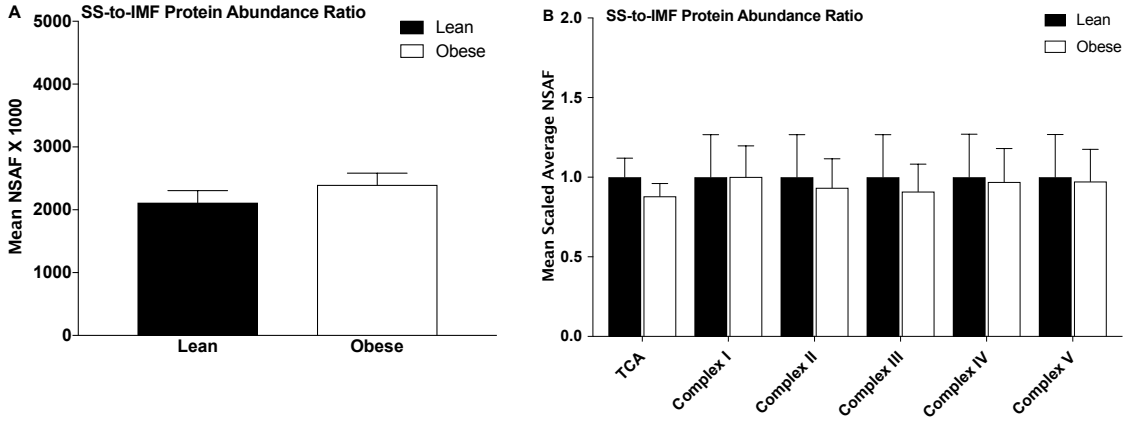

Figure 4-12. Average normalized spectral abundance for SS-to-IMF protein abundance ratios between lean and obese groups. SS-to-IMF protein abundances ratios for 290 overlapping mitochondria protein identifications were averaged within each group. Mean scaled average NSAF SS-to-IMF protein abundance ratios for manually curated proteins sets assigned to the citric acid cycle (TCA), the electron transport chain (complex I – IV), and ATP synthase (complex V). Data is presented as mean NSAF SS-to-IMF protein abundance ratio ± SEM; Statistical significance determined using Unpaired, parametric t test; ($P > 0.05$)

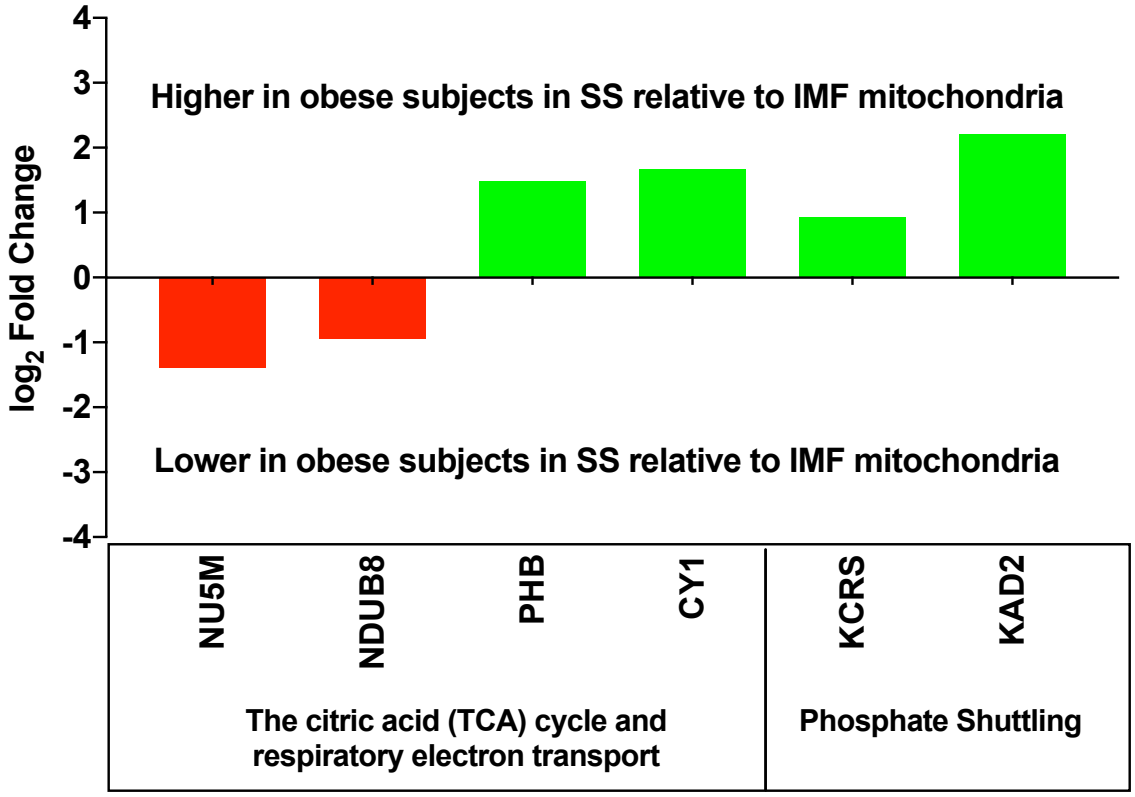

Figure 4-13. Mitochondria proteins with different SS-to-IMF protein abundance ratios from obese relative to lean controls are shown. There were 6 among 290 overlapping protein identifications in SS and IMF mitochondria with significantly different SS-to-IMF protein abundance ratios in obese, compared to lean controls. SS-to-IMF protein abundance ratios of obese relative to lean expressed as $\log_2$ fold change and grouped according to biological processes and pathway as revealed by REACTOME analysis; NADH dehydrogenase subunit 5, (NU5M), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, (NDUB8), Creatine kinase S-type, mitochondrial, (KCRS), Prohibitin, (PHB), Cytochrome c1, heme protein, mitochondrial, (CY1), Adenylate kinase 2, mitochondrial, (KAD2).

Table 4-2. Mapped pathways for proteins with significantly different SS-to-IMF protein abundance ratios between lean and obese subjects based on REACTOME analysis.

Pathway identifier	Pathway name	#Entities found	Entities pValue	Entities FDR	Submitted entities found
R-HSA-1430728	Metabolism	5	0.0013	0.0064	KAD2, CY1, KCRS, NDUB8, NU5M
R-HSA-611105	Respiratory electron transport	3	0.0000	0.0004	CY1, NDUB8, NU5M
R-HSA-163200	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	3	0.0000	0.0004	CY1, NDUB8, NU5M
R-HSA-1428517	The citric acid (TCA) cycle and respiratory electron transport	3	0.0001	0.0008	CY1, NDUB8, NU5M
R-HSA-6799198	Complex I biogenesis	2	0.0004	0.0030	NDUB8, NU5M
R-HSA-1268020	Mitochondrial protein import	2	0.0005	0.0030	CY1, NDUB8
R-HSA-392499	Metabolism of proteins	2	0.3019	0.3019	CY1, NDUB8

Table 4-2. NADH dehydrogenase subunit 5, (NU5M); NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, (NDUB8); Creatine kinase S-type, mitochondrial, (KCRS); Prohibitin, (PHB); Cytochrome c1, heme protein, mitochondrial, (CY1); Adenylate kinase 2, mitochondrial, (KAD2).

Discussion

The primary goal of this work was to investigate the proteome differences in skeletal muscle mitochondria between lean and obese individuals. For that purpose, we used label free quantitative proteomics along with a comprehensive protein-protein interaction and functional pathway analysis approaches. In the present study, we identified 1,449 and 1,016 proteins in human skeletal muscle subsarcolemmal and intermyofibrillar mitochondria, respectively. Among these, 539 and 301 SS and IMF mitochondrial proteins, respectively, were detected in >50% of the subjects in the lean and obese groups. Comparing the total abundance of normalized spectra from the 539 proteins in SS mitochondria revealed that the total abundance of proteins was 2% lower in obese compared to lean controls. We also compared the 301 proteins in IMF mitochondria and determined that the total abundance of these proteins was 9% lower in the obese subjects when compared to the lean controls. This finding is consistent with previous reports of reduced muscle mitochondrial content in obese humans (Kurt Hojlund et al., 2008; Kelley et al., 2002; Mogensen et al., 2007; Ritov et al., 2005). We showed no difference in the citrate synthase activities, used as an index of mitochondrial content (Larsen et al., 2012) as indicated in chapter 3, in either SS or IMF mitochondria isolation preparations between lean and obese, suggesting no difference in the muscle mitochondria content between groups; this apparent discrepancy between the study in chapter 3 and chapter 4 might be explained as follows: citrate synthase activities, described in chapter 3, were normalized to total protein (mg) and determined by method of Lowry (Lowry et al., 1951), an absorbance assay dependent on peptide bonds. We also showed in chapter 2, that the proteins in the final mitochondrial preparations isolated from mouse muscle, contain both mitochondrial and non-mitochondrial assigned protein

identifications, as determined by mass spectrometry. Therefore using label free quantitative mass spectrometry allowed us to identify mitochondria-specific proteins in the final SS and IMF mitochondrial preparations for the final analysis performed in this study. This, in turn, increased the sensitivity of our measurements, consequently resulting in the reported differences in SS and IMF mitochondria between lean and obese individuals due to the increased signal to noise ratio.

The reduced abundance of SS and IMF mitochondria of obese compared to lean controls, lead us to investigate groups of proteins associated with key biological processes involved with regulating energy metabolism in skeletal muscle. We manually curated sets of proteins using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (http://david.abcc. ncifcrf.gov) (DAVID) and gene ontologies. This allowed us to group together the following sets of proteins: (1) the electron transport chain (complex I, II, III, and IV); (2) ATP synthase; and (3) citric acid cycle (TCA). We sought to determine if skeletal muscle mitochondria from obese subjects have a reduced abundance of proteins associated with citric acid cycle, electron transport chain, and ATP synthase, proteins well known for their association with the capacity to generate ATP in muscle.

Our results revealed that the total abundance of spectra assigned to proteins associated with TCA and the electron transport chain, and ATP synthase, were not different between lean and obese groups. However, the total abundance of spectra from all 539 identified proteins in SS mitochondria was lower by 2% in obese ($P < 0.01$). To reconcile this difference, we sought to compare the total abundance of spectra from other proteins not assigned to TCA, electron transport and ATP synthase in SS mitochondria

between lean and obese. This analysis revealed that there was significantly lower abundance of spectra assigned to proteins not assigned to TCA, electron transport or ATP synthase in SS mitochondria of obese versus lean controls (314.4 ± 3.1, n=17 vs. 325.7 ± 3.0, n=16, ∑NSAF X 1000; P = 0.0136). We analyzed the 539 protein identifications in SS mitochondria by REACTOME to reveal that among the 539 protein identifications in SS mitochondria, 127 were involved in metabolism of proteins (Pathway ID: R-HSA-392499) (Appendix A). Among the 127 protein identifications involved in metabolism of proteins, 120 were not previously assigned to TCA, electron transport or ATP synthase. The total abundance of normalized spectra from these 120 proteins involved in metabolism of proteins was significantly lower in SS mitochondria of obese versus lean controls as determined by Unpaired parametric t test; (53.8 ± 1.3, n=17 vs. 60.7 ± 1.7, n=16, ∑NSAF X 1000; P = 0.0032). This is consistent with previous reports of lower rates of protein synthesis in muscle mitochondria from obese subjects (Guillet et al., 2009; Tran et al., 2016).

On the contrary, the abundance of mitochondria protein identifications assigned to TCA along with citrate synthase was increased by over 10% and 54%, respectively, in IMF mitochondria of obese subjects when compared to lean controls, providing further evidence to support the increased abundance of citrate synthase in muscle mitochondria in obese rodent models (Turner et al., 2007). The observation of higher abundance of proteins assigned to TCA in parallel with our findings of reduced total abundance of proteins assigned to complex I, III and V in IMF mitochondria of obese subjects, suggests a difference in protein composition in IMF mitochondria related to the capacity to generate ATP, compared to lean controls. Furthermore, this observation of significantly

reduced protein abundance of the electron transport chain and ATP synthase in IMF mitochondria of obese subjects, suggests a significant reduction in the relative capacity to produce ATP, since IMF mitochondria comprise approximately 80% of the total mitochondrial volume in skeletal muscle mitochondrial reticulum (Abdul-Ghani et al., 2009; Chomentowski, Coen, Radiková, Goodpaster, & Toledo, 2011; Ferreira et al., 2010). We also sought to compare the total abundance of spectra from other proteins not assigned to TCA, electron transport and ATP synthase in IMF mitochondria between lean and obese to determine other pathways based on REACTOME analysis may be contributing to the 9% lower abundance of total normalized spectra in IMF mitochondria. This analysis revealed that there was significantly lower abundance of spectra assigned to proteins not assigned to TCA, electron transport or ATP synthase in IMF mitochondria of obese versus lean controls (298.0 ± 6.4, n=17 vs. 307.1 ± 20.1, n=16, ∑NSAF X 1000; P = 0.0136). As expected, REACTOME analysis of the 301 protein identifications in IMF mitochondria revealed that 107 protein identifications were involved in the citric acid (TCA) cycle and respiratory electron transport (Pathway ID: R-HSA-1428517) (Appendix B).

Among the 539 mitochondria protein identifications in SS mitochondria, 73 were significantly differentially expressed between lean and obese individuals. Among 301 mitochondria protein identifications in IMF mitochondria, 41 were significantly differentially expressed between lean and obese individuals. Bioinformatic interrogation of the differentially expressed proteins in SS and IMF mitochondria of obese subjects, revealed the following key biological processes by gene ontology related to energy metabolism in muscle: oxidation-reduction process; (GO:0055114), cellular respiration; (GO:0045333), small

molecule metabolic process; (GO:0044281), carboxylic acid metabolic process; (GO:0019752), respiratory electron transport chain; (GO:0022904), ATP metabolic process; (GO:0046034), oxidative phosphorylation; (GO:0006119), monocarboxylic acid metabolic process; (GO:0032787), mitochondrial electron transport, NADH to ubiquinone; (GO:0006120), mitochondrial ATP synthesis coupled electron transport; (GO:0042775), phosphorus metabolism; (GO:0006793), and primary metabolism; (GO:0044238) (Appendix E and F).

REACTOME analysis of the 73 significantly DEP in SS mitochondria, revealed categories reflective of key biological processes associated with regulation of energy and metabolism in skeletal muscle including the citric acid cycle (TCA), electron transport, ATP synthesis, mitochondrial protein import, mitochondrial translation, complex I biogenesis, pyruvate metabolism, and metabolism of amino acids. Performing the same analysis on the 41 DEP in IMF mitochondria, revealed categories reflective of key biological processes associated TCA, electron transport, ATP synthesis, complex I biogenesis, and fatty acid metabolism. Consequently, the difference in protein composition, as it relates to mitochondrial protein import, translation and complex I biogenesis in SS and IMF mitochondria between lean and obese, suggest the possibility of dysregulation of distinctive key biological processes of protein synthesis (Guillet et al., 2009; Tran et al., 2016), and complex I biogenesis (Lefort et al., 2010; Ritov et al., 2010) in muscle of obese subjects. Furthermore, our results provide support of previous reports of biochemically distinct subpopulations of mitochondria in skeletal muscle (Palmer et al., 1985) with respect to SS mitochondria having a protein composition that supports membrane processes, such as protein import (Cogswell et al., 1993; D. A. Hood, 2001). Overall, our data show that there

are differentially expressed proteins between lean and obese subjects, associated with pathways related to mitochondrial translation and metabolism of amino acids localized in SS mitochondria and fatty acid metabolism localized in IMF mitochondria. In terms of obesity, the pathway and functional annotation analyses reveals that DEPs associated with mitochondrial translation, TCA, electron transport, as well as complex I biogenesis, are down-regulated in both SS mitochondria and IMF mitochondria of obese subjects. However, differentially expressed mitochondrial protein identifications associated with fatty acid metabolism are significantly up-regulated in IMF mitochondria of obese subjects compared to lean controls as evidence by a 33% average fold change for the 7 differentially expressed mitochondria proteins (ACADM, MCAT, ACSF2, ACOT9, AL3A2, ACSL1, CACP) assigned to fatty acid metabolism based on REACTOME analysis (Figure 4-9B). These results are in line with previous findings, which suggest fatty acid metabolism is up-regulated in obese rodent models (Turner et al., 2007).

Interestingly, muscle from obese subjects had a higher SS-to-IMF protein abundance ratio for creatine kinase S-type, mitochondrial (KCRS), and adenylate kinase 2, mitochondrial (KAD2). These results are consistent with previous findings that have shown 30% increase in enzymatic activity of creatine kinase, which regulates energy metabolism in muscle, in obese/overweight women (Hittel, Hathout, Hoffman, & Houmard, 2005). The increased enzymatic activity of adenylate kinase in the obese subjects was also reported by this same group and was proposed as a representative compensatory glycolytic drift to counteract reduced muscle mitochondrial function with the progression of obesity (Hittel et al., 2005). The results from this study are in line with these previous findings and further show that the higher SS-to-IMF protein abundance ratio of KAD2 and KCRS suggests a

redistribution of energy metabolism related to phosphate regulation toward the periphery of myocytes in obese individuals (Janssen, Terzic, Wieringa, & Dzeja, 2003).

This study also provides evidence suggesting that the reduced abundance of proteins in IMF mitochondria, can at least in part, be explained by the reduced abundance of proteins assigned to the electron transport chain and ATP synthase. Notably, two key catalytic enzymes responsible for the phosphorylation of ADP, ATP synthase subunits beta and alpha (ATP5B, ATP5A), which have been previously shown as altered in muscle of obese subjects (Højlund et al., 2003; K. Hojlund et al., 2010; Tran et al., 2016) were significantly down-regulated in IMF mitochondria of obese subjects in the current study. Consequently, these results suggest that the muscle of obese humans having significantly reduced capacity to generate ATP may localize in IMF mitochondria. Conversely, muscle from obese subjects may have a higher catalytic potential in the matrix of IMF mitochondria of obese as evidence by the significantly higher abundance of protein identifications assigned to the citric acid cycle, including the mitochondrial enzyme marker citrate synthase, which suggests the possibility of increased reactive oxygen species with the decreased abundance of subunits from complex I, as previously reported (Lefort et al., 2010).

Overall, differentially expressed proteins associated with up-regulation of fatty acid metabolism were localized in IMF mitochondria, while mitochondrial protein identifications associated with the down-regulation of protein synthesis were localized in SS mitochondria. Finally, while we showed that the overall abundance of mitochondria protein identifications associated with the citric acid cycle and respiratory electron transport in SS mitochondria of obese were not different compared to lean controls, we

also showed a reduced abundance of identified proteins from ATP synthase or complex V, specifically in IMF mitochondria of obese subjects. This significant reduction in protein abundance of ATP synthase, a well known protein complex responsible for using the proton-motive force in generating ATP in muscle, might explain the significantly reduced ATP capacity in skeletal muscle of these subjects (Abdul-Ghani et al., 2009), which in turn may result in a progressive decrease in muscle mitochondrial function, exacerbating the state of obesity

Although previous studies have reported lower muscle mitochondrial content as determined by the activity or abundance of the mitochondrial enzyme marker citrate synthase (Kelley et al., 2002; Lefort et al., 2010; Mogensen et al., 2007; Ritov et al., 2010; Simoneau & Kelley, 1997), our findings suggest the contrary. In fact, normalized spectral abundance of the enzyme marker was not different in SS mitochondria of obese and nearly 54% higher in IMF mitochondria of obese subjects; FDR ≤ 0.05; ($p = \textit{0.054}$) (Appendix B). The possible increase in abundance of citrate synthase in the IMF mitochondria of obese subjects compared to lean controls, is in concert with the higher coexpression of up-regulated mitochondrial proteins associated with the citric acid cycle and specifically the elements of the electron transport chain (succinate dehydrogenase: complex II) responsible for the oxidation of reduced flavin adenine dinucleotide ($FADH_2$) in IMF mitochondria of the obese, and agrees with previously reported up-regulation of citrate synthase in skeletal muscle of obese animal models (Benton et al., 2008; Jain et al., 2014). It is also worth noting that the abundance of proteins associated with complex II was significantly higher in IMF mitochondria of obese compared to lean controls. Other groups have reported differences in complex II in muscle mitochondria of obese subjects related to fiber type switching (Tanner

et al., 2002). Since IMF mitochondria are normally associated with higher activity of oxidative phosphorylation (Ferreira et al., 2010) and we found that the IMF mitochondria of obese subjects have a reduced abundance of mitochondria proteins assigned to the electron transport chain, it is warranted to consider the following: muscle IMF mitochondria of obese may have the ability to up-regulate succinate dehydrogenase or complex II, to accept electron transfer of NADH: ubiquinone oxidoreductase (i.e. complex I), which may be in part, due to nutrient overload observed in the obese (Iossa et al., 2004). This in turn could result in a reduced capacity for reactive oxygen species generation originating at the site of complex I (Pryde & Hirst, 2011).

In summary, this study shows the total abundance of normalized spectra of mitochondria proteins was reduced by a modest 2% in SS, but to a greater degree of 9% in IMF mitochondria of obese compared to lean controls. The differences in protein composition of SS and IMF mitochondria between lean and obese subjects confirmed the biochemically distinct nature of the two subpopulations with respect to function (i.e. mitochondrial protein import localized in SS and fatty acid metabolism in IMF mitochondria). Furthermore, this research presents novel findings in obesity, with respect to regulating energy metabolism in skeletal muscle, as well as reveals possible mechanisms to explain the reduced capacity of ATP in skeletal muscle of the obese. Overall, these findings show that obesity is associated with differences in protein composition in both SS and IMF mitochondria of obese related to key biological processes responsible for regulating energy metabolism in skeletal muscle.

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CHAPTER 5: CONCLUSION

In this dissertation, we set out to reveal the functional and proteome differences in skeletal muscle mitochondria between lean and obese humans. Using oxygen electrode polarography and label free quantitative mass spectrometry, we were able to test our specific hypotheses, and discover novel findings in obesity. We also were able to investigate specifically the differences in protein composition and function of two subpopulations of skeletal muscle mitochondria located in the periphery, subsarcolemmal (SS) mitochondria, and in the interior of the cell, intermyofibrillar (IMF) mitochondria, as well as investigate any differences in protein composition and function associated with obesity. Lastly, we were able to demonstrate a significant difference in protein compositions in both the SS and IMF mitochondria, between lean and obese subjects, associated with regulating energy metabolism in skeletal muscle.

Our first study investigated the function of skeletal muscle mitochondria in mouse muscle, when using a protease to isolate mitochondria from skeletal muscle. Using a protease, such as the bacterial protease nagarse, is a common technique employed in preparations of mitochondria from tough tissues such as skeletal muscle (Dow, 1967; Idell-Wenger, Grotyohann, & Neely, 1982; Palmer, Tandler, & Hoppel, 1977; Thakar & Ashmore, 1975). We showed that using a protease during isolation preparations of skeletal muscle mitochondria reduces the abundance of proteins, by nearly 25%, in the final mitochondrial suspension; the majority of contaminating proteins that were reduced as a result of the protease treatment, were proteins not assigned to mitochondria. Overall, the data indicated that skeletal muscle mitochondria preparations isolated using protease effectively removes a significant abundance of non-mitochondrial proteins and

substantially increased mitochondrial catalytic potential and energetic coupling. This study was important to our overall work to demonstrate, like others previously (Palmer et al., 1977; Palmer, Tandler, & Hoppel, 1985), preparations of skeletal muscle mitochondria exposed to protease, results in mitochondria isolates exhibiting high function, but more importantly, demonstrated a confounding difference between preparations of isolated SS mitochondria treated and not treated with nagarse. Since future studies in this dissertation involved the study of two subpopulations of skeletal muscle mitochondria, SS and IMF mitochondria, we concluded that we could not directly compare functional and proteome differences between SS and IMF mitochondria, when one subpopulation of skeletal muscle mitochondria is isolated with protease. Aware of this confounding effect of using nagarse to isolate mitochondria from skeletal muscle, the studies detailed in chapters 3 and 4 emphasized differences in SS mitochondria between lean and obese subjects, independently and separately, from the compared differences in IMF mitochondria between lean and obese groups.

In the second study, we investigated the differences in skeletal muscle mitochondrial function between lean (BMI < 25 kg/m2; 37 ± 3 yrs; n = 10) and obese (BMI > 30 kg/m2; 35 ± 3 yrs; n = 11) individuals in response to amino acids infusion. Amino acids are well known for stimulating mitochondrial ATP production in SS mitochondria of healthy lean subjects (Tatpati et al., 2010). In chapters 3 and 4 of this dissertation, we successfully isolated two subpopulations of skeletal muscle mitochondria, SS and IMF mitochondria, using previously established techniques (Kras et al., 2016; Palmer et al., 1977, 1985). Subsequently, we demonstrated that a four-hour infusion of amino acids (AA) enhanced the capacity for respiration and ATP production

of muscle SS, but not IMF, mitochondria in lean individuals in parallel with increased uncoupled respiration. However, neither of these parameters increased in SS or IMF mitochondria in obese individuals. We also established that treating isolated SS mitochondria with amino acids elicits a similar response, which was in agreement to what we observed in the human isolated SS mitochondria in vivo (i.e. increased in state 3 Jo oxygen consumption and ATP production). Our in-vitro assays of isolated SS mitochondria from mouse muscle treated with amino acids in chapter 3 revealed a significant increase in the capacity of SS mitochondria to generate ATP in response to amino acids treatment.

The second study of this dissertation also described that amino acids infusion stimulated proton leak (state 2 Jo) of SS mitochondria from lean subjects, where amino acids did not affect the proton leak rate of SS mitochondria of the obese subjects. Higher rates of proton leak may suggest the activation of uncoupling proteins (Crescenzo et al., 2014), a well-defined proton leak pathway (Crescenzo et al., 2014; Lambert & Brand, 2004). Thus, these results show reduced activation of proton leak pathways in SS mitochondria from obese humans in the presence of increased plasma amino acid concentrations.

We concluded from the second study that AA infusion stimulated skeletal muscle ATP production in the lean but not the obese. The data further suggest mitochondria from obese may be overstimulated at rest and AA treatment improves ADP/O coupling. In turn, there may be physiological mechanisms which allow skeletal muscle mitochondria to adjust oxygen consumption rates in subsarcolemmal mitochondria during states of increased availability of amino acids, which may not be present in the intermyofibrillar

mitochondria populations of both lean and obese individuals. Moreover, the uncoupling affect, or higher respiratory control ratios, observed in subsarcolemmal mitochondria after AA further supports that mitochondria in the periphery of myocytes, which seem particularly affected by obesity, may be more adept to control proton flux, while simultaneously, maintaining ATP production capacity. Future studies are needed to investigate the exact mechanisms by which AA stimulates ATP production through complex-I and complex-II enzyme pathways, and results in oxidative phosphorylation uncoupling of subsarcolemmal mitochondria in lean but not obese individuals and the potential effects of chronic amino acid supplementation to treat obesity.

In chapter 4, we determined differences in proteomes of SS and IMF mitochondria between lean and obese individuals. The overall findings suggested that obesity is associated with changes in protein composition within the skeletal muscle mitochondria reticulum, which results in a reduced capacity of ATP production. We showed a reduced total abundance of normalized spectra in both SS and IMF mitochondria of obese relative to lean controls. We further demonstrated that among the 539 and 301 mitochondria protein identifications in SS and IMF mitochondria, respectively, 73 and 41 were differentially expressed in either the SS or IMF mitochondria of obese subjects and were associated with biological processes that regulate electron transport chain ($P<0.0001$), citric acid cycle ($P<0.0001$), oxidative phosphorylation ($P<0.001$), branched-chain amino acid degradation, ($P<0.0001$), and fatty acid degradation ($P<0.001$). We provided evidence that supports the biochemical distinct nature of SS and IMF mitochondria. We also showed differential abundance and redistribution of proteins involved in citric acid cycle, electron transport and phosphate

shuttling as evidenced by the higher SS-to-IMF protein abundance ratios for KAD2, KCRS, CY1, along with prohibitin (PHB), a protein which may play a role in regulating mitochondrial respiration activity. Work with respect to Chapter 4 is summarized in Figure 5-1, showing that obesity is associated with an overall differential abundance of mitochondrial proteins, evident particularly in the IMF subpopulation in skeletal muscle. Specifically, IMF mitochondria have reduced expression of complex I, complex III and complex V proteins, but increased expression of proteins related to the TCA cycle.

The overall findings of this research work in lean versus obese humans are important with respect to: (1) understanding physiological changes associated with the dysregulation of energy metabolism in skeletal muscle of obese subjects; (2) revealing novel findings in obesity with respect to the distribution of protein composition in myocytes related to regulating energy metabolism; and (3) focusing future studies on assessing ways to improve the capacity of ATP production in skeletal muscle mitochondria of the obese, which predictably would result in a higher daily expenditure of energy, whereby lowering the risks associated with obesity in humans. These results support and extend the concept that obesity is associated with skeletal muscle mitochondria dysfunction and disruptions in the regulation of energy metabolism. These studies pave the way for future investigations that elucidate the mechanisms responsible for the reduced response to amino acid stimulation in SS mitochondria from obese individuals, specifically related to respiration not coupled to generating ATP (i.e. uncoupled respiration). These studies also provide us with a better understanding of the mitochondrial protein composition in SS and IMF mitochondria in healthy lean and relatively healthy obese humans. Future investigations are needed to identify the

mechanisms responsible for the reduced abundance of proteins involved in protein import and protein translation in the periphery of muscle cells, as well as the reduced abundance of proteins associated with the electron transport chain (i.e. complex I and complex III) and ATP synthase (complex V) in the interior of muscle cells during obesity. Understanding the protein compositional changes in skeletal muscle mitochondria in obesity is a crucial functional interface between mechanisms involved in regulating whole-body energy expenditure, and improving the regulation of body weight.

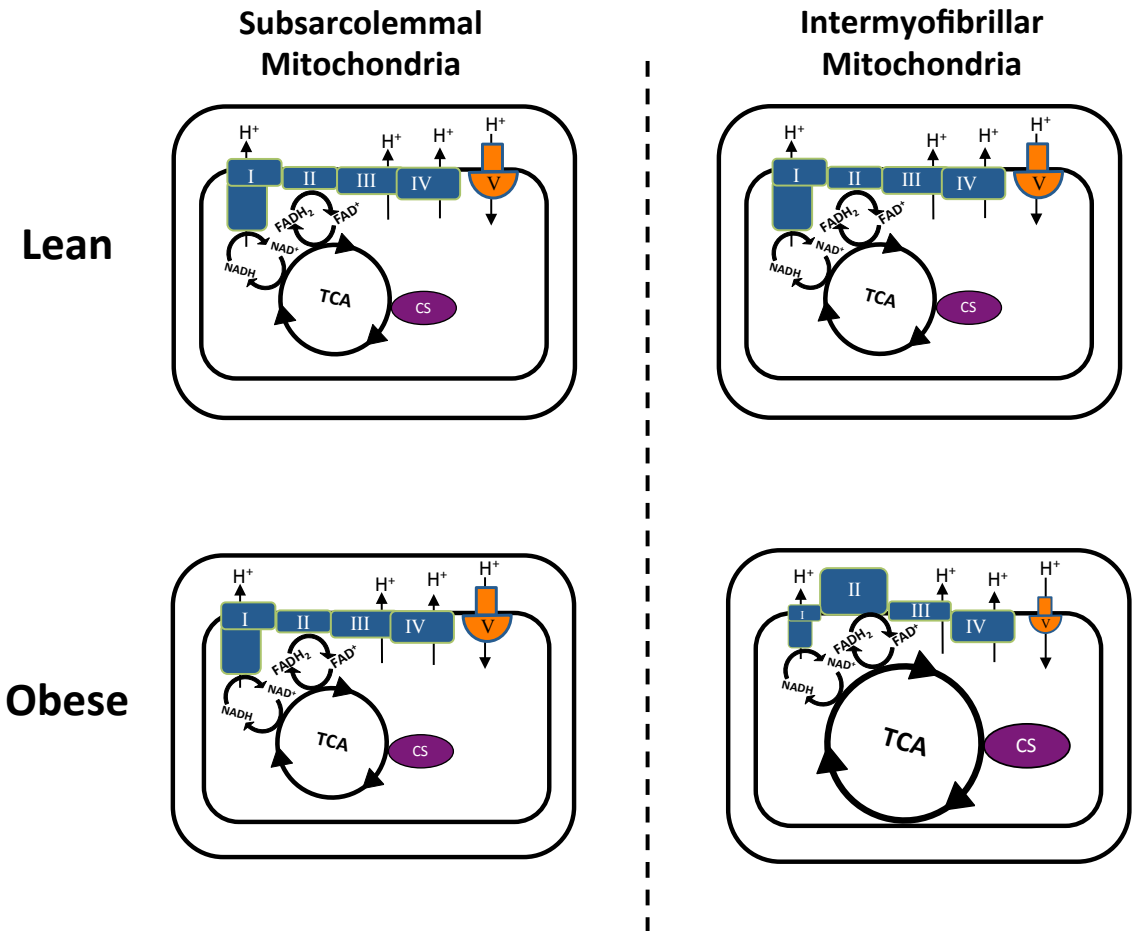

Figure 5-1. Model depicting protein abundance in human skeletal muscle mitochondria of lean and obese individuals. Citrate synthase (CS); Tricarboxylic acid (TCA) cycle; NADH: ubiquinone oxidoreductase (complex I); Succinate-coenzyme Q reductase (complex II); Cytochrome c – oxidoreductase (complex III); cytochrome c oxidase (complex IV); and ATP synthase (complex V). Increased catalytic potential in IMF mitochondria of obese subjects, as it is evident by the higher abundance of proteins associated with the TCA cycle. A lower abundance of proteins involved in electron transport and ATP synthase in IMF Mitochondria of obese subjects suggests an overall reduced capacity of ATP generation in muscle of obese subjects.

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APPENDIX A

THE 539 SUBSARCOLEMMAL MITOCHONDRIA PROTEIN IDENTIFICATIONS ASSIGNED WITH 99% CONFIDENCE IN >50% OF SUBJECTS WITH ≥ 2 IDENTIFIED PEPTIDES

[CONSULT ATTACHED FILES]

APPENDIX B

THE 301 INTERMYOFIBRILLAR MITOCHONDRIA PROTEIN IDENTIFICATIONS ASSIGNED WITH 99% CONFIDENCE IN >50% OF SUBJECTS WITH ≥ 2 IDENTIFIED PEPTIDES

[CONSULT ATTACHED FILES]

APPENDIX C

DIFFERENTIALLY EXPRESSED PROTEINS IN SUBSARCOLEMMAL MITOCHONDRIA OF OBESE SUBJECTS MAPPED TO PATHWAYS AND OVER-REPRESENTATION ANALYSIS BASED ON REACTOME THAT MET FALSE DISCOVERY RATE (FDR) CORRECTION CRITERIA OF $P \leq 0.05$

[CONSULT ATTACHED FILES]

APPENDIX D

DIFFERENTIALLY EXPRESSED PROTEINS IN INTERMYOFIBRILLAR MITOCHONDRIA OF OBESE SUBJECTS MAPPED TO PATHWAYS AND OVER-REPRESENTATION ANALYSIS BASED ON REACTOME THAT MET FALSE DISCOVERY RATE (FDR) CORRECTION CRITERIA OF $P \leq 0.05$

[CONSULT ATTACHED FILES]

APPENDIX E

KEY BIOLOGICAL PROCESSES BY GENE ONTOLOGY ASSOCIATED WITH SIGNIFICANTLY DIFFERENTIALLY EXPRESSED PROTEINS IN SUBSARCOLEMMAL MITOCHONDRIA FROM OBESE SUBJECTS THAT MET FALSE DISCOVERY RATE (FDR) CORRECTION CRITERIA OF $P<0.05$

[CONSULT ATTACHED FILES]

APPENDIX F

KEY BIOLOGICAL PROCESSES BY GENE ONTOLOGY ASSOCIATED WITH SIGNIFICANTLY DIFFERENTIALLY EXPRESSED PROTEINS IN INTERMYOFIBRILLAR MITOCHONDRIA OF OBESE SUBJECTS THAT MET FALSE DISCOVERY RATE (FDR) CORRECTION CRITERIA OF $P<0.05$

[CONSULT ATTACHED FILES]

APPENDIX G

KEY BIOLOGICAL PROCESSES BY GENE ONTOLOGY ASSOCIATED WITH PROTEINS EXPRESSING SIGNIFICANTLY DIFFERENT SS-TO-IMF PROTEIN ABUNDANCE RATIOS BETWEEN LEAN AND OBESE SUBJECTS THAT MET FALSE DISCOVERY RATE CRITERIA ($P<0.05$)

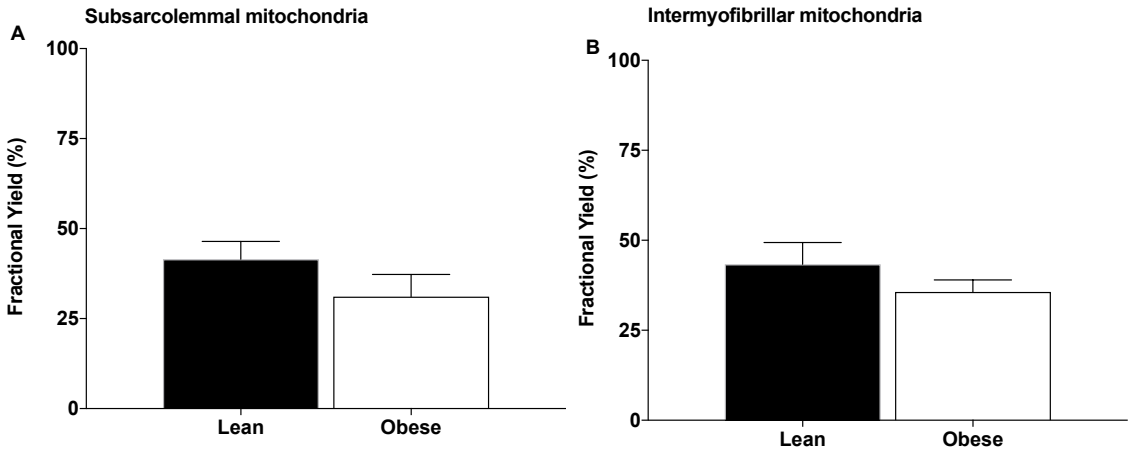
[CONSULT ATTACHED FILES]

APPENDIX H

FEATURES OF ISOLATED MITOCHONDRIAL PREPARATION

Features of isolated skeletal muscle mitochondrial preparations

The preparation of skeletal muscle from either SS mitochondria or IMF mitochondria between lean and obese (n = 3), measured in triplicate, provided similar yields as determined by Unpaired t tests; SS mitochondria, lean versus obese (38±5% versus 32±12%; *P>0.05*); IMF mitochondria, lean versus obese (43±13% versus 33± 1%; *P>0.05*)



The fractional yield of isolated subsarcolemmal and intermyofibrillar mitochondria preparations from muscle of lean and obese individuals. The present preparation of SS and IMF mitochondria from muscle of lean (n=3) and obese (n=3); A) SS mitochondria fractional yield from lean and obese; 41.5 ± 4.9% versus 31.1 ± 6.1%; *P > 0.05;* B) IMF mitochondria fractional yield from lean and obese; 43.3 ± 6.0% versus 35.6 ± 3.3%; *P > 0.05*; assayed as citrate synthase activity in the final preparation of SS and IMF mitochondria fractions relative to the total citrate synthase activity recovered following whole muscle homogenization; Data presented are ±SEM; significance determined by parametric, Unpaired t test; $P < 0.05$.

APPENDIX I

PERMISSIONS STATEMENT

All co-authors have granted permission for the use of the articles presented in this dissertation.