THE ROLE OF LONG-CHAIN ACYL-COENZYME A SYNTHETASE 1 (ACSL-1) IN LIPID METABOLISM IN HUMAN SKELETAL MUSCLE PRIMARY MYOTUBES.

by

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Obesity is considered a major health threat to the U.S. due to being a strong risk factor for developing type 2 diabetes and other metabolic diseases. The prevalence and severity of obesity is even greater among some subpopulations in the U.S. (African-American Women). In this regard, metabolic dysfunction may be associated with an impairment of mitochondrial fatty acid oxidation (mtFAO) which can lead to over accumulation of bioactive lipids such as fatty acyl-CoA species. While reductions in mitochondrial content may be a precipitating variable, reductions in key enzymes that lead to partitioning fatty acids towards mitochondrial oxidation may also be a contributing factor. Recently, reductions of acyl-CoA synthetase (ACS) activity have been identified in skeletal muscle. Long chain acyl-CoA synthetase (ACSL) exists as five different isoforms, the roles of which are to activate fatty acids to acyl-CoAs in the initial step of fatty acid metabolism (synthesis or oxidation). In liver of rodents, ACSL-1 has been thought to direct fatty acids toward mtFAO, but little data exists in human skeletal muscle. The purpose of this study was to understand the potential role of ACSL-1 activity in lipid metabolism in human skeletal muscle. To address the purpose of the study, we employed a model of underexpression/knockdown (UEX/KD) of ACSL-1 in primary human skeletal muscle cells (HSKM). Based on data from our laboratory, ACSL-1 overexpression significantly increased mtFAO in HSKM cells from obese individuals. Therefore, we hypothesized that ACSL-1

UEX/KD would reduce mtFAO in this tissue. To address our hypothesis, we conducted fatty acid oxidation and lipid synthesis experiments following 48 h of lipid exposure in HSKM primary myotubes obtained from percutaneous biopsies of the vastus lateralis transfected with either shRNA (KD) or scrambled RNA (control) plasmid vectors. Results demonstrated that ACSL-1 was significantly reduced ($P<0.05$) following KD vs. control. However, following ACSL-1 KD, we observed an absence of change in complete (CO_2) and acid soluble metabolites (ASM) incomplete metabolites oxidation palmitate. In addition, we also reported no alterations of total lipid synthesis and esterification of acyl-CoA toward MAG, DAG, and TAG synthesis despite the supply of exogenous lipids in our cell model. This is the first report of successful transfection and ACSL-1 KD in HSKM cells. Given the inconsistent findings with our original hypothesis, we now hypothesize the presence of compensatory mechanisms that exist following UEX/KD of ACSL-1 to offset the negative effects of ACSL-1 KD. Alternatives include upregulation of additional ACSL isoforms (e.g., ACSL-5) and/or elevations in peroxisomal activity.

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DEFINITIONS, SYMBOLS and ABBREVIATIONS

1. Obesity: increased body weight caused by excessive accumulation of fat or those with a BMI (Body Mass Index) expressed as body weight per height (kg/m^2) ≥ 30.
2. AAW: African American Women
3. CW: Caucasian Women
4. CPT-I: Carnitine palmitoyl transferase I. Transporter that allows entrance of long-chain fatty acid into the mitochondrial matrix.
5. ACS: Acyl-CoA synthetase. Mitochondria outer membrane protein that activates long-chain fatty acids by ligation of coenzyme A to fatty acyl lipids.
6. ACSL: long-chain Acyl-Coenzyme A synthetase. Its product, acyl-CoA is the "activated" substrate for CPT-I regulated fatty acid mitochondrial important for β-oxidation or partitioning toward lipid synthesis at and within the endoplasmic reticulum. Thus, the products of ACSLs are acetyl-CoA via β-oxidation and CO_2 from the Krebs Cycle or triacylglycerol (TAG), phospholipids (PL), and cholesteryl esters (CE).
7. ACSL-1: Isoform highly expressed in liver, heart, adipose tissue, express at moderate levels in skeletal muscle and at low levels in lung tissues.
8. ACSL-5: Isoform highly expressed in liver, adipose, and duodenal tissues and at very low levels in skeletal muscle.
9. shRNA: short hairpin RNA or small hairpin RNA. Plasmids encoding shRNA enter the cell via lipid-based transfection. shRNA plasmids are capable of stable inhibition of gene expression.
10. Mitochondria: organelle that functions in energy production. Site of β-oxidation and oxidative phosphorylation.
11. *In vitro*: Procedure performed in a controlled environment outside the living organism.

12. β-oxidation: Mitochondrial (Matrix) pathway responsible for the removal of carbons from a long-chain fatty acid to acetate (acetyl-CoA) units for entrance into the Krebs Cycle.
13. Nucleofection: Transfection technology based on the momentary creation of small pores in cell membranes by applying electrical pulses.
14. Gene knockdown (KD): Molecular technique used to reduce the expression of a gene's activity through genetic modification or by treatment with a reagent such as short DNA or RNA oligonucleotide.
15. Western blotting: A technique used to detect a specific protein by using antibodies specific for epitopes on protein structure and subsequently identified following PAGE separation, transfer and bound to a membrane; and finally detected by Horse Radish Peroxidase driven chemiluminesce methods.
16. Cell viability: A determination of living or dead cells based on a total cell sample.
17. Myotube: Differentiated myocell derived from satellite cell precursors obtained from tissue biopsies. The differentiated cell is an elongated, multinucleated myotube.
18. Myoblast: An undifferentiated cell capable of giving rise to muscle cells.
19. Tryacylglycerol o triglyceride (TG): A naturally occurring ester of three fatty acids and a glycerol that is the chief constituent of stored fats and oils within the cell cytoplasm.
20. Esterification: A chemical reaction resulting in the formation of at least one ester product.

CHAPTER I

INTRODUCTION

In a human, obesity is defined by an individual possessing a body mass index (BMI) greater than or equal to 30 kg/m^2. More specifically, BMI is classified as normal (18.5 to less than 25 kg/m^2), overweight (25 to less than 30 kg/m^2) and obese (30 kg/m^2 or greater) (Flegal, Graubard, Williamson, & Gail, 2005). BMI is calculated as height (m^2) divided by body weight (in kg) and is used as an indicator of total body fat according to Centers for Disease Control in the United States (U.S.) (CDC, 1985-2008).

In general, human obesity is the result of an energy imbalance (positive energy balance) due to an excess in energy consumption above that needed for daily physiological requirements (e.g., basal, resting metabolism plus that needed for physical activity) and reductions in energy expenditure. Thus and more recently, it is recognized by health officials that the rise in those incurring a positive energy balance in Western Society is also due to a reduction in energy expenditure due to less daily physical activity. A potential explanation for this observed positive energy balance is thought to be an ever growing dependence on technology leading to a reduction in recreational/health activities and less energy expenditure (Booth, Gordon, Carlson, & Hamilton, 2000). Consequently, it is not surprising that the incidence of childhood obesity is also on the rise in the U.S. In 2003-2004, 17.1 % of children and adolescents were overweight (Ogden, Carroll, Curtin, Lamb, & Flegal, 2010).

Obesity is strongly linked with the development of metabolic related diseases known as the Metabolic Syndrome manifested as elevated triglycerides, hypertension, whole body inflammation, skeletal muscle and liver insulin resistance, elevated fasting plasma glucose, and

reduced high density lipoprotein cholesterol (HDL-C), (Nesto, Nelinson, & Pagotto, 2009). Also not surprising is the link between the high incidence of obesity and the presence of type 2 diabetes in the U.S. which has increased dramatically over the past two decades. In 2002, an estimated 6.3% (18.2 million people) of the U.S. population was diagnosed with diabetes (CDC, 2003). When additional demographic data were linked to type 2 diabetes, the CDC defined this disease and its complications as the seventh leading cause of death in the U.S. with many states reporting statistics of over 10% of their population was diagnosed with type 2 diabetes (CDC, 2007)

While admittedly changes in lifestyle and environmental factors are likely to play a significant part for the rise in incidence and metabolic outcomes of obesity and type 2 diabetes, biological factors are also hypothesized to be contributors in a cause and effect fashion (Bouchard, 2007; Wing et al., 2001). This appears to be true not only for obesity in general, but may biological differences also account (at least in part) for differences among U.S. subpopulations. For example, the stark differences in the prevalence and severity of obesity between African-American Women (AAW) vs. Caucasian Women (CW) (Kumanyika, 1987). In general, lower mitochondrial content has been reported in skeletal muscle from obese and diabetic individuals compared to lean participants (Kelley, He, Menshikova, & Ritov, 2002) as well as the more recent finding that obese AAW appear to possess a reduced skeletal muscle capacity to oxidize exogenous fatty acids under maximal stimulated conditions compared to CW (Cortright, et al., 2006; Privette, Hickner, MacDonald, Pories, & Barakat, 2003). This may, in part, explain the finding that AAW have a two fold higher incidence and severity of obesity when compared to CW of similar age and socio-economic status (CDC, 2006-2008).

Given the above discussion, for some time now, alterations in mitochondrial handling of fatty acids have been proposed to be linked with the obese state and insulin resistance. For

example, mitochondrial dysfunction due to a deficiency or dysfunction in key regulatory proteins involved in the β-oxidative spiral for lipid oxidation leads to an increase of triglyceride (TAG) storage and/or acumulation of "bioactive lipid species" in both mouse skeletal muscle and liver causing severe hepatic and muscle insulin resistance (Zhang, et al., 2007). An extension of the developed insulin resistance in these tissues is heightened blood glycemia, endothelial glycosylation leading to reduction in peripheral blood flow to tissues, and pathological outcomes such as tissue necrosis/amputation, and retinopathy/blindness on top of neuropathies (Engelgau et al., 2004).

To extend, obese and type 2 diabetic individuals have been associated with a phenomenon known as lipid metabolic inflexibility (Storlien, Oakes, & Kelley, 2004). For example, as seen at the whole body level, following a high fat meal or during endurance exercise, the normal metabolic response of skeletal muscle is to "switch" metabolic processes to a greater reliance on lipids (oxidation) versus stored glycogen or blood glucose. This phenomenon does not occur in obese or diabetic individuals. The inflexibility of fatty acid oxidation is observed at the level of the mitochondria as evidenced by a low activity of Carnitine-Palmitoyl Transferase I (CPT-I) which is an entry step for fatty acyl-CoAs into the mitochondria for oxidation, and is considered the rate limiting step in the bioenergetic use of fatty acids (J.Y. Kim, Hickner, Cortright, Dohm, & Houmard, 2000). As evidence, fatty acid oxidation was observed to be significantly lower in skeletal muscle of extremely obese individuals under maximal stimulated conditions (Hulver et al., 2003). In terms of lipid balance, elevated serum lipid levels and increased intracellular uptake, coupled with a decrease in mitochondrial utilization of cellular fatty acids can lead to increased intramyocellular metabolites such as fatty acyl-CoAs and diacylglycerols (DAG) and the creation of a lipotoxic cellular environment (Li, Klett, & Coleman, 2010).

More recently, another candidate, long-chain acyl-CoA synthetase (ACSL), has been identified as a potential contributor to the reduced mitochondrial oxidative process of fatty acid disposal in skeletal muscle from obese individuals. ACSL is the required activator of fatty acyl long-chain molecules which act as the utilizable substrate for CPT-I (Coleman, Lewin, Van Horn, & Gonzalez-Baró, 2002). For example, it has been observed in AAW that CPT-I content and activity is significantly lower compared to CW of similar BMI (Cortright et al., 2006; Privette et al., 2003). ACSL is the focus of the present investigation.

There are five identified mammalian ACSL isoforms which utilize fatty acid chain lengths from 12 to 20 carbons (Soupene, E. and Kuypers, F.A., 2008). The five isoforms of the ACSL family consists of ACSL-1, ACSL-3, ACSL-4, ACSL-5 and ACSL-6. Each of the ACSL isoforms differ in their organelle localization, substrate preference, and enzyme kinetics (Mashek, Li, & Coleman, 2006); The function of the ACSL enzyme is required for activating acyl-CoA units towards either storage in the form of TAG at the level of the endoplasmic reticulum, or for fatty acid oxidation by the mitochondria (Digel, Ehehalt, Stremmel, & Fullekrug, 2009).

Despite the progress in our understanding of ACSL's role in cellular and mitochondrial partitioning and cellular derived fatty acids, this understanding is based almost exclusively on studies in rodent derived liver. Little is known about their activity and function in skeletal muscle, specifically human tissues. Clinically, this may be extremely important as skeletal muscle accounts for the majority of lipid oxidation and ~85% of glucose disposal following a meal (DeFronzo, 2010); a condition that can be impaired in the insulin resistant state associated with reduced skeletal muscle lipid metabolism. Thus changes in ACSL function may be linked, either directly or indirectly to lipid induced insulin resistance and the progression towards type 2 diabetes.

Most recently, studies using a transgenic model to knockout ACSL-1 (ACSL-$1^{L-/-}$) showed an accumulation of hepatic fat in ACSL-$1^{L-/-}$ mice compared to wildtype controls. Eliminating ACSL-1 in the liver of ACSL-$1^{L-/-}$ mice resulted in lower long chain acyl-CoA content, lower incorporation of oleate into hepatic TAG and reduced oleate oxidation, suggesting that hepatic ACSL-1 is important for mitochondrial β-oxidation (Li, et al., 2009). An accumulation of fat in the liver can lead to hepatic steatosis and disruption of hepatic insulin signalling as observed in Li et al. (2009) study. The high content of intracellular fatty acids resulted in an elevation of malonyl-CoA which inhibits CPT-I activity and β-oxidation (Schrauwen & Hesselink, 2004). Additionally, accumulation of hepatic fat in mouse models stimulate an increase in fatty acid metabolites (fatty acyl-CoA, DAG, ceramide, and glycosphingolipid), leading to TAG storage and activating cellular kinases that can reduce insulin action (Nagle, Klett, & Coleman, 2009). The same occurs in a defect with human skeletal muscle fatty acid oxidation of obese individuals leading to accumulation of not only intramyocellular triacylglycerols (IMTG) but in bioactive lipid species that are known activators of kinases known to attenuate the insulin signaling cascade and induce insulin resistance in this tissue (Shulman, 2000).

In summary, the goal of this research was to understand the role of ACSL-1 activity on fatty acid oxidation in cells derived from HSKM cells. To address this goal, we employed a model to underexpress/knockdown ACSL-1 (ACSL-1 KD) using short hairpin RNA (shRNA) technology. Our hypothesis was that ACSL-1 plays a major role in skeletal muscle fatty acid oxidation, and may be important for overall lipid dynamics; therefore reducing ACSL-1 activity would lead to decrements in the entrance of fatty acids into the mitochodria for oxidation. Although beyond the scope of the current project, we suspected that the biological consequence of ACSL-1 KD would be an accumulation of bioactive lipid species in skeletal muscle (e.g.,

DAG, fatty acyl-CoA, ceramides) (Hulver et al., 2003) leading to impaired insulin signaling and induction of insulin resistance, contributing to the etiology of type 2 diabetes.

Statement of the Problem : Obesity is considered a major health threat in the U.S. and a strong risk factor for developing type 2 diabetes and other metabolic diseases. Common observations in skeletal muscle in the obese and diabetic state include reductions in CPT-I activity/content and decrements in mitochondrial oxidative capacity for fatty acids which can led to an accumulation of bioactive lipid species and ultimately insulin resistance. Although the rate limiting step in lipid oxidation is considered to be at the level of CPT-I, limitations in substrate availability for CPT-I (acyl-CoAs) could also limit fatty acid oxidation. Given recent reports of decrements in total ACSL activity in skeletal muscle in the obese state as well as the observations from animal studies, the present research focused on the role of ACSL-1 isoform on skeletal muscle fatty acid oxidation in humans. The ACSL-1 isoform was chosen as the target based on its suspected association with the mitochondria.

Purpose of the Study: To establish a role of ACSL-1 in human skeletal muscle (HSKM) fatty acid oxidation.

Researh hypothesis: Based on previous results from our laboratory, ACSL-1 overexpression significantly elevated mitochondrial fatty acid oxidation approximately 2-fold ($P < 0.05$) in HSKM from obese subjects (data courtesy of Dr. H.B. Kwak; Appendix H). Therefore, we hypothesized that ACSL-1 isoform functions in the oxidation of fatty acids in human skeletal muscle and that a reduction of ACSL-1 would reduce mitochondria fatty acid oxidation in this tissue. We addressed the proposed hypothesis by using an shRNA transfection model in HSKM primary myotubes to underexpress/KD ACSL-1 protein content.

Delimitations (Self selected boundaries for the study)

1. The use of ACSL-1 isoform specific gene knockdown (KD) in human primary myotubes obtained from the vastus lateralis muscle group.
2. Participants involved in the study were lean Caucasian women.
3. Effects of ACSL-1 KD were extrapolated from Western Blotting assays.
4. Use of shRNA transfection by electroporation technology vs. adenoviral KD RNAi approaches as is common for studies of this type.
5. ACSL-1 KD by shRNA transfection occurred at the myoblast stage.
6. The control samples used to compare oxidation rates following ACSL-1 KD were transfected myoblasts with scrambled RNA.
7. Transfected cells were incubated with oleate and palmitate for 48 h prior to measurements of fatty acid oxidation and evaluation of lipid synthesis.

Limitations (The researcher has no control)

1. Primary myotube's metabolic response to the transfection technique.
2. The conclusions derived from the research design were limited to data from *in vitro* studies.
3. The potential for ACSL-1 KD on myotube growth and differentiation.
4. Survival rate of transfected cells.
5. Unequal transfection among myoblast cells.

CHAPTER II

REVIEW OF LITERATURE

Overview

The incidence of obesity continues to escalate in the Western world. Obesity, although in many cases is a preventable or treatable disease, it remains a continuing major health problem in the U.S. and the occurrence continues to escalate. As such, obesity remains a serious health threat in the U.S. due to its link with the major life threatening pathologies such as coronary heart disease, promotion of insulin resistance, and hypertension (Garrison, Higgins, & Kannel, 1996). According to recent CDC statistical data obtained from Behavioral Risk Factor Survelliance System (BRFSS) in 2005, 60.5 % of the total U.S. adult population is overweight, 23.9 % obese, and 3.0 % morbidly obese (CDC, 2005). When obesity rates were compared according to ethnicity, obesity prevalence was higher in Non-Hispanic Blacks (35.7 %), followed by Hispanics (28.7 %) and non-Hispanic Whites (23.7 %). When obesity was compared by gender, non-Hispanic Black women had greater occurrence (39.2 %), followed by non-Hispanic Black men (31.6 %), Hispanic women (29.4%), Hispanic men (27.8 %), non-Hispanic White men (25.4 %) and non-Hispanic White women (21.8 %) (CDC, 2006-2008). In the past years, there have been 111, 909 reported deaths associated with obesity, with the majority occuring in individuals younger than 70 years old (Flegal et al., 2005). If the epidemic of obesity as a threat to the health of the U.S. is not enough of a concern, the associated economic burden that obesity imposes on the U.S. health care system continues to rise in parallel. In 1998, medical cost of obesity was $ 78.5 billion, in 2008, the cost has risen to $ 147 billion and continue to escalate. (Finkelstein, Trogdon, Cohen, & Dietz, 2009).

Obesity has been identified as a strong risk factor for the development of type 2 diabetes, (Nesto et al., 2009). In 2006, diabetes was reported to represent the seventh leading cause of death in the U.S. Some of the cost associated with diabetes are heart disease, stroke, hypertension, blindness, kidney disease, nervous system disease, among others (CDC, 2007).

With regard to type 2 diabetes as a comorbidity with obesity, the total estimated cost of diabetes alone in 2007 was $174 billion. Medical costs attributed to diabetes were estimated to be around $27 billion for care to directly treat diabetes, $58 billion to treat the portion of related chronic complications that were attributed to diabetes, and $31 billon in excess general medical costs (CDC, 2007). Together, these statitistics make obesity and type 2 diabetes one of the most economically burdensome health concerns for the nation.

How might the obese state be associated with type 2 diabetes? Among many scientific strategies used to answer this question, it was noted early that the accumulation of TAGs and other lipids in skeletal muscle was associated with insulin resistance in this tissue (Pan et al., 1997). Logically, then it was speculated that excess lipid storage, not only in adipose tissue but in skeletal muscle was occuring as a result of a chronic positive energy balance due not only to high dietary fat consumption, but also reduced energy expenditure (Coppack, Jensen, & Miles, 1994), as exemplified by the low levels of physical activity reported in present day society. According to NHANES 2003-2004 data, physical activity decreases between childhood and adolescence. For example, 42% of children ages 6-11 years old accumulated one hour of physical activity on most days of the week, but only 8% of adolescentes achieved these guidelines (Troiano et al., 2008). Given these statistics, it became evident that there was an absence of a mechanistic answer for the relationship between tissue (skeletal muscle) lipid accumulation, reductions in physical activity, and a disruption of cellular homeostasis. However, what slowly began to emerge was that an over accumulation of cellular lipids appeared to have a

cause and effect relationship between altered lipid metabolism and insulin resistance. The end result of the latter was the creation of a chronic state of hyperglycemia and eventual overstimulation and loss of function of pancreatic insulin secretion (i.e., type 2 diabetes).

Thus, current wisdom linking obesity with skeletal muscle insulin resistance, and eventually diabetes, centers on investigations that study disturbances in lipid homeostasis in skeletal muscle of obese individuals. Refering to a lipid substrate balance analogy, increased adiposity can occur under chronic conditions of consumption of a high fat/energy meals coupled with reductions in energy demand (e.g., reduced physical activity and hence ATP production demand). The end result would be increased adiposity visually realized to the naked eye or by one of several measures of adiposity such as DEXA scanning, skinfold, girth assessments, or BMI calculations (Booth et al., 2000). Environmental factors such as behavior or the lipid content in Western foods could account for the obesity/diabetes epidemic (Wing et al., 2001). That is, fat cells enlarge to accommodate high circulating levels of lipid but when their storage capacity is exceeded, spillover occurs, one site being skeletal muscle. The end result is increased TAG storage and flux of more bioactive lipid species in this tissue (Lieberman & Marks, 2009).

The above scenario is undoubtedly a contributing factor to the etiology of obesity and the accumulation of skeletal muscle lipids. However, the other side of the substrate balance equation must also be considered, that being the metabolism of cellular lipids. This requires a competent mitochondrial mass with a capacity to oxidize fatty acids that meets or exceeds cytosolic fatty acid concentrations. The question then arises, do mitochondria function normally in skeletal muscle from obese subjects? In response to this querie, our laboratory and others have demonstrated a decrease in fatty acid oxidation in skeletal muscle of morbidly obese women under maximally stimulated conditions (Hulver et al., 2003; Privette, et al., 2003). Similarly, Kelley, Goodpaster, Wing and Simoneau (1999) reported that fatty acid oxidation measured in

obese and lean subjects by indirect calorimetry was lower in the obese individuals under maximally stimulated conditions. Likewise, J.Y. Kim et al. (2000) demonstrated that the rate of palmitate oxidation in skeletal muscle of obese women was lower compared to lean women and this reduction was due (in part) to low activity of CPT-I, the protein complex required for fatty acid uptake into the mitochondrial matrix for oxidation (J.Y. Kim et al., 2000). Thus, in a physiological condition that leads to an elevation of plasma free fatty acids (high fat diet and/or inactivity), skeletal muscle would increase its lipid content due to increased uptake as with obese and diabetic individuals. This in combination with a diminished capacity to oxidize fatty acids under maximally stimulated conditions could lead to a further accumulation of not only neutral lipids (i.e., IMTG) but also bioactive lipid metabolites (i.e., DAG, acyl-CoA and ceramides) in liver, heart, and the topic of this research, skeletal muscle. The accumulation of intramuscular lipids has been shown to correlate with insulin resistance (Schrauwen & Hesselink, 2004). This impairment could be due to a number of sites of dysfunction: increased sarcolemmal transporters, mitochondrial CPT-I, and the electron transport chain. The impairment of mitochondrial fatty acid oxidation could be due to lower mitochondrial and microsomal acyl-CoA synthetase in skeletal muscle (the enzyme that ligates coenzyme A to long-chain fatty acids which is the required substrate for CPT-I and hence obligatory entrance into the mitochondrial matrix for oxidation). In summary, these metabolic defects appear to be associated with obesity leading to insulin resistance in skeletal muscle of obese individuals and the development of type 2 diabetes (Pan, et al., 1997).

With this brief overview in mind, the global purpose of the proposed research is to further understand the metabolic regulators of skeletal muscle lipid metabolism as it relates to insulin resistance and obesity. Our intent is to add scientific knowledge about mechanisms that contribute to the development of these diseases along with gaining insight towards more

effective interventions for those affected with obesity and insulin resistance and associated pathologies. We addressed these goals by the described research studies designed to further understand the effect of a recently identified reduction in the protein responsible for activating fatty acids, namely acyl-CoA synthetase (ACS), for storage or oxidation in human skeletal muscle (Privette et al., 2003). Our approach is to compliment ongoing studies in our laboratory by knocking down the presence of a specific isoform of ACSL, ACSL-1, which has been associated with mitochondrial oxidation of fatty acids and observed the consequence of that knockdown in HSKM cells.

The Role of Skeletal Muscle in Fatty Acid Metabolism.

Skeletal muscle is the major site of not only insulin stimulated glucose disposal following a meal (~ 85% of the blood clearance is due to skeletal muscle uptake) (DeFronzo, 2010) but also is the major site of fatty acid oxidation. A cause and effect relationship between excess intra-myofibrillar lipid load and insulin resistance has been supported by many studies in the literature. To understand the relationship, it is first important to understand the normal metabolic handling of fatty acids by skeletal muscle in the healthy state in order to discern sites of dysregulation that occur in the obese, insulin resistant condition.

Fatty acids are transported to skeletal muscle from the liver as very long density lipoprotein (VLDL) (de novo lipid synthesis), from the diet as chylomicrons, or esterified to albumin following lipolysis of adipocyte triglyceride (Lieberman & Marks, 2009). Afterwards, they are taken up to the skeletal muscle by active and passive transport (CD36 fatty acid transporter) (Schwenk, Holloway, Luiken, Bonen, & Glatz, 2010). Subsequently, fatty acids can be partitioned toward TAG formation by the endoplasmic reticulum, or they can be chaperoned

to the mitochondrial for transport into the matrix for β-oxidation and subsequent production of reducing equivalents (NADH, $FADH_2$) to support oxidative-phosphorylation and maintenance of energy charge in the cell (Lieberman & Marks, 2009). This research proposal focuses on the mitochondrial arm of the lipid partitioning pathways.

As depicted in Figure 1, long chain fatty acids (LCFA) are activated by the thioester linkage of coenzyme A (CoA) by acyl-CoA synthetase enzyme to create LCFA-CoA (Mashek, Li, & Coleman, 2007). These activated lipid species are then made available to the mitochondria organelle at the site of CPT-I which enzymatically exchanges the CoA moiety for cytosolic carnitine. On the matrix side, LCFA-carnitine is activated back to LCFA-CoA by the analog to CPT-I, namely CPT-II, which converts the translocated acyl-Carnitine back to acyl-CoA (McGarry, & Brown, 1997). Finally, fatty acids are oxidized by the β-oxidation system generating acetyl-CoA for entrance into the tricarboxylic acid cycle (TCA). CPT-I allows entrance of LCFAs into the mitochondrial matrix and towards β-oxidation. The main biological regulator of CPT-I activity is malonyl-CoA. Malonyl–CoA is synthesized from acetyl-CoA by the enzyme acetyl-CoA carboxylase-2 (ACC2). Studies have shown that mice lacking ACC2 have increased skeletal muscle oxidation. In contrast, an excess of malonyl-CoA disrupts CPT-I activity and results in the accumulation of fatty acids (Lieberman & Marks, 2009).

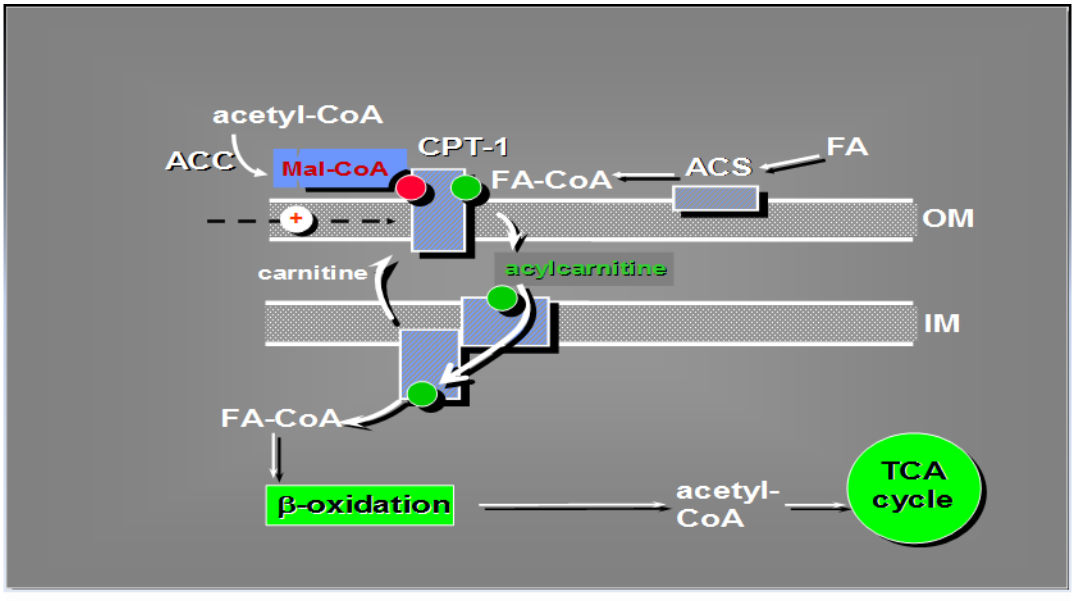


Figure 1. The Role of Mitochondrial Fatty Acid Oxidation in Skeletal Muscle (courtesy of Muoio, D. Ph.D.). Cytosolic long-chain fatty acid (LCFA) is activated by acyl-CoA synthetase (ACSL). ACSL is necessary for enzymatically ligating coenzyme A (CoA) to cytosolic LCFA and its activated form is required for transport into the mitochondria by CPT-I. CPT-II exchanges carnitine for CoA gaining access inside the mitochondria matrix to undergo β-oxidation. In opposition, acetyl-CoA carboxylase converts mitochondrial derived acetyl-CoA (from TCA citrate) to malonyl-CoA. Malonyl-CoA is the biological inhibitor of CPT-I and thus reduces mitochondrial β-oxidation via inhibition of fatty acid transport to the matrix.

The disruption of the above pathway can lead to an accumulation of IMTG or bioactive lipid species which has been demonstrated to disturb cellular homeostasis in non- adipose tissues such as heart, liver, and skeletal muscle (i.e., creation of a cellular lipotoxic environment). This begs the question as to what is or are the major sites of lipid partitioning regulation in the myocyte? As alluded above, to date the rate limiting step in fatty acid oxidation has been assumed to be CPT-I (Wolfgang et al., 2008). Disruption of CPT-I activity by its inhibitors malonyl-CoA (its naturally occurring biological inhibitor) or etomoxir (synthetic inhibitor of CPT-I) leads to more than a 90% reduction of mitochondrial fatty acid oxidation (Noland et al., 2007; Sebastián et al., 2009). Up to now, the metabolic step prior to CPT-I transport, production of CPT-I substrate acyl-CoA by ACSL, as described above, was not considered rate limiting for

fatty acid oxidation. Recently however, potential dysfunction or reductions in activity of ACSL in the obese state has been suggested (described below). ACSL-1 has been thought to activate fatty acids destined to lipid synthesis but this data is based solely on studies in the liver from rodents (Li et al., 2009). A potential role for ACSL-1 in mitochondrial fatty acid oxidation in skeletal muscle is only now emerging based on data generated in rat, mice liver and heart (Muoio, Lewin, Wiedmer, & Coleman, 2000; Li et al. 2009; Chiu et al., 2001)

Mitochondrial Changes and Skeletal Muscle Lipid Accumulation in Obesity and Diabetes

The reduction of fatty acid oxidation in the extremely obese participants contributes to an accumulation of intramyocellular long chain fatty acyl-CoA due to impairment in skeletal muscle lipid metabolism (Hulver et al., 2003). In contrast, there is considerable evidence to associate other lipid species derived from altered skeletal muscle lipid metabolism such as DAGs, fatty-acylCoA/phospholipid species and ceramides (Yu et al., 2002). Raising plasma free fatty acid levels during euglycemic-hyperinsulinemic clamp in HSKM showed an increase in DAGs levels and protein kinase C (PKC) signaling, but no increase was observed in intracellular ceramide (Itani, Ruderman, Schmieder, & Boden, 2002). What is hypothesized to lead to the accumulation of "bioactive lipid species" is an alteration in the lipid partitioning pathways of lipid metabolism (Schrauwen & Hesselink, 2004). For example, changes in mitochondrial handling of cellular lipid flux, which is elevated in the obese-insulin resistance condition, is hypothesized to be a mitigating factor leading to fatty acid accumulation within skeletal muscle in this population (Blaak, 2004). What is currently unknown is the exact mechanism by which the mitochondrial capacity to oxidize lipids becomes insufficient to lessen the cytosolic lipid accumulation leading to reduced insulin action. Evidence for reductions in mitochondrial mass has been presented (Ritov et al., 2005). For example, Ritov et al. (2005) indicated that electron transport chain

activity in subsarcolemmal mitochondria (located underneath skeletal muscle membrane) was reduced in obese and type 2 diabetic individuals compared to lean subjects. This reduction was confirmed by transmission electron microscopy. Kelley et al. (2002) measured skeletal muscle mitochondria bioenergetic function in lean, obese, and type 2 diabetic individuals. They reported dysfunction of mitochondria of type 2 diabetic participants. These subjects had a lower capacity of electron transport chain measured by NADH:O_2 oxidoreductase (measures the overall activity of mitochondrial electron transport chain), and it was highest in lean volunteers. Kelley et al. (2002) also observed skeletal muscle mitochondria was reduced by 35% in obese and type 2 diabetic individuals compared to lean counterparts. These findings lead to the contention that impairment of mitochondrial function in skeletal muscle contribute to the development insulin resistance. Certainly, reductions in mitochondrial oxidation of fatty acids stemming from lower mitochondrial enzyme content could result in accumulation of fatty acids; however, the literature is still lacking as to whether this is an early stage of lipid associated metabolic disease, or whether it is the result of chronic overload of lipids leading to a lipid toxic environment that eventually results in mitoptosis (Schrauwen & Hessellink, 2004). In this regard, increased free radical production due to an imbalance between lipid flux and a low energy demand (reduced electron flow to complex IV of the electron transport chain) has been proposed as the initial lesion in the excess lipid induced insulin resistance in skeletal muscle (Anderson et al., 2009; J.A. Kim, Wei, & Sowers, 2008). Although beyond the scope of the present investigation, studies of this sort are being pursued in our and other laboratories (Anderson et al., 2009). Alternatively, reduction in particular mitochondrial proteins upstream of the electron transport chain have been investigated, albeit on a limited basis in human skeletal muscle. Early and current findings are discussed below.

Evidence for Reduced Fatty Acid Oxidation Activity in the Obese State.

Our laboratory has measured muscle lipid oxidation by analyzing skeletal muscle homogenates of obese and lean individuals (J.Y. Kim et al., 2000). The results show a decrease in muscle lipid oxidation of obese individuals under maximally stimulated conditions. The substrates utilized in the studies to measure fatty acid oxidation rates were palmitate (C16 long-chain fatty acid requiring ACSL activity for deriving CPT-I substrate), palmitoyl carnitine (molecule generated by CPT-I; hence CPT-I independence), octanoate (C8 medium-chain fatty acid; independent of CPT-I activity) and as well as determination of CPT-I activity/content. Palmitate oxidation was significantly lower by 62 % in obese participants compared to lean counterparts. Palmitoyl carnitine oxidation was also decreased with obesity by ~45% and octanoate was reduced by ~69 %. Palmitate oxidation after correction for mitochondrial volume (palmitate oxidation/citrate synthase activity) was also reduced with obesity by 48 %. In summary, the results indicate that skeletal muscle of obese individuals have a decreased ability to oxidize lipids under maximally stimulated conditions and directs them toward synthesis. This is at least in part by a reduction of CPT-I activity and likely reflects, at least partially, a reduced mitochondrial content as reported by Ritov et al. (2005) and Kelley et al. (2002). More recently however, additional insights gained from our laboratory also indicate that reductions in mitochondrial fatty acid oxidation may exist at the level of ACSL.

Acyl-CoA Synthetase (ACSL) Enzyme Function in Metabolism.

ACSL is divided into five sub-families based on its chain length of acyl groups: acyl-CoA synthetase short chain (ACSS) C2 to C4; medium-chain (ACSM) C4 to C12; long chain (ACSL) C12 to C20; bubblegum (ACSBG) C14 to C24; and very long chain, annotated as a solute carrier family 27A (SLC27A) C18 to C26 (Soupene & Kuypers, 2008).

Based on studies in rodents, there is a family of mammalian long chain Acyl-CoA synthetase (ACSL) isoforms that consist of five members, ACSL-1, ACSL-3, ACSL-4, ACSL-5 and ACSL-6. Each of the ACSL isoforms differs in their organelle localization, substrate preference and enzyme kinetics (Mashek et al., 2006). ACSL-1 has been reported to be located in the liver mitochondrial membrane (Distler, Kerner, & Hoppel, 2007), plasma membrane and mitochondria of adipocytes (Soupene & Kuypers, 2008), adipocyte GLUT-4 vesicles (Sleeman, Donegan, Heller-Harrison, Lane & Czech, 1998), and endoplasmic reticulum (Lewin, Kim, Granger, Vance & Coleman, 2001). ACSL-1 mRNA in rodents has been identified to be richly present in adipose tissue, liver, and heart, at moderate levels in skeletal muscle, and at low levels in lung, kidney and adrenal organs (Mashek et al., 2006). Overexpression of ACSL-1 in mouse heart increases myocardial lipids, such as TAG, and causes dysfunction of β-oxidation leading to cardiac hypertrophy, heart failure, left ventricular dysfunction, and sudden death (Chiu et al., 2001). Overexpression of ACSL-1 in the liver leads to TAG synthesis (Parkes et al., 2006). Overexpressing ACSL-1 in NIH-3T3 fibroblasts or PC12 neurons also increases oleic acid incorporation into TAG (Mashek et al., 2007). ACSL-3 was predominantly found in the brain and testis (Mashek et al., 2006). It is believed to be required for the synthesis of fatty acids important for brain metabolism (Van Horn et al., 2005). ASCL-4 was expresed abundantly in liver and adrenal gland (Mashek et al. 2006). ACSL-5 is mostly expressed in brown adipose tissue, duodenal mucosa, and liver tissue. Its overexpression in hepatic cells partitions exogenous

fatty acid towards synthesis pathways (Mashek et al., 2006). ACSL-6 was found at moderate levels in skeletal muscle, testis and mostly in the brain. The overexpression of clonned ACSL-6 from rat brain increased fatty acid synthesis towards specific lipids. (Van Horn et al., 2005).

The Role of ACSL-1 in Rodent Liver

The fate of acyl-CoA depends on the acyl-CoA synthetase's (ACS) location. ACSL-1 can direct acyl-CoA towards synthesis of TAG, phospholipids and β-oxidation, recycling pathways or cholesterol and retinal esterification. Acyl-CoA can also alter cell signals such as insulin secretion, apoptosis, glucose transport and metabolism. Evidence shows that each of the ACSL isoforms channels fatty acid into different metabolic pathways (Coleman et al., 2002). Studies in rats suggest that in liver, acyl-CoAs do not move freely but are channeled toward specific pathways. This was delineated by using the ACSL inhibitor triacsin C in isolated rat hepatocytes. Triacsin C inhibited TAG synthesis, but had little effect on oleate incorporation into cholesterol esters, phospholipids, or the end products of β-oxidation. When rat hepatocytes where studied in the fed state, triacsin C inhibited TAG synthesis more than in starved rat hepatocytes indicating that acyl-CoA synthetase has different metabolic pathways at least in the liver. It was also found that ACSL-1 was found mostly in microsomes of rat hepatocytes indicating its involvement with TAG synthesis and not β-oxidation (Muoio et al., 2000). Another study knocking out ACSL-1 observed a reduced presence of long chain acyl-carnitine and its metabolite products from long chain fatty acids from mitochondrial β-oxidation in liver. The decrease of long chain acyl-carnitine and acid soluble metabolites (ASM; requires acyl-CoA entrance into the mitochondrial matrix but represents incomplete β-oxidation/TCA metabolism) suggested that long chain fatty acid oxidation in liver was impaired by ACSL-1 deficiency (Li, et al., 2009). The low activity of

enzyme ACSL-1 led to the accumulation fatty acids in the liver and impaired its function. Overall, the absence or presence of acyl-CoA synthetases changed the intracellular concentrations of fatty acids and acyl-CoA content with the latter being more important in signaling molecules as stated above (Digel et al., 2009).

The Role of ACSL-1 in Rodent Skeletal Muscle

At present, there is little information with regard to the metabolic pathway(s) regulated by ACSL-1 in skeletal muscle. As stated, it is known from studies in the liver that ACSL catalyzes the first step in fatty acid metabolism by converting long chain fatty acids into acyl-CoA thioesters. Acyl-CoAs enter both anabolic and catabolic pathways and their altered content can be linked to insulin resistance and other disorders (Li et al., 2006). It has been hypothesized that the absence of ACSL-1 in skeletal muscle would lead to an accumulation of IMTG and other lipid species due to an impairment of mitochondria's ability to activate long-chain fatty acids for subsequent mitochondrial transport and oxidation. It has been observed that mice with a conditional knockdown of ACSL-1 (ACSL-$1^{T-/-}$) have a 70-90 % reduction in total ACS activity in gastrocnemius, soleus, quadriceps, and extensor digitorum longus. This indicates that skeletal muscle oxidation of palmitate in ACSL-$1^{T-/-}$ is impaired in skeletal muscle compared to the control group (data courtesy of Dr. Rosalind A. Coleman, unpublished findings). Furthermore, recent data from our laboratory suggested that ACSL-1 overexpression in skeletal muscle increases fatty acid oxidation, H.B. Kwak (personal communication; Appendix H). Primary myoblasts isolated from vastus lateralis of obese subjects (N=5) were transfected with ACSL-1 plasmid DNA, differentiated into myotubes, and harvested (7 d) to measure FAO ([1-^{14}C] palmitate), Radioactivity of CO_2 (complete palmitate oxidation) and ASM (acid soluble metabolites; incomplete palmitate oxidation) was determined by [1-^{14}C] and liquid scintillation.

Both complete (CO_2) and incomplete (ASM) FAO increased by approximately 2-fold ($P < 0.05$) in ACSL-1 overexpression.

Cellular Lipid Accumulation and Insulin Action in Skeletal Muscle

It has been proposed that the accumulation of intramuscular lipids could disrupt insulin signaling by changing the dynamic flux of lipid species such as acyl-CoAs and DAGs and thus up regulate certain PKC serine/kinase isoforms (i.e., PKC θ) which have been demonstrated to impair insulin receptor tyrosine kinase and IRS-1 activity; the result would be an attenuation of glucose transporter 4 (GLUT 4) translocation to the cell surface membrane leading to impaired glucose uptake (skeletal muscle insulin resistance; the hallmark of the Metabolic Syndrome) (Lowell & Shulman, 2005). The later outcome would be chronic reductions in glucose uptake which would then overstimulate the pancreatic β cells as an attempt to offset the insulin resistance at the level of skeletal muscle in an attempt to clear blood glucose. Eventually, chronic overstimulation would lead to β-cell failure and manifestation of overt type 2 diabetes (Kasuga, 2006). As observed by Kelley et al. (1999) lipid accumulation in obese skeletal muscle reduces fatty acid oxidation under maximally stimulated conditions which has been associated with insulin resistance.

In summary, a change in function of several key lipid-mitochondrial proteins can have profound effects on mitochondrial handling of long-chain fatty acids and cellular bioenergetics. In the past, a strong association between lipid overload, reductions in mitochondrial oxidative capacity and insulin signaling have been hypothesized (J.Y. Kim et al., 2000). Several cause and effect relationships have been suggested including lipid induced activation of certain PKC isoforms and consequently induction of the serine/threonine phosphorylation of key elements in the signaling cascade with subsequent reduction in GLUT 4 recruitment and induction of skeletal

muscle insulin resistance. The eventual outcome would lead to pancreatic β cell failure and Frank type 2 diabetes and related pathologies (Itani, Zhou, Pories, MacDonald, & Dohm, 2000). Those mechanisms which could induce reductions in the insulin signaling cascade and insulin action could be initiated by a reduction in the mitochondrial oxidation of fatty acids. In the past, it is believed that CPT-I was the main regulator of mitochondrial oxidation as it is responsible for fatty acid transport into the matrix for subsequent oxidation (McGarry & Brown, 1997). More recently however, other potential regulators have been identified to change in function under altered conditions of lipid metabolism. Relevant to the current research is the function of acyl-CoA synthetases in lipid trafficking toward synthesis or oxidation of fatty acids (Coleman et al., 2002). Acyl-CoA synthetase activity is necessary for activation of acyl-lipids in order to serve as CPT-I substrate or for lipid synthesis in the endoplasmic reticulum. Altered function of this enzyme could therefore alter mitochondrial oxidation of fatty acids and drive an imbalanced partitioning toward synthesis and metabolism to bioactive lipids which could, hypothetically, induce insulin resistance (J.Y. Kim et al., 2000). As such, ACSL-1 is an essential enzyme that contributes to the regulation of mitochondria bioenergetics in the regulation of fatty acids and could help aid in devising mechanistic based therapies for combating the ever rising incidence and severity of obesity induced skeletal muscle insulin resistance and progression towards type 2 diabetes. The presence and action of ACSL-1 is being delineated in the liver, but little is understood about its potential role in skeletal muscle, the major organ for lipid oxidation/disposal and blood clearance of dietary glucose. Based on data from rodent liver and preliminary data from our laboratory, we hypothesize that ACSL-1 is present in human skeletal muscle and its decrease in activity could lead to a decrease in mitochondria fatty acid oxidation.

CHAPTER III

METHODOLOGY

Experimental Design

The aim of this study was to test the metabolic effect of ACSL-1 underexpression/KD on skeletal muscle cell's ability to oxidize fatty acids. To address this aim, we harvested skeletal muscle cells obtained from the vastus lateralis and induced a KD condition of ACSL-1 by cell transfection using shRNA scramble as a negative control and ACSL-1 shRNA plasmids to create inhibition of the target gene expression. We used the transfected cells to test our hypothesis that ACSL-1 KD would result in a decrease of HSKM fatty acid oxidation. In order to study the role of ACSL-1 KD expression in primary HSKM cells several experimental approaches were employed 1) Participants were screened at baseline (Appendix C) and assessed for fasting blood glucose, insulin action, and caloric levels. 2) A series of control experiments were performed to detect transfection efficiency 3) specificity of our ACSL-1 antibody was tested on several tissues with known ACSL-1 activity and this data also provided us with a proper approach towards gel loading of protein (μg) for Western Blotting 4) We conducted Western Blotting assays to confirm the effectiveness of our shRNA plasmid transfection model on ACSL-1 gene knockdown at protein level 5) We conducted measurement assays on *in vitro* rates of fatty acid oxidation in primary, differentiated myotubes obtained from vastus lateralis muscle biopsies and finally 6) assays were conducted to the effect(s) of ACSL-1 KD on total lipid synthesis and presence of subclasses of lipids in transfected cells.

Participants

Six non-obese Caucasian (BMI < 27 kg/m^2) women (ages 25-45 yr) were recruited to participate in this investigation. HSKM cells were cultured from non-obese Caucasian women because they posses "normal" ACSL activity compared to obese individuals. All participants were premenopausal women who were non-smokers. Inclusion/exclusion criteria included subjects that were: non-diabetic, without metabolic disease known to affect glucose and lipid metabolism, and the absence of any medications (e.g., synthroid) known to alter metabolism (Appendix C). Participants received both oral and written information about the experimental procedures before giving their informed consent (Appendix B). The experiments were approved by the Institutional Review Board of East Carolina University (Appendix A). Participants were screened and their diet history was evaluated prior to entrance into the study using a 3-day (two non-consecutive week days and one weekend day) food record (Appendix D) and 24-h recall diets were evaluated for estimated total energy, fat carbohydrate, and protein intake (nutritionist 5 software). Prior to commencing the study, participants were evaluated for body composition by DEXA, aerobic capacity (VO2 peak) using a cycle ergometer. On the day of the experiment, participants reported to the laboratory following an overnight fast (approximately 10 h). Height, body weight and menstrual cycle (Appendix E) data were recorded. Skeletal muscle biopsies were obtained from the lateral aspect of the vastus lateralis by the percutaneous needle biopsy technique under local subcutaneous anesthesia (1% lidocaine) (Evans, Phinney, & Young, 1982).

Cell Culture Procedures

The culture technique used in the laboratory is schematically outlined in Figure 2. The typical mass of the skeletal muscle fibers obtained was ~120 mg from the lateral portion of the vastus lateralis. Approximately 60-70 mg was used for cell culture procedures. The sample was collected in cold low glucose DMEM. At the cell culture laboratory under sterilize conditions, sterilize forceps and scalpel were used to mince muscle in a petri dish containing 2 mL DMEM to remove blood clots, fat, and connective tissue. Minced muscle was transferred into 50 mL conical tube rinsing with hanks buffer saline (HBS) as necessary. The sample was centrifuged at 500 g for 10 min, the supernatant was removed and sample was resuspended in 5 mL trypsin collagenase cocktail, warmed to 37°C in a slow shaking water bath for 30 min. Afterwards, 1 mL (5% conc) FBS (fetal bovine serum) was added to stop trypsin action. The sample was again centrifuged at 500 g for 10 min. Supernatant was then removed and sample was transferred to non-collagen coated T-25 flask containing warmed growth media and placed in the incubator for 3 h. Sample was then transferred to collagen coated T-25 flask containing growth media and 24 h later 2 mL growth media was added to the flask inside the incubator. Growth media was changed every 5 days until cells reached 80% confluence; this process took ~6 weeks. Cells were transferred to collagen coated T-75 flask; first growth media was removed from T-25 flask and washed gently with 5 mL hanks solution. Cells were detached with 2 mL (0.05%) trypsin EDTA for 2 min. Trypsin was stopped by adding 10 mL growth media and transferred to conical tube. The T-25 Flask was rinsed with hanks and sample was centrifuged at 500 g for 10 min. Supernatant was removed and cells were resuspended in 12 mL growth media and transferred to T-75 collagen coated flask containing growth media and allowed to reach 80 % confluence or cells were frozen down in cryovial containing 5% DMSO (dimethyl sulfoxide) and stored in liquid nitrogen for further studies. NOTE: We were concern during the growth and differentiation processes of the cells that

adipocyte cells may have also be stimulated to grow along with the myotubes. However, repeated examination using microscopy has led us to believe that this was not the case. Our laboratory has not observed the presence of adipocytes or lipid droplets associated with differentiated myotubes.

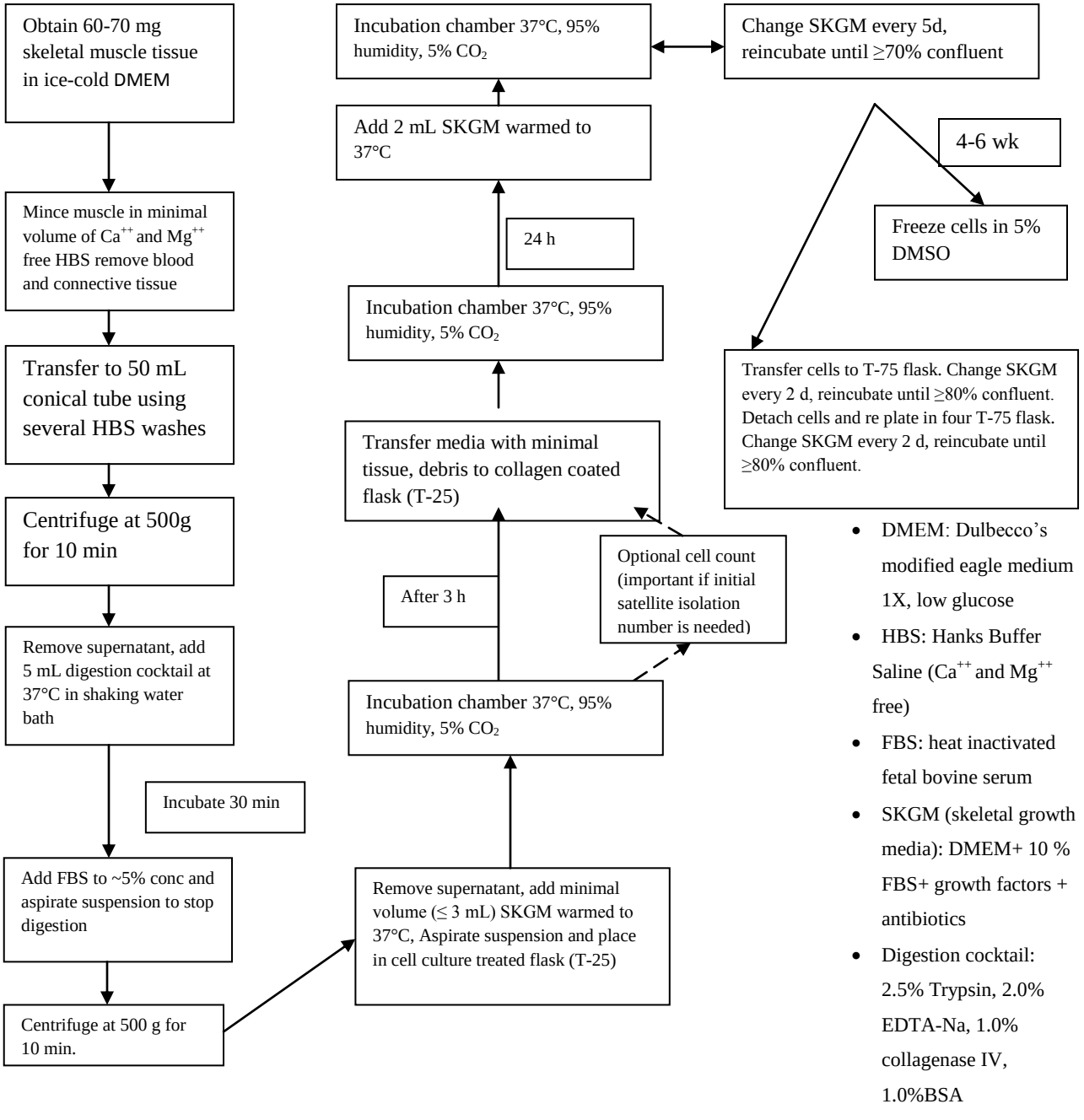


Figure 2: Schematic of human skeletal muscle cell isolation, propagation, and storage.

Berggren, J.R., Tanner, C.J., and Houmard J.A. (2007)

Control Experiments to Detect Transfection Efficiency and ACSL-1 Antibody Specificity.

Transfection efficiency was measured by green fluorescent protein (GFP) and a series of control tissue samples were used in order to determine ACSL-1 antibody specificity prior to initiate experiments.

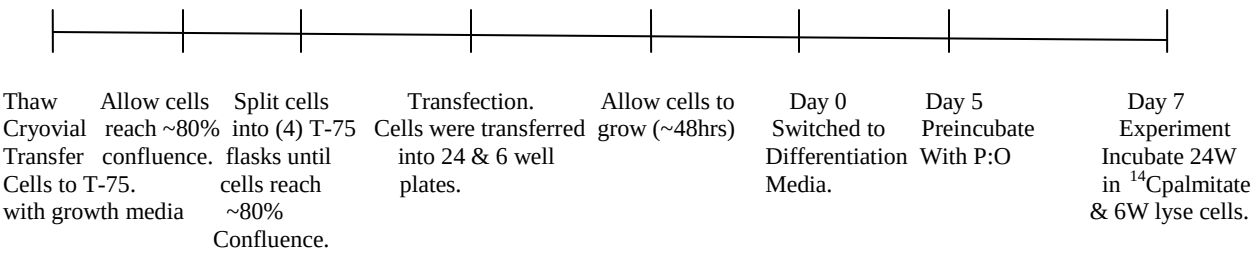
Earlier, our laboratory, had assessed transfection efficiency into myoblasts (technique performed by H.B. Kwak, Appendix F) harvested and grown from satellite cells obtained from vastus lateralis biopsies. Myoblasts ($1x10^6$ cells) were resuspended in 100 µl Nucleofector solution (Lonza, Walkersville, MD) combined with 3 µg ACSL plasmid DNA (Invitrogen: Carlsbad, CA) and 2 µg pmax GFP (green fluorescent protein) (Lonza: Walkersville, MD).

ACSL-1 antibody was measured on rat tissues and mouse liver. Rat adrenal, brain, and lung organs were collected in lyses buffer containing 50 mM HEPES, 10 mM EDTA, 100 mM NaF, 50 mM Na pyrophosphate, 10 mM Na orthovanadate, and 1% Triton X-100 supplemented with phosphatase and protease inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Frozen mice liver was powder and homogenized on lyses buffer. Rat and mice samples were sonicated two times for 10 sec and placed in cold room to rotate for 2 hours and vortex every 15 min. Consequently, samples were centrifuged at 15 000 rpm for 15 min. Supernatant was collected, bicinchoninic acid BCA (Pierce Biotechnology, Rockford, IL) was performed and samples were stored at -80°C. On the day of Western Blotting experiments, samples were diluted to obtain the desired final protein concentration.

Transfection Procedures

Once myoblasts have reached 80% confluence, they were split into four T-75 flasks and allowed once again to reach 80% confluence. Cells were carefully detached from T-75 flasks as described under "Cell Culture Procedures." Cell counting determinations were performed using a Vi Cell ^{TM} Cell Viability Analyzer (Beckman Coulter: Hialeah, FL). During this time frame, myoblast cells were centrifuged at 90 g for 10 min. Cells (1.3×10^6), gently resuspended in 100 µl room temperature Nucleofector solution (Lonza, Walkersville, MD) combined with 2 µg scramble shRNA/ACSL-1 shRNA plasmids (Santa Cruz Biotechnology: Santa Cruz, CA) and transfected by Amaxa's Nucleofector Technology (Lonza: Walkersville, MD). We chose shRNA methods over siRNA transfection because sRNA has greater efficiency to underexpress the desired gene and long lasting effect whereas siRNA transfection is transient to maximal 72 h (Dykxhoorn, Novina, & Sharp, 2003). After transfection, myoblasts cells were transferred to pre-equilibrated growth media and cell plated into 24 well plates and 6 well plates at 37°C in a humidified 5% CO_2 and 95% O_2 incubator for ~48 hours to reach 80% confluence and growth media was then switched to differentiation media (Appendix I). Figure 3, illustrates a timetable for the experiments from cell growth to day 7 experimentation for palmitate oxidation and cell lysate collection for Western Blotting analysis.

Figure 3: Experiment Timetable



Myotube Cells on Differentiation Media

Once myoblast cells reached 80% confluency, cells were placed in differentiation media to reach myotube stage for 7 days. On Day 5 of differentiation, primary human myotubes from 24 and 6 well plates were incubated for 48 h in lipid media containing 100 µM palmitate: oleate, 0.5% BSA, 1.0 mM carnitine, 0.1 mM CoA and differentiation media. On day 7 of differentiation, 6 well plate cells were harvested (Refer to Cell Lyses Procedures), BCA (Pierce Biotechonology, Rockford, IL) analysis was done on cells and samples were aliquoted and frozen at -80°C for further protein analysis. Cells differentiated on 24 well plates were incubated in radioactive media for fatty acid oxidation experiment (Refer to Measurement of Fatty Acid Oxidation Procedures). Protein concentrations and the rate of fatty acid oxidation were determined by using BCA assay kit (Pierce Biotechonology, Rockford, IL); data was expressed as pmol/µg protein/hr.

Cell Lyses Procedures

On day 7 of differentiation, lipid media (palmitate:oleate) was removed from 6 well plate cells, and cells were gently washed twice with 2 mL PBS (phosphate buffer saline). Myotubes were harvested in lyses buffer. Cells were sonicated for 3 sec. Samples were placed on ice and vortex every 10 min for 2 h. Afterwards, cells were centrifuged for 15 min at 14,000 rpm. Supernatant was collected and protein content was determined using BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Aliquots of total proteins (15 µg) were stored at -80°C for further analysis.

Western Immunoblot Analysis

On the day of Western blotting, frozen samples were slow thawed on ice water and prepared in loading buffer (4x Laemmli and DL-Dithiothreitol). Fifteen µg of protein from myotube cell lysates samples were denatured at 95°C for 5 min and then equally loaded into 10 % Tris HCl polyacrylamide gels, and electrophoresed at 100V. The gel was then transferred at 100V for 2 h onto a nitrocellulose membrane for binding proteins. Afterwards, the membrane was blocked in 5% nonfat milk in TBS with 0.1% Tween-20 for 2 h. After blocking, the membrane was cut at 50 kDa lane and the top part was incubated at 4°C in blocking buffer overnight in cold room with the appropriate primary antibody rabbit polyclonal ACSL-1 (1:1000, Cell Signaling: Beverly, MA). The bottom part of the blot cut was incubated separately overnight with primary antibody monoclonal mouse GAPDH to verify equal loading among lanes (1:10000 Advanced Immunochemical: Long Beach, CA). Next day, the membranes were retrieved and washed four times in TBS with 0.1% Tween-20 for 5 min. The membranes were incubated at room temperature for 1 h in 5% nonfat dry milk blocking buffer with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signaling: Beverly, MA and Millipore: Billerica, MA). Following 4 washes in TBS with 0.1% Tween-20, an enhanced chemiluminescence (ECL) detection system (Amersham: Piscataway, NJ) was used for visualization. Densitometry (as area times grayscale relative to background) was performed using a Kodak film cartridge and film, a scanner interfaced with a microcomputer, and the NIH Image Analysis 1.62 software program. All bands were normalized to mouse liver control band density. Equal loading of sample protein was confirmed by GAPDH band densities for each treatment sample and controls.

Measurement of Fatty Acid Oxidation Procedures

Following incubation of differentiating cells from days 5-7 with lipid media (described above), cells plated on 24 well plate were incubated with radioactive media utilizing a radiolabeled free fatty acid as a tracer, [1-^{14}C] palmitate (Perkin Elmer: Boston, MA), on day 7 to measure fatty acid oxidation in primary human skeletal myotubes cells according to the methods of J.Y. Kim et al. (2000). In brief, differentiated HSKM cells with scrambled or shRNA ACSL-1 plasmids were incubated at 37°C in a humidified 5% CO_2 and 95% O_2 incubator for 3 hours in differentiation media containing 100 µM palmitate, 12.5 mM HEPES, 0.25% BSA, 1.0 mM carnitine, 0.1 mM CoA and 1µCi/ml [1-^{14}C] palmitate (Perkin Elmer: Boston, MA). Following the incubation period, the medium was transferred to 48 well plate to measure radioactivity of CO_2 (complete palmitate oxidation) and acid soluble metabolites, ASM, (incomplete palmitate oxidation) fractions by liquid scintillation counting using 4 ml of Uniscint BD (National Diagnostics, Atlanta, GA). The remaining cell pellets were washed twice with ice-cold phosphate-buffered saline, harvested in 200 µl 0.05% SDS, and cell lysates were stored at -80°C for subsequent protein determination and fractional and total lipid content (Refer to Total Lipid and Lipid Fraction Determination Procedures).

Total Lipid and Lipid Fraction Determination Procedures

Total Lipid Analysis

Cells followed [1-^{14}C] palmitate incubation were retrieved from -80°C for BCA analysis. On the day of experimentation, samples following [1-^{14}C] palmitate incubation were slowly thawed on ice water and transferred 150 µL from each sample into glass tubes. Then 1:2 chloroform ($CHCl_3$) : Methanol (CH_3OH) was added and vortex. Next, 625 µL of $CHCl_3$ was added to each sample and vortex, and 625 µL ddH_2O was added to each sample and vortex one more time. Samples were then centrifuged at 1,000 rpm for 5 min at room temperature to separate the phases. The organic layer was obtained with a glass Pasteur pipette and transferred to a glass scintillation vial. Samples were dried overnight with the caps off. The next day, each sample was resuspended in 500 µL of 2:1 ($CHCl_3$: CH_3OH) and vortex. Then, 50 µL were taken out from each sample and placed in scintillation vials with 4 mL of scintillation fluid to determine total lipid synthesis. The remaining 450 µL were left on glass scintillation vials with caps off to dry overnight.

Assessment of Mono-, Di-, and Tri- acyl lipid Species by Thin Layer Chromatography Procedures

On the day after assessment of total lipids, samples were resuspended in 50 µL of 2:1 ($CHCl_3$: CH_3OH) and enriched with 20 µL of each standard monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG). Each sample was spotted in respective lanes on an oven-dried TLC plate (Analtech Inc.: Newark, DE) which was then placed in a sealed tank containing solvent (60:40:3, heptane-isopropyl ether-acetic acid). Samples were allowed to migrate on the thin layer chromatography (TLC) plate until ¼ inch from the top; afterwards, the TLC plate was removed from the tank and allowed to dry overnight. The next day, dried TLC plates were

exposed to iodine crystals until the bands depicting MAG, DAG and TAG were visualized. Finally, each individual band was scraped and place in separate scintillation vials with scintillation fluid and counted. Data was expressed as pmol/µg protein/hr.

Statistical Approach

Students paired *t-test* analysis was used to evaluate statistical differences between the means (scrambled vs. ACSL-1 shRNA transfected myotubes) on rates of fatty acid oxidation, ACSL-1 protein content, total and fractional lipid content. Data was presented as mean ± standard error of the mean (SEM), with significance set *a priori* at $p<0.05$. Statistical analysis was performed using Graph Pad Prism 5 (San Diego, CA).

CHAPTER IV

RESULTS

Overview

The major findings from this hypothesis driven research conducted in primary skeletal muscle cells obtained from lean Caucasian females were as follow: 1) Control experiments demonstrated a transfection efficiency of 50 % at 24 h after transfection in myoblast by Amaxa's Nucleofector technology using a GFP plasmid probe compared to other studies that demonstrated exceeding 50% transfection efficiency of proliferated myoblasts in rodent models (Neuhuber, Huang, Daniels, & Torgan, 2002) 2) Western Blot analysis demonstrated antibody specificity for ACSL-1 protein in human skeletal muscle myotubes as confirmed by a) the presence of distinct Western Blot bands present at the correct molecular weight b) and the presence of distinct bands for mouse liver at the expected molecular weight highly expressed in this tissue (Distler et al., 2007), human HepG2 lysates (positive control graciously provided by Cell Signaling: Beverly, MA) and human skeletal muscle homogenates, rodent lysates from lung tissue (which served as confirmatory negative control; protein expression was low in these tissues) 3) successful underexpression/knockdown of ACSL-1 protein was verified by Western Blotting. ACSL-1 KD was significantly reduced 31.6 % in shRNA transfected myotube cells (P= 0.03) (Figure 5) vs. control lysates (myoblasts transfected with scrambled RNA 4) Rates of palmitate oxidation in KD ACSL-1 myotube cells after 48 h palmitate:oleate incubation remained unchanged (Figure 6). 5) Finally, lipid analysis was performed on cells after [1^{-14}C] palmitate incubation. Total lipid synthesis and lipid specific measurement of MAG, DAG, and TAG remained unchanged as well (Figures 7 and 8).

Participants

Participant's demographic data: age (y), height (m), weight (kg), and BMI (kg/m^2) from six lean CW are presented in Table 1. Participants were between 37 and 45 years old and they were lean (BMI 23.2 ± 0.93). Five of the six participants completed the personal history questionnaire and confirmed that participants were premenopausal, sedentary and were not taking medications known to affect lipid metabolism (Appendix C). All participants filled out a menstrual cycle questionnaire (Appendix E) indicating the use and type of oral contraceptives during the study (Kane et al., 2010). Additionally, a 3-d dietary record was completed by five participants during baseline and their diets were evaluated for total calories (kJ/Kg), carbohydrate (g/Kg), fat (g/Kg), and protein (g/kg) to evaluate participants' dietary intake of macronutrients prior to commencement of the study as illustrated in Table 2. According to the American Diabetes Association (ADA) guidelines, the Recommended Dietary Allowances (RDAs) for carbohydrate is 130 $g \cdot day^{-1}$, for protein is 0.8 $g \cdot kg\ body\ wt^{-1} \cdot day^{-1}$ and total fat 65 $g \cdot day^{-1}$ based on a 2000 calorie diet. In comparison to the U.S. Recommended Dietary Intake (RDI) guidelines for total energy intake of macronutrients (45-65% carbohydrate, 20-35% as fats, and 15-20% protein/46 $g \cdot day^{-1}$) participants were within the recommended values of % carbohydrate (54.3 ± 5.2), % fat (30.0 ± 4.7), and % protein (15.0 ± 0.8) as highlighted in Table 2.(Bantle et al., 2008). In addition, assessment of fasting blood glucose (mg/dL), fasting insulin (μU/mL), and insulin resistance by homeostatic model assessment of insulin resistance (HOMA-IR) was performed in five of the six participants before the study, as shown in Table 3. Fasting blood glucose (FBG) levels were determined according to the criteria of the ADA guidelines and insulin sensitivity levels were defined by Stern et al. (2005). According to the ADA guidelines, a normal FBG is between 70 and 100 mg/dL, and a FBG > 126 mg/dL is the standard diagnose for diabetes. According to Stern et al. (2005), the levels for the diagnosis of diabetes by HOMA-IR < 3.60 is considered the criterion

for insulin sensitivity and HOMA-IR > 3.60 is considered the criterion for insulin resistance. HOMA was calculated from values of fasting blood glucose and insulin by using the following formula: fasting glucose (mg/dL) X fasting insulin (µU/mL) / 405. According to HOMA values, participants were not considered insulin resistance (1.08 ± 0.2). Note: data on the questionnaires and blood analysis is missing for subject six as she needed to be recruited after the original subject's cell sample was eliminated due to low viability of an original sample.

Participants' Characteristics

Lean Caucasian Women	
Number of Participants	**(n= 6)**
	Mean ± SEM
Age	36.8 ± 2.5
Height (m)	1.64 ± 0.02
Weight (Kg)	63.2 ± 2.36
BMI (kg/m^2)	23.2 ± 0.93

Table 1. Data are expressed as mean ± SEM for six lean Caucasian females; age, height, weight, and BMI (Body Mass Index**)**

Total Calories, Carbohydrate, Fat, and Protein

Lean Caucasian Women	
Number of Participants	**(n= 5)**
	Mean ± SEM
Total calories (kJ/Kg)	135.3 ± 14.6
Carbohydrate (g/Kg)	1.9 ± 0.2
Fat (g/Kg)	1.14 ± 0.2
Protein (g/Kg)	1.25 ± 0.2
Carbohydrate (%)	54.3 ± 5.2
Fat (%)	30.0 ± 4.7
Protein (%)	15.0 ± 0.8

Table 2. Date are expressed as mean ± SEM for five lean Caucasian females. Total calories, carbohydrate, fat, and protein were evaluated prior to enter the study using a 3 day food record (two non-consecutive days and one weekend day).

Fasting Glucose, Insulin, and Insulin Resistance (HOMA)

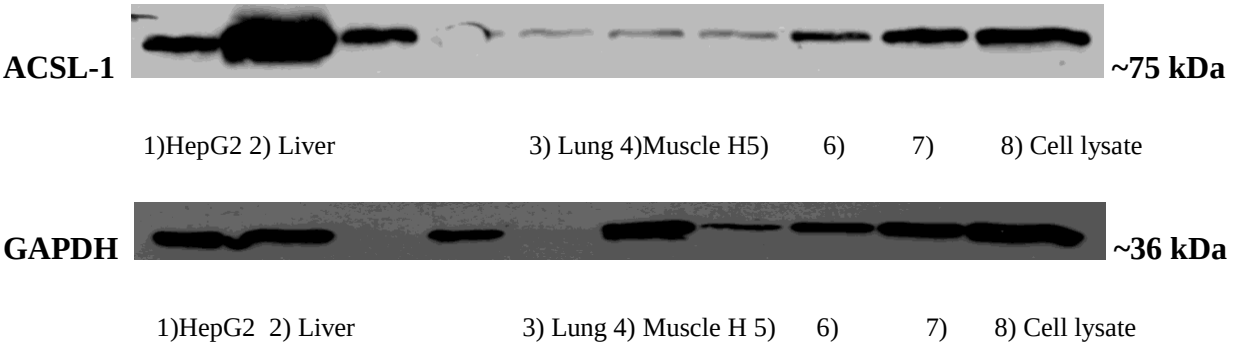
	Lean Caucasian Women
Number of Participants	**(n= 5)**
	Mean ± SEM
Glucose (mg/dL)	86.3 ± 3.0
Insulin (µU/mL)	4.9 ± 1.1
HOMA	1.08 ± 0.2

Table 3. Data are expressed as mean ± SEM. Data are expressed as glucose (mg/dL), insulin (µU/mL)**,** and insulin sensitivity (HOMA) values in five lean Caucasian females prior to muscle biopsy. Fasting glucose, insulin, and HOMA values are within range.

Control Experiments

In order to determine transfection efficiency using Amaxa Nucleofector technology, primary human skeletal myoblasts were transfected by GFP plasmid DNA. Cell viability was approximately 40% and approximately 50% transfection efficiency at 24 h post transfection (Appendix F). Seven days after differentiation GFP showed fully differentiated cells as being fully elongated and multinucleated morphology of myotubes. This data indicated successful transfection by GFP plasmid DNA in differentiated primary HSKM cells (data courtesy of Dr. H.B. Kwak, personal communication, Appendix F).

In order to determine that the antibody expression was specific to ACSL-1 isoform, the protein of interest was blotted for several tissues (Figure 4). Rat and liver protein levels were chosen for Western Blotting to serve as positive controls. ACSL-1 mRNA abundance in mouse models have shown to be highly expressed in liver, adipose, heart tissues; at moderate levels in gastrocnemius and soleus muscles, and at very low levels in lung, kidney, and adrenal organs (Mashek et al., 2006). In addition a dose response of human cell lysates was used to determine the optimal protein (µg) to load into the gels. The protein specific bands for ACSL-1 were observed at ~75kDa; this is in agreement with the study by Li et al. (2009).



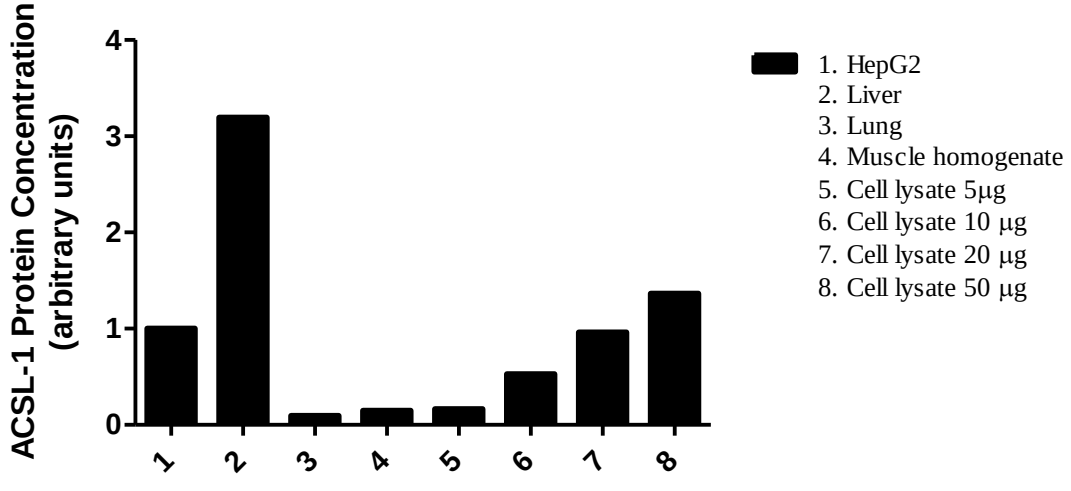
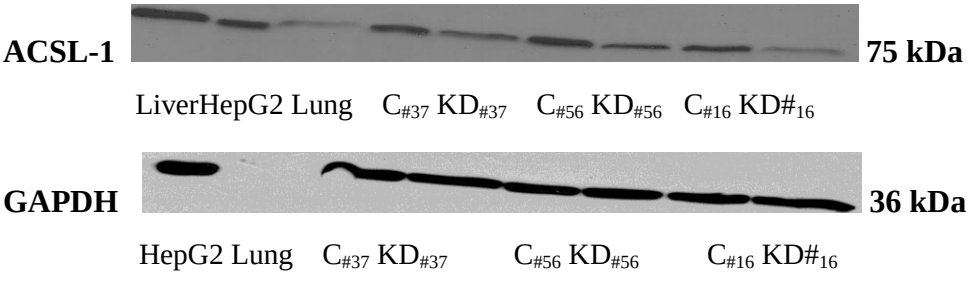


Figure 4. Dose Response. A dose response of human myotube lysate was performed to determine adequate amount of protein specific and visible to ACSL-1 antibody. In addition, antibody specificity was confirmed by using positive controls (HepG2 cells, mice liver tissue and skeletal muscle homogenates) and negative control (lung tissues). Bands were visible at ~75 kDa. The amount of protein used was as followed HepG2 (20µg), $Liver_M$ (20µg), $Lung_R$ (20µg), Muscle homogenates (20µg), Cell Lysate (5µg, 10µg, 20µg, and 50µg). Each band was normalized to HepG2 band.

ACSL-1 KD Efficacy Determined by Western Immunoblot Analysis

The generation of ACSL-1 KD was done by introducing ACSL-1 shRNA plasmid into myoblast cells. Control cells were transfected with a scramble shRNA sequence that did not lead to degradation of ACSL-1 gene. The lysates were prepared for Western Blotting following transfection procedures using AMAXA's Nucleofector Technology (Lonza: Walkersville, MD) as described under "Transfection Procedures." Protein bands were detected at the correct molecular weight at ~75 kDa band for mouse liver and lung samples as well as for control HepG2 lysates. Most importantly, Western Blot analysis confirmed successful reduction of ACSL-1 protein expression following transfection procedures. ACSL-1 protein levels verified by Western Blot analysis were reduced by 31.6 % (P= 0.03) Figure 5 following transfection with shRNA plasmid transfection. Furthermore, Western Blot probing for GAPDH confirmed equal loading of protein for treatment and all control samples (Appendix G).

A)



B)

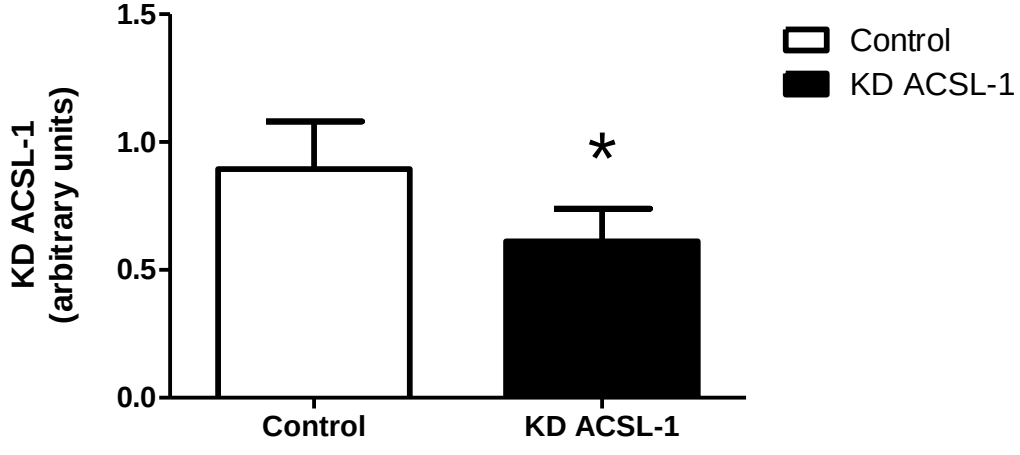


Figure 5. Transfection efficiency of ACSL-1 KD shRNA. Cell lyses obtained from participants who were 6 lean Caucasian women. A) Western blot bands were $Liver_M$ (1 µg), $Lung_R$ (15 µg), and 3 samples of ACSL-1 KD (15 µg). Bands were normalized to $Liver_M$ (1µg). GAPDH shows equal loading of samples. B) Open bars represent control cells transfected with an empty vector shRNA, closed bars represent ACSL-1 transfected cells with ACSL-1 shRNA. ACSL-1 gene was KD by 31.6 % (P=0.03). Data are presented as mean ± SEM (n=6).

In vitro Rates of Fatty Acid Oxidation

The analysis of *in vitro* fatty acid oxidation following ACSL-1 transfected KD and control samples were measured as collected radioactivity of $^{14}CO_2$ (complete fatty acid oxidation), ASM (incomplete fatty acid oxidation) and total fatty acid oxidation ($^{14}CO_2$ and ASM levels) by liquid scintillation counting. According to CO_2 [1^{-14} C] palmitic radioactivity levels there was no change in fatty acid oxidation in the KD ACSL-1 transfected myotube cells (P= 0.10), ASM (P= 0.10) or total fatty acid oxidation (P=0.09) vs. control lysates. The rates of fatty acid oxidation were expressed as pmol/µg protein/ hr (Figure 6).

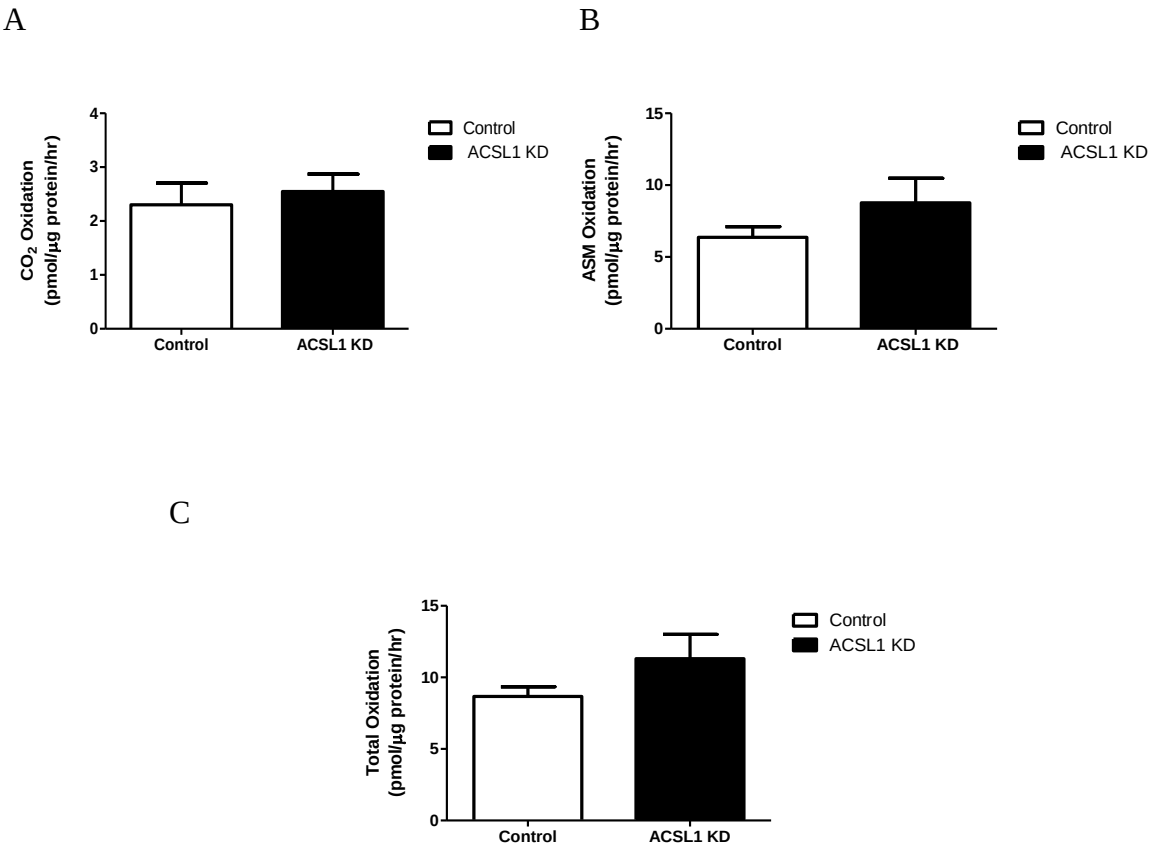


Figure 6. Effect of ACSL-1 KD *in vitro* fatty acid oxidation after 48h lipid incubation

Participants were six lean Caucasian females who were biopsied from the vastus lateralis muscle following an overnight fast. Satellite cells were cultured and differentiated into myotubes and then transfected with scrambled RNA or shRNA plasmid vectors. Oxidation studies were performed according to methods described above. Fatty acid oxidation was measured by ^{14}C-leveled radioactivity of CO_2 (**A**) ASM (**B**) and total lipid oxidation **(C)** by liquid scintillation counting (open bar, control cells; closed bar, KD ACSL cells). Data are presented as mean ± SEM (n=6).

Lipid Synthesis and lipid Accumulation in ACSL-1 KD Myotubes

ACSL isoforms deliver Acyl-CoA substrates for lipid oxidation, synthesis and intermediates that act on cell signaling (Nagle et al., 2009). Several studies have suggested that intermediates such as ceramides, DAG, and Acyl-CoAs affect insulin sensitivity in skeletal muscle (Lowell & Shulman, 2005). In order to determine the rates of fatty acid incorporation into several lipid species the extraction of cellular lipids was performed as described under "Total lipid and TLC fraction determination procedures." We examined that ACSL-1 KD cells after 48 h incubation of 1:1 palmitate: oleate (100μM). Results demonstrated an absence of change for the accumulation of total lipids in KD vs. control samples (P= 0.21) (Figure 7). Further analysis for MAG, DAG, and TAG also demonstrated an absence of significant differences in the means between control and ACSL-1 shRNA transfected cells (P= 0.14, P=0.19, and P=0.38 respectively) (Figure 8). Measurements for total lipid synthesis, MAG, DAG, and TAG were expressed as pmol/μg protein/hr.

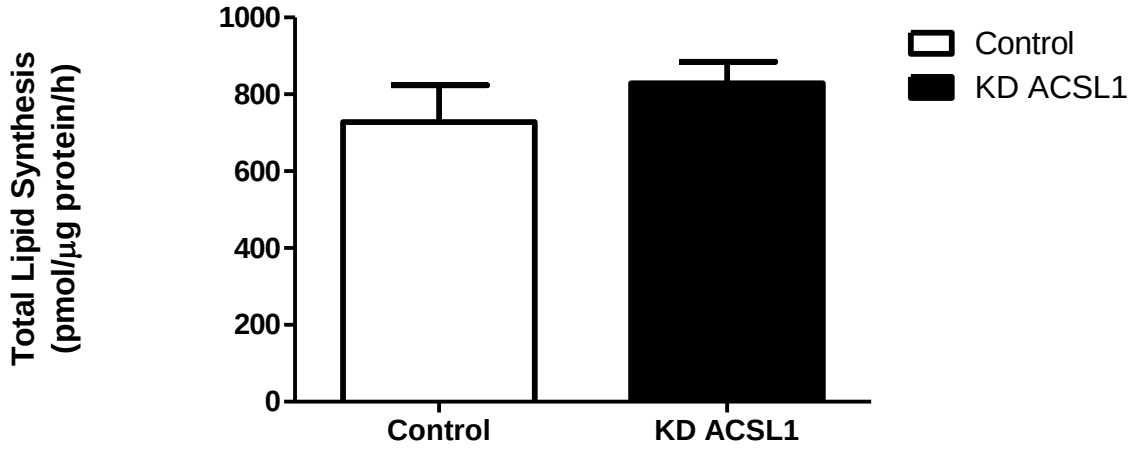


Figure 7. Total Lipid Synthesis. Extraction of cellular total lipids performed in cell lysates incubated with 1:1 palmitate: oleate and after [1^{-14}C] palmitic incorporation. Data are represented as mean ± SEM (n=6)

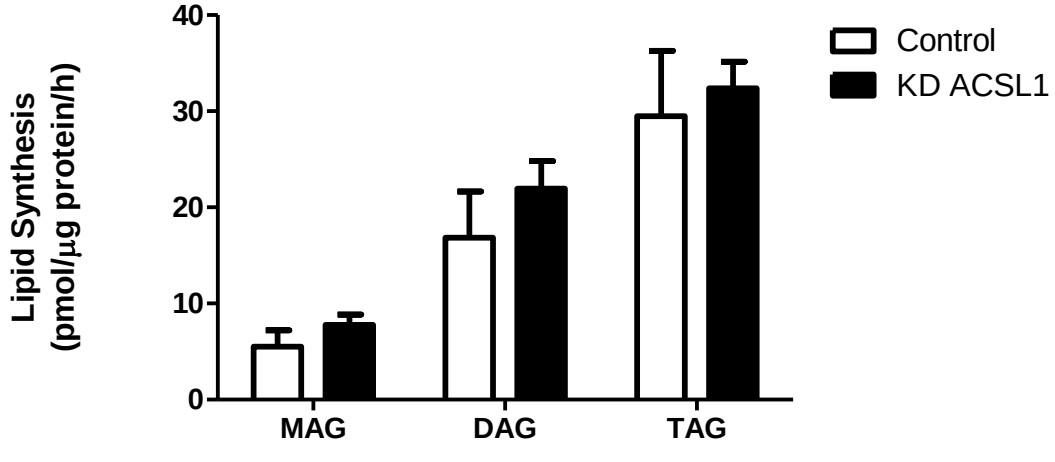


Figure 8. Lipid synthesis (MAG, DAG, and TAG). Lipid synthesis of monoacylglycerols, diacylglycerols, and triacylglycerols performed in cell lysates incubated with 1:1 palmitate: oleate and after [1^{-14}C] palmitic incorporation. Data are represented as mean ± SEM (n=6)

CHAPTER V

DISCUSSION

Transfection efficiency of our ACSL-1 shRNA plasmid vector into human primary skeletal muscle cells obtained from human skeletal muscle biopsies was verified by Western Blot analysis. Transfection efficiency from AMAXA device was measured by GFP technology and demonstrated a ~50% efficiency in our ability to successfully utilize a relatively new technology for this process (Appendix F). As expected, we provided clear evidence for the ability of the chosen antibody to detect protein at the correct molecular weight and with very high expression in our positive controls, rodent liver, HepG2 lysates, and human skeletal muscle homogenates, as well as the expected outcome of low expression in lung tissue as predicted by the literature (Mashek et al., 2006). Results from the laboratory have demonstrated that overexpression of ACSL-5 isoform in our primary HSKM cell model does not result in elevation of complete and incomplete fatty acid oxidation nor does underexpression/KD of ACSL-1, resulting in alterations of lipids. To our knowledge this is the first report to demonstrate the presence of ACSL-1 in primary human skeletal muscle myotubes, the ability to reduce its expression in our model, and to report the effect on rates of fatty acid oxidation and lipid synthesis.

The participants involved in the study were lean Caucasian women (n=6; BMI < 25 kg/m^2) with a mean age of 36 yr. All participants were premenopausal, considered healthy and sedentary after filling out a personal history form questionnaire (Appendix C). According to serum levels of fasting glucose, fasting insulin, and HOMA-IR participants reported no presence of insulin resistance (0.9 ± 0.7). Our premenopausal participants also fill out a menstrual cycle recall questionnaire (Appendix E) to determine type of birth control used, length of cycle and the effects of menstrual status and female sex steroids on mitochondrial bioenergetics. Results were

reported in a recently published manuscript (Kane et al., 2010). Overall, subjects met our inclusion criteria established *a priori*.

In addition, a food diary form (based on Kg/body mass; Appendix D) was completed for five of the participants prior to the beginning of the study and before their muscle biopsy. Participants selected 2 weekdays and 1 weekend day for their food diary form. This was done to determine the major fuel oxidized in the participants. In comparison to the U.S. Recommended Dietary Intake (RDI) guidelines for total energy intake of macronutrients (45-65% carbohydrate, 20-35% as fats, and 15-20% protein/46 $g \cdot day^{-1}$) participants were within the recommended values of % carbohydrate (54.3 ± 5.2), % fat (30.0 ± 4.7), and % protein (15.0 ± 0.8) Table 2.

The ability to transfer exogenous shRNA into cultured primary human skeletal muscle myoblast cells to knockdown ACSL-1 gene was achieved by electroporation. This technique enabled us to study the regulation of fatty acid oxidation and lipid synthesis in our model. The first step was to determine transfection efficiency of AMAXA device. To determine transfection efficiency by Amaxa's Nucleofector device, primary human skeletal myoblasts were transfected by GFP plasmid DNA. Based on methodology developed prior to this study, cell viability was approximately 40% and there was approximately 50% transfection efficiency at 24 h after transfection of myoblasts compared to exceeding 50% efficiency transfection of myoblasts cultured from rodent models (Neuhuber et al., 2002). After, 7 days of differentiation, GFP showed a strong fluorescence signal with fully differentiated cells being elongated and multinucleated. These data indicated successful transfection by GFP plasmid DNA in differentiated primary HSKM cells and supports the use of this technology for introduction of our selected plasmid in our cell culture model (data courtesy of Dr. H.B. Kwak, personal communication, Appendix F).

Additional control experiments were conducted using Western Blotting procedures to determine that the antibody was specific to our target protein, ACSL-1, in order for us to monitor the extent of our underexpression efforts (Figure 4). Earlier literature reported that ACSL-1 has been found to express strongly in liver and lower in lung tissues (Mashek et al., 2006). Therefore, we chose to do a series of Western Blotting experiments that detected ACSL-1 in these tissues. According to our molecular weight ladder, ACSL-1 antibody was detected at ~75 kDa and highly in positive controls of mouse liver, human skeletal muscle homogenates and HepG2 and at lower levels in negative control (mouse lung extracts). Furthermore, we also blotted for human skeletal muscle cells at different protein levels (5 µg, 10 µg, 20 µg, and 50 µg). The bands showed a dose response of increasing protein content. After these preliminary experiments, we felt confident that our chosen antibody was specific for our target protein (Figure 4)

After successful confirmation of antibody specificity, we progressed toward the transfection studies. We chose shRNA over small interfering RNA (siRNA) because transfection of siRNAs leads to transient gene silencing and might not result in sustained interference for genes that encode proteins with long half-lives or for experiments that would be ongoing for greater than 36 h (Dykxhoorn et al., 2003). In the first step of the underexpression process using double-stranded RNA (dsRNA) is cleaved by an RNAase III family member, Dicer, into 21-23 nucleotide siRNAs in an ATP dependent reaction. These siRNAs are incorporated into the RNA-inducing silencing complex (RISC), and then the activated RISC targets the complementary mRNA for degradation (Dykxhoorn et al., 2003). The amount of shRNA used in this model to perform effective gene knockdown was 2 ug of shRNA ACSL-1 and equal amounts of scrambled RNA as control. Using the procedures established early in this specific project, the ACSL-1 gene was successfully underexpressed/KD as evidenced by a 31.6% reduction in normalized protein

levels (P= 0.03) and verified by Western Blotting from lysates of differentiated primary HSKM cells vs. controls derived from the same subject.

In order to determine the rates of fatty acid oxidation in our model, we recapitulated the human, obese AAW phenotype by underexpression ACSL-1 in primary HSKM cells from lean CW. We incubated the myotubes with palmitate: oleate (100 µM) for 48 h on day 5 of differentiation. We hypothesized that ACSL-1 KD would reduce fatty acid oxidation levels based on earlier studies from our laboratory which overexpressed ACSL-1 in primary HSKM (Appendix H). In these earlier studies, palmitate complete CO_2 oxidation was approximately 75% higher in ACSL-1 overexpression compared with control. Similarly, palmitate incomplete ASM oxidation was 61.7 % higher in ACSL-1 overexpression compare to control ($P < 0.05$). (data courtesy of Dr. H. B. Kwak, Appendix H). This suggested that the ACSL-1 isoform indeed has a role in mitochondrial fatty acid oxidation in HSKM. Unexpectedly however, following ACSL-1 KD, fatty acid oxidation in our model, results following transfection demonstrated an absence of a reduced effect on complete (P= 0.10) and incomplete fatty acid oxidation (P=0.10).

Given these unexpected findings, we proposed the following alternatives to assist in interpreting our data. First, it can be speculated that another isoform located on the outer mitochondrial membrane could have served as a compensatory mechanisms to offset the negative effects of ACSL-1 KD on mitochondrial fatty acid oxidation. For example, ACSL-5 protein has been detected at 76 kDa in the mitochondrial fraction, 73 and 74.5 kDa levels in endoplasmic reticulum (ER), and 74.5 kDa in cytosol and mitochondria-associated membrane (MAM) (Lewin et al., 2001). In support, we have conducted earlier experiments in our laboratory examining the overexpression of ACSL-5 on fatty acid oxidation in human primary myotubes. According to captured [1-^{14}C] label CO_2 measurements, complete palmitate oxidation was higher (+112%; $P<0.05$) compared to control cells. Measured [1-^{14}C] labeled ASM radioactivity from

ACSL-5 overexpressing cells also resulted in a significant increase (+71%; $P<0.05$) following a 3 h incubation protocol with 100 μM palmitate when compared with control cells. This study demonstrated that ACSL-5 overexpression can lead to an increase in complete and incomplete fatty acid oxidation in HSKM (data courtesy of Dr. H.B. Kwak, Appendix H). The increase of ACSL-5 role in fatty acid oxidation suggested to us that this particular isoform is a likely candidate to respond when ACSL-1 is underexpressed or demonstrates reduced activity. Future studies are required to investigate the protein expression of ACSL-5 in myotubes that underexpresses ACSL-5. These experiments would provide for intriguing insights into the regulation of mitochondrial fatty acid oxidation by the ACSL family and their effects of skeletal muscle lipid dynamics not only in the normal/health condition but also under circumstances of pathology such as evidenced with the obese and diabetic state. Co-expression studies might also provide a fruitful avenue to pursue with regard to gene therapy approaches toward resolving decrements in lipid oxidation in skeletal muscle from obese and/or type 2 diabetic patients.

Given the above unexpected findings with our fatty acid oxidation experiments following knockdown of ACSL-1, the next logical approach was to assess potential alterations in the lipid synthesizing arm of fatty acid partitioning in our cell model. Despite the potential for compensation of ACSL-1 KD by ACSL-5 on fat oxidation, it still may be that either or both isoforms could potentiate the partitioning of activated acyl-CoA units toward lipid synthesis. Results however did not support this possibility. Our model system and approach reported no alterations of total lipid synthesis. Similarly, despite the high content of exogenous lipids supplied to the ACSL-1 KD transfected cells, esterification of MAG, DAG, and TAG were unchanged. This suggested that ACSL-1 and/or increases in compensatory expression of additional isoforms may not be a major regulator(s) for activation of fatty acids towards lipid synthesis in the skeletal muscle from healthy, lean individuals. Alternatively, although ACSL-1

and/or ACSL-5 appears to be involved in mitochondrial fatty acid oxidation, it may be that that mitochondrial regulation of fatty acid oxidation is at the level of CPT-I as supported by the majority of the literature (J.Y. Kim et al., 2000; Wolfgang et al., 2008). Perhaps, as observed in skeletal muscle of obese AAW, ACSL may only be limiting for fatty acid oxidation in the pathological condition and perhaps only in certain racial subpopulations.

To end our discussion, It was reasonable to speculate on an additional pathway which could serve as a compensatory mechanism during conditions of skeletal muscle ACSL-1 reductions in activity; namely peroxisome handling of fatty acid oxidation. Studied most extensively in the liver using rodent models, it is conclusive that peroxisomes play an intimate and essential role in cellular lipid dynamics. For example, the peroxisome is the only organelle capable of oxidizing very long-chain fatty acids (>22 C). In contrast to the mitochondria however, the β-oxidative system of the organelle is incomplete. Accordingly, peroxisomal oxidative products are chain shortened down to C6 units but not lower (Reddy & Hashimoto, 2001). Interestingly, evidence exists that fatty acids oxidized by the peroxisomal pathway are exported as acyl-carnitine units. This may have significant implications for mitochondrial fatty acid oxidation as these lipid products can enter into the mitochondria independent of ACSL and/or CPT-I. Once arriving in the matrix, exposure to CPT-II would reestablish these lipid peroxisomal export products as acyl-CoA substrates for oxidation. In this regard, the first evidence for the existence of a peroxisomal-mitochondrial interactive pathway to handle long-chain fatty acid metabolism in skeletal muscle was established by our laboratory. Noland et al. (2007) demonstrated that the peroxisome organelle can contribute to complete lipid disposal in skeletal muscle by interacting with the mitochondria. However, an interesting caveat emerged from this study. When skeletal muscle from lean rats was assessed for peroxisomal oxidation of long-chain or very-long chain fatty acids, no evidence for their activity was realized. In stark contrast, when the obese counterpart

the lean Zucker (the insulin resistant fatty fa/fa $^{-/-}$) animal was studied, a great abundance of activity was noted. In fact, when the very long-chain fatty acid, lignoceric acid, was radiolabeled with [1-^{14}C] oxidation to [1-^{14}] CO_2 was observed only in the skeletal muscle of the fatty (fa/fa $^{+/+}$) animal. Furthermore, [1-^{14}C] palmitate oxidation was also observed despite incubating with malonyl-CoA, the natural inhibitor of CPT-I. Thus, under conditions of elevated lipid supply, as occurs in the obese insulin resistant condition, or in our model following 48 h of lipid incubation, results demonstrated that the peroxisomal β-oxidation of long-chain fatty acids in the presence and absence of malonyl-CoA can enter mitochondria independently of CPT-I. Given these recent findings, we proposed the following testable hypothesis. In the presence of reduced ACSL activity (ACSL-1 or otherwise), the overflow of fatty acids following reduced uptake by the mitochondria (due to the reduction of acyl-CoA substrate from ACSL), the peroxisome would accept long-chain acyl fatty acids and chain shorten them for ACSL/CPT-I independent oxidation by the mitochondria. As such, in the present experiments, it may be that with reduced activity of ACSL-1, a compensatory mechanism emerged in that peroxisomal activity was elevated (perhaps with or without increased ACSL-5 compensation) and thereby neutralized the negative effects of ACSL-1 underexpression. This could at least explain the absence of an effect of ACSL-1 KD on fatty acid oxidation of palmitate. True, this may not necessarily explain why we failed to visualize differences in lipid synthesis. However, it may be that other ACSL isoforms more specific for synthetic pathways were upregulated. Clearly, many additional studies are needed to verify either or both of our alternative explanations for our findings. Nonetheless, they do represent intriguing possibilities that will likely render fruitful toward our understanding of lipid dynamics in skeletal muscle under the healthy and pathological condition.

In the context of the above findings, it is important to discuss potential limitations of our study. First, common to all cell culture transfection studies, there is the potential for unequal

transfection among all of the myotubes in our model. We admit this possibility because we did not investigate the actual transmission of ACSL-1. We assumed that the efficiency would be similar to that observed for GFP. Future studies should be conducted to directly measured transfection efficiency of our target protein using the AMAXA electroporation technology. In the worst case scenario, we may have had only underexpressed our protein in a portion of the myoblast that were studied in our assay. To address this issue, additional studies would be necessary using a greater content of shRNA plasmid during our transfection procedures. Thus, we suggest assays to assess a run a dose respond series of experiments using increasing amounts of shRNA to observe whether the 2 ug employed in the current research design was the optimal amount to gain the maximum underexpression obtainable. In this regard however, the trade off of greater underexpression of the target protein may not mimic the true physiological condition as it is highly unlikely that full loss of ACSL-1 activity exists in human skeletal muscle, even in the pathological condition.

A second limitation in our study was that our cells in ACSL-1 underexpression were not in state III respiration when energy demand is at its highest (e.g., during muscle contractions such as that which occurs with physical exertion/exercise). In our model, cells where studied under state IV condition when adenosine triphosphate (ATP) requirements are low. Therefore, we suggest additional future studies to examine mitochondrial respiration under conditions of elevated, state III respiration. In this regard, we suggested the use of the newer experimental approaches using the permeabilized fiber assay where mitochondrial function can be observed under state III conditions. In this regard, we were able to induce a 30% KD for ACSL-1 in the present study which may have little consequences under low energy demand near state IV respiration. However, if we had be able to elevate energy demand to that required by the cell during contractions, a significant effect of ACSL-1 KD on cellular bioenergetics and rates of

respiration/synthesis may have emerged. Therefore, at present it may be erroneous to conclude that ACSL-1 is not important for fatty acid oxidation in situations of high energy demand. An additional approach could also include a cell model in which contractions could be induced such as would occur under conditions of electrical stimulation. This technology is being work out in our laboratory and may demonstrate to be very fruitful in the future to understand the role of ACSL-1 in lipid metabolism during state III respiration (physical activity).

In conclusion, we are the first to successfully underexpress ACSL-1 in human skeletal muscle. Contrary to our hypothesis however, the results of the current research did not support our prediction that ACSL-1 KD would reduce fatty acid oxidation in primary HSKM cells. However, recent data from our laboratory showed that ACSL-1 overexpression in primary HSKM cells do demonstrate an increased on fatty acid oxidation (data courtesy of Dr. H.B.Kwak, Appendix H). This indicates that ACSL-1 isoform has a role in fatty acid oxidation in skeletal muscle but that role could not be identified in our model and research design of ACSL-1 KD. Several alternatives have been proposed which require further investigation such as an additional isoform induced upregulation and compensation for underexpression of ACSL-1. For studies to test the possibility of this alternative hypothesis, co-expression experiments would be one logical approach. In addition, we propose based on reports from our own laboratory that the peroxisome may act as in an inducible and dynamic fashion in response to changes in lipid dynamics following reduction in ACSL-1 activity. Finally, we also propose future studies using permeabilized fiber and high resolution oximetry to determine the effects of ACSL-1 underexpression under state III condition which may serve to “unmask” the repercussions of lowered skeletal muscle ACSL-1 activity during times of heightened energy demand. Therefore, we close with the following; although much is understood about lipid regulation in skeletal muscle, it still represents a new and exciting frontier for understanding the role of lipid biology

in health and disease, and as such provides hope for devising new approaches toward the treatment of metabolic diseases as observed in the obese and diabetic condition.

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Appendix A

Institutional Review Board Approved letter

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
1L-09 Brody Medical Sciences Building• 600 Moye Boulevard • Greenville, NC 27834
Office **252-744-2914** • Fax **252-744-2284** • **www.ecu.edu/irb**

TO: Ronald Cortright, PhD, Department of EXSS, ECU, 371 Ward Sports Medicine Bldg.

FROM: UMCIRB

DATE: August 11, 2010

RE: Full Committee Approval for Continuing Review of a Research Study

TITLE: Impaired Acyl-CoA Synthetase-Muscle Lipid Oxidation in African-American Women

UMCIRB #07-0135

The above referenced research study was initially reviewed by the convened University and Medical Center Institutional Review Board (UMCIRB) on **4/25/07.** The research study underwent a subsequent continuing review for approval on **8/11/10** by the convened UMCIRB. The UMCIRB deemed this **NIH/NIDDK** sponsored study **more than minimal risk** requiring a continuing review in **12 months**. Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The above referenced research study has been given approval for the period of **8/11/10** to **8/10/11**. The approval includes the following items:

- Continuing Review Form (dated 07/27/2010)
- Protocol (version date 03/19/2007)
- Protocol Summary (version date 02/04/2010)
- Informed consent – Single Day Biopsy Procedure (version date 02/04/2010)
- Informed consent – Effects of 8 Weeks of Exercise (version date 02/04/2010)
- Informed consent – Effects of 10 Days of Exercise (version date 02/04/2010)
- Informed consent - Assessment of Insulin Sensitivity and Nutritional Survey (version date 02/04/2010)
- Study Outlines – Single Biopsy Day/10 Days/8 Weeks (version date 02/04/2010)
- Study Flyer – Research Subjects Needed (version date 03/29/2010)
- Study Flyer – Research Subjects Needed (version date 05/05/2008)
- Personal History Form (version date 02/07/2007)
- Menstrual Cycle Recall (version date 02/07/2007)
- Food Diary and Food Log (version date 02/07/2007)
- Food Frequency Questionnaire (version 08/22/2007)
- Data Safety Monitoring Memo (dated 07/26/2010)

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

NOTE: The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: None

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.

Appendix B

Institutional Review Board Approved Inform Consent

Informed Consent

Title of Project: Impaired Acyl-CoA Synthetase-Muscle Lipid Oxidation in African-American Women (UMCIRB # 07-0135).
Subtitle of Project: Acyl-CoA Synthetase Activity-Muscle Lipid Oxidation in African-American Women under Sedentary Conditions (single day biopsy procedure).

Principal Investigator: Ronald N. Cortright, Ph.D
Co-Investigators: Robert C. Hickner, Ph.D.; Joseph Houmard, Ph.D.; Hisham Barakat, Ph.D.; and James deVente, M.D., PhD.

Institution: Human Performance Laboratory, East Carolina University
Address: 371 Ward Sports Medicine Building, Greenville, NC
Telephone: (252) Office:737-4678, lab:252.744-2934, or home:756-7735

This consent document may contain words that you do not understand. You should ask the study doctor or the study coordinator to explain any words or information in this consent form that you do not understand.

Introduction: You have been asked to participate in a research study being conducted by **Dr. Ronald N. Cortright, Dr. Robert C. Hickner, Dr. Joseph Houmard, Dr. Hisham Barakat, Dr. James deVente** and fellow researchers at East Carolina University.

The **purpose** of this study is to determine why obese people utilize fat to a lesser extent and why African-American women have a greater tendency to gain weight and develop diabetes than do Caucasian women.

Obesity has reached epidemic proportions in the United States and is threatening to become a global epidemic. Obesity represents a serious health threat because of the increased risk of developing chronic diseases such as diabetes and cardiovascular disease. According to recent estimates, the prevalence of obesity is greater among African-American than Caucasian women in the United States. African-American women gain weight at an earlier age and remain heavier than Caucasian women at the same age. This racial difference is important because obesity is strongly associated with skeletal muscle insulin resistance (inability of muscle to take in sugar from the blood) and supports the existing data demonstrating that African-American women have twice the incidence of type 2 diabetes compared with Caucasian women. Although environmental factors such as socioeconomic status, diet, and level of activity may influence the greater prevalence of obesity and diabetes, it is becoming increasingly evident that inherent physiological and biochemical differences underlie the increased incidence of these diseases in African-American women. We have demonstrated that obese African-American women have a reduced ability to release fat from sites of storage (the adipocyte) and to use fat by skeletal muscle to make energy when compared to Caucasian women of similar age and weight. This is fundamentally important because the reduced ability to release and "burn" fat can result in its increased accumulation within the fat and muscle cells, the latter which is strongly linked with insulin resistance in obese individuals. Newer information suggests that African-American women who are not obese may be more likely to gain weight when compared to non-obese Caucasian women. For example, our lab has noticed that non-obese African-American women have a greater difficulty using fat to make energy when they are at rest and during exercise. Despite the negative implications of these findings for health however, the cellular mechanisms to explain this race/ethnic metabolic

Subjects Initials _____

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difference in the propensity toward obesity and diabetes has scarcely been studied in African-American women.

We hope to apply the knowledge gained from this research to better understand why obese women in general, and more so, African-American women, cannot use fat as effectively for energy production as non-obese Caucasian women. These studies could lead to more specific (dietary, activity, or pharmacological) treatments for obesity and diabetes, especially for African-American women.

You should understand that you will be one of approximately 144 women (over 3-4 years) in the research study (ages 18 – 45 years). Subjects for this particular part of the study will be 24 lean (BMI $\leq$ 26 kg/m^2) African-American (AAW; N=12) and Caucasian Women (CW; N=12) and 24 obese (BMI > 30 kg/m^2) AAW (N=12) and CW (N=12).

The total numbers of days for your participation will be 3 days. Your first visit will take approximately 90 minutes during which time we will determine your body composition (in terms of fat and lean weight) by DEXA and your aerobic exercise capacity. On the same day, the researchers will also teach you how to select, measure, and record the foods that you eat for two-three day periods during the study. On another day of your choice, you will be asked to report to the office of The Leo Jenkins Cancer Center for a CT scan to determine your body's sites of trunk fat storage. On a separate and third day, the biopsy procedures and blood draw will occur. The length of time will be approximately 1 hour. The total hourly commitment for your participation in the study is approximately 3.5 hours (day 1 assessments = ~ 1.5 hours; CT scan = ~ 1 hour; 2 biopsies and blood draw = ~ 1 hour).

On the day of the biopsies, you will be asked to report in the morning (after an overnight fast-no food after 10:00 PM the night before) to room 2377 of the East Carolina Heart Institute (ECHI) for the muscle biopsy procedure. The procedure consists of application of a local anesthetic and two muscle biopsies, one from each thigh. From the small biopsy samples (~ 75 milligrams each; the size of a pencil eraser) the investigators will determine your muscle's ability to burn fat. We will also determine which muscle genetic factors are important in determining fat utilization, why fat utilization may be less in obese individuals, and why African-American women may differ from Caucasian women. This information will help us to improve drug and physical activity strategies for individuals prone to obesity and diabetes.

In order to further determine physiological factors that lead to obesity and associated diabetes, we will collect blood from your arm on the day of the biopsy procedure. The total amount collected will be small (about 30-40 milliliters = ~ 2-4 tablespoons).

Details of each procedure are described below.

Plan and Procedures

My participation will involve:

The following are screening procedures and assessment of metabolism, fitness, and body composition:

1. **Preliminary Assessments: First Visit.**

- **Health History and Other Forms** (FITT building). You will be asked to complete a health history questionnaire to help determine if you are suitable for this study (e.g., types of medications used if

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any). In addition, you will be asked to record the history of your recent menstrual cycle and whether you are currently taking birth control pills.

- **Body Composition** (FITT Building): **DEXA.** Your body composition (relative amounts of fat and lean tissue) will be determined by using an FDA-approved bone density instrument (Prodigy Advanced, GE Lunar Corp., Madison, WI). The procedure is called Dual Energy X-ray Absorptiometry (DEXA). A person trained for the use of the DEXA will perform all testing, and you will need to report to the Human Performance Laboratory for the scan. One benefit of this testing is that it provides the most accurate assessment of body composition available. You will be asked to lie face up, on a padded table for 7 minutes while the scanner arm of the DEXA machine passes over your entire body. The scanner will not enclose or touch you, and you can wear regular clothing (no metal allowed). The results of the DEXA Scan will be confirmed by skinfold determination of body composition. Several sites on your body will be measured for skinfold thickness. This is a painless procedure that involves the use of a caliper that determines the thickness of the skin and fat located under the skin at the site. The information gained from both procedures will allow us to use equations to estimate your percent body fat and percent lean body weight.

- **CT or CAT Scan.** Computed (Axial) Tomography (CT) is a routine method that provides very clear pictures of structures inside the body. The CT scan device uses sophisticated computers and a safe amount of X-rays. It will be used to assess the regional fat content in your trunk area. An appointment will be made for you at The Leo Jenkins Cancer Center. The visit will take approximately one hour. The CT scan device looks like a giant donut. You will be asked to lay down on a table and an instrument will be used to scan your middle body area. The test takes approximately 30 minutes. This information will help us to interpret the lipid metabolism data gained from your blood and muscle biopsies.

Females with ANY chance of being pregnant should not undergo DEXA or CT scanning. If you become pregnant during the course of this study, you should immediately inform the staff.

- **Maximal Exercise Test.** Fitness test (FITT Building - Human Performance Laboratory). The procedure will determine your maximal ability to use the oxygen (air) you breath to make energy from the food you eat. It will also allow the study investigators to set your workload for the submaximal exercise test described above. You will perform cycling (cyclists) exercise for ~10 minutes. You will begin cycling at light intensity (you will not breathe hard) for 2 minutes. The workload will be increased every 2 min. until you can no longer continue. This will allow us to determine your maximal exercise capacity.

2. **The muscle biopsy procedure.** The biopsy procedure will take place at the East Carolina Heart Institute. The procedure will occur under sterile conditions and a physician will be available during the entire time of the procedure. You will report to room 2377 at 7:30 AM following an overnight fast (no food after 10:00 PM the night before). For this procedure, a small amount of anesthesia (3 cc of 1% Lidocaine) will be injected in a ½ inch area under the skin of your thigh. A small (1/4 inch) incision will then be made through the skin, fat, and fibrous layer that lies over the muscle. A biopsy needle (about ½ the width of a pencil) is then inserted through the incision ½ to 1 inch into the muscle. A small piece of muscle (1/2 the size of an erasure at the end of a pencil) is then clipped out with the biopsy needle. The needle is withdrawn and the muscle sample is prepared for analysis. You will undergo two muscle biopsies. A separate incision will be made for each of the biopsies, one per leg. The first biopsy will be taken from the left leg and the second will be taken from the right leg.

The muscle biopsies will be taken by Robert Hickner, Ph.D., Joseph Houmard, Ph.D., or Ronald N. Cortright, Ph.D. The muscle samples will be assessed for the ability to metabolize fat and to control the metabolism of energy by assessing the levels of certain muscle factors (the expression of mitochondria and genes that regulate fat metabolism in skeletal muscle; e.g, PGC-1, PPARs, uncoupling proteins, etc.). In addition, a portion of the muscle biopsy may be used to culture cells to understand the portion of lipid metabolism that is inherited.

3. **Blood samples** (a total of 6 samples for the entire study) will be obtained on the day of the biopsies. Blood will be drawn from a small catheter placed in your arm vein. The total amount of blood will be approximately 30-40 ml (~ 4 tablespoons). Blood will be drawn from your arm vein. In addition, we will measure blood insulin and glucose. Other fat metabolism related molecules and hormones such as Leptin, Adiponectin, Ghrelin (hormones released by the fat cells which are associated with the regulation of fat and blood sugar) will also measured. Determining blood lipids will also help us to determine the relationship between these blood variables and factors indicating your muscle's lipid metabolic capacity and diabetic status. The blood samples will be taken by Dr. Robert Hickner, Dr. Ronald Cortright, Dr. Joseph Houmard, or a trained research nurse at the East Carolina Heart Institute.

Potential Risk and Discomforts

Certain risks and discomforts may be associated with this research. They include:

- The DEXA is a safe procedure for assessing body composition. The scanner will not enclose or touch you, and you can wear regular clothing (no metal allowed). You will be exposed to minimal radiation (DEXA: ~0.4 microSieverts per whole body scan) that is within an acceptable range as provided by "North Carolina Regulations for Protection Against Radiation". (30 miliSieverts) For example, one would receive radiation exposure of approximately 80 microSieverts on a transatlantic airline flight of 8 hours, 50 microSieverts living in Denver, Colorado, at an elevation of 5,000 feet for approximately 4 weeks, or 30 to 40 microSieverts during a typical chest x-ray. However, even this minimal exposure to X-ray radiation my have negative effects on the unborn fetus. Therefore, you will be screened for menstrual cycle status by questionnaire and queried to be sure they are not pregnant prior to commencing the study.
- For the CT scan, the amount of radiation (~75 miliSieverts per abdominal CT scan) that you will be exposed to falls within the national acceptable range for CT scans. A normal CT scan consists of multiple slices yielding a total expose of 500 -650 mGY (500-650 mSV). Therefore, you will be exposed to 1/20th of the typical diagnostic CT scan. However, even this minimal exposure to X-ray radiation my have negative effects on the unborn fetus. Therefore, you will be screened for menstrual cycle status by questionnaire and queried to be sure you are not pregnant prior to commencing the study. You will be exempt from post training CT scans (eight week training study only) if pregnancy is detected. The CT scan will be performed in a way that will minimize your exposure to radiation.
- The total amount of blood drawn for lipid metabolism measurements (~ 4 tablespoons) is very small compared to the total amount (about a gallon) of blood that you have. There is an extremely small risk of local bruising or infection associated with insertion of the sterile needle (we draw blood through these) into your arm.
- Risks associated with the exercise protocols are dizziness, ventricular arrhythmia (odd heart beats), and in very rare instances death. These risks are very small, with an incidence of fewer than 1 in 10,000 deaths in patients who are known to, or suspected of, having heart disease. The risk is expectedly much smaller than this in a group of younger, healthy subjects. To minimize this risk, we will have a physician present and a heart (ECG) monitoring device will also be used during the exercise tests. The physician will be trained to recognize heart problems during exercise and trained to revive

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people in the event of serious heart problems during the exercise test. The exercise tests will be stopped if you feel dizzy, are having chest pain, are having serious shortness of breath, or ask that the test be ended. The test will also be stopped if the physician detects (from the ECG) heart function that is not normal. All of the necessary emergency equipment (including crash cart for heart problems) will be in the room. If you experience a cardiovascular event or pass out, then Pitt County Emergency Services will be contacted.

- Dr. James deVente, M.D., PhD. or other attending physicians will be provided medical coverage for the maximal exercise test and the muscle biopsies performed at the East Carolina Heart Institute or the Human Performance Laboratory. With respect to the muscle biopsy procedure, there is a small risk of hematoma (bruise) or infection around the biopsy site. This risk will be minimized by using sterile procedures and applying pressure to the biopsy site for 10 minutes, or until bleeding is stopped if longer than 10 minutes, following biopsy. A steri-strip (thin bandage) will be applied over the incision and will remain in place for at least 4 days to close the incision during healing. A pressure wrap will also be placed around the biopsied limb and will remain for 8 hours following biopsy. There is an extremely remote risk of allergic reaction to the Lidocaine anesthesia. This risk will be minimized by using subjects who have had prior exposure to Lidocaine or Novocaine anesthesia; this precaution should eliminate this risk. Dr. James deVente, M.D., PhD (or other physicians associated with the study) will initiate any medical treatment necessary during or following any adverse event from the biopsy procedure.

Exclusions

To the best of your knowledge, you are not allergic to Novocaine/lidocaine. For example, you have not had an allergic reaction to an injection at the dentist's office. To your knowledge, you do not possess any condition which would result in excessive bleeding. You do not have known kidney disease, and you do not have know heart disease (i.e., had a heart attack). Other exclusion criteria include: individuals who are ill or taking medications, individuals who are known diabetics, individuals who currently smoke, African-Americans that are not of at least second generation African-American decent, individuals who are pregnant, and individuals who are exercise training or who have exercise trained regularly within the last 6 months.

Potential Benefits

1) You will receive information concerning your health risk due to your level of obesity and insulin resistance.
2) You will benefit from gaining knowledge of your body composition and aerobic fitness level.
3) You will receive information about their skeletal muscle fiber type.
4) Society and medical science may benefit from gaining the knowledge resulting form this investigation.

Termination of Participation

Your participation in this research study may be terminated without your consent if the investigators believe that these procedures will pose unnecessary risk to you. You may also be terminated from participation if you do not adhere to the study protocol.

Cost and Compensation

The policy of East Carolina University does not provide for compensation or medical treatment for subjects because of physical or other injury resulting from this research activity. However, every effort will be made to make the facilities of the School of Medicine/ECHI available for treatment in the event of such injury.

You will receive $100.00 for your time and efforts for participating in the muscle biopsy procedures ($50.00 for each biopsy). You will receive, free of charge, the body composition and maximal aerobic capacity analysis.

You do not give up any legal rights as a research participant by signing this consent form.

Confidentiality

Only the investigators associated with this study will have access to the data obtained. The data gathered from the study will be stored on a computer hard drive which will be accessible only by the investigators or technical staff. Numeric coding will protect the identity of the subjects. No identifying information will be released. The information and insights gained from the study may be presented at scientific conferences and/or published. In both instances, you will not be identified by name.

Voluntary Participation

The nature and purpose of the procedures, the known risks involved, and the possibility of complications have been explained to you. No guarantee of assurance has been given by anyone as to the results that may be obtained. You know that being in this study is of your own free will. You know that you can decide not to be in this study after you have already started. You may stop at any time without losing benefits that you would have received before being in the research study.

Persons to Contact with Questions

The investigators will be available to answer any questions concerning this research, now and in the future. You may contact the investigators, **Ronald Cortright Ph.D.** (work: 737-4678/office or home: 756-7735), **Robert Hickner, Ph.D.** (work: 737-4677 or home: 353-5556), or **Joe Houmard, Ph.D.** (work: 737.4688/737-4617). Drs. Cortright, Hickner and Houmard are found in the Human Performance Laboratory, Ward Sports Medicine Building, ECU. Also, if questions arise about your rights as a research subject, you may contact the Chairman of the University and Medical Center Institutional Review Board at phone number 252-744-2914 (days).

Research Participant Authorization To Use And Disclose Information

Federal laws require that researchers and health care providers protect your identifiable health information. Federal laws also require that researchers get your permission to use collected health information for research. The identifiable information we will collect from subjects in this research project will include:

*General Medical History including: Family health history, medications, nutrition, physical activity levels, menstrual history, nutritional history, and body weight history.

*Muscle biopsy information, body composition information, blood levels of insulin, glucose, and other compounds related to muscle and fat cell lipid metabolism.

The members of our research team that will have access to your information will include the Principle investigator, co-investigators, as well as technical and nursing personnel involved in this project. Information about you will be used and released in such a way that will protect your identity as much as possible; however, confidentiality cannot be absolutely guaranteed. We will only share your information with those individuals listed above. If we need to share information with other individuals other than those listed, we will request your permission a second time.

Subjects Initials _____

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You will be given a signed copy of your authorization to release medical information for your records. You can limit the amount and type of information that is shared and you must make this request in writing; however, the researcher is able to use any and all information collected prior to the request not to disclose information. Although you can limit the release of your medical information, withholding some information may cause you to become ineligible for this research project. Because research information continues to be looked at after a study is finished, it is difficult to say when the use of your information will stop. There is currently not an expiration date for the use and disclosure of your information for this study.

If you have questions related to the sharing of information, please call Ronald N. Cortright, Ph.D. at 252-737-4678. You may also telephone the University and Medical Center Institutional Review Board at 252-744-2914. In addition, if you have concerns about confidentiality and privacy rights, you may phone the Privacy Officer at Pitt County Memorial Hospital at 252-847-6545 or at East Carolina University at 252-744-2030.

FUTURE TESTING OF BLOOD/MUSCLE SAMPLES

Upon termination of this study, the blood and muscle samples collected for this study will be stored for up to 7 years to research scientific questions specifically related to obesity, muscle/fat cell lipid metabolism and insulin resistance/diabetes in African-American vs. Caucasian women. You will continue to be the owner of the samples and retain the right to have the sample material destroyed at any time during this study by contacting the study principal investigator Ronald N. Cortright, at Office:252.737.4678 or lab: 252.744.2934. During this study, the samples will be stored with number identifiers only; however, the number identifier will be linked to a specific name and will be kept on file in the possession of the principal investigator. The linked file will be stored password protected on the Principal Investigator's computer with CD backup. No other individuals will have access to these identifying materials unless the principal investigator is required by law to provide such identifying information. Data will not be publicly available and participants will not be identified or linked to the samples in publication. If a commercial product is developed from this research project, you will not profit financially from such a product. Furthermore, there are no plans for the investigators to profit financially from such a product.

Subjects Initials _____

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Title of Project: Impaired Acyl-CoA Synthetase-Muscle Lipid Oxidation in African-American Women (UMCIRB # 07-0135)

CONSENT TO PARTICIPATE

I have read all of the above information, asked questions and have received satisfactory answers in areas I did not understand. (A copy of this signed and dated consent form will be given to the person signing this form as the participant or as the participant authorized representative.)

Participant's Name **(PRINT)**	**Signature**	**Date**	**Time**

Guardian's Name **(PRINT)**	**Signature**	**Date**	**Time**

WITNESS: I confirm that the contents of this consent document were orally presented, the participant or guardian indicates all questions have been answered to his or her satisfaction, and the participant or guardian has signed the document.

Witness's Name **(PRINT)**	**Signature**	**Date**	**Time**

PERSON ADMINISTERING CONSENT: I have conducted the consent process and orally reviewed the contents of the consent document. I believe the participant understands the research.

Person Obtaining consent **(PRINT)**	**Signature**	**Date**	**Time**

Principal Investigator's **(PRINT)**	**Signature**	**Date**	**Time**

Subjects Initials _____

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Appendix C

Personal History Form

PERSONAL HISTORY FORM

Technician_____ Contract_____ ID_____

PLEASE PRINT AND FILL OUT COMPLETELY

1. **Name**: _____ Date: _____
   Phone#: (home) _____ (work) _____
   Address: _____
   City: _____ State _____ Zip _____
   e-mail address (if available):_____
2. **Employer**: _____
   Occupation: _____

3. Date of Birth: _____ Sex**: _____** Age: **_____** Race: _____

4. **General Medical History**

Circle one

Any medical complaints presently? (if yes, explain) .... yes no

_____
_____
_____

Any major illnesses in the past? (if yes, explain) .....(date) _____ yes no

_____
_____
_____

Any hospitalization or surgery? (if yes, explain) ..... (date) ____yes no

_____
_____
_____

Have you ever had an EKG (electrocardiogram) ? ..... (date) _____ yes no

Are you diabetic? ....If yes, at what age did you develop diabetes: _____ yes no

Are you currently taking any medications? ..... yes no

Medication	Dosage	Reason	Times taken per day

Have you ever been exposed to Lidocaine or Novocaine? yes no

Have you ever had an allergic reaction to Lidocaine or Novocaine? yes no

5. **Family History**

	Age if alive	Age of death	Cause of death
Father	_____	_____	_____
Mother	_____	_____	_____

Do you have a family history of: (Blood relatives only: give age of occurrence if applicable)

	Relationship	Age of occurrence
--High blood pressure ..... yes no		_____
--Heart attack.....yes no		_____
--By-pass surgery.....yes no		_____
--Stroke.....yes no		_____
--Diabetes.....yes no		_____
--Gout.....yes no		_____
--Obesity.....yes no		_____

6. **Tobacco History** (check one)

_____ None
_____ Quit months/years ago
_____ Cigarette
_____ Snuff
_____ Chewing tobacco
_____ Pipe

Total years of tobacco use? _____

Cigarette history
_____ 1-10 daily
_____ 11-20 "
_____ 21-30 "
_____ 31-40 "
_____ more than 40

Snuff history
_____ < 0.5 cans daily
_____ 0.5-2.5 cans "
_____ > 2.5 cans "

Chewing history
_____ < 0.5 pouches daily
_____ 0.5-2.5 pouches "
_____ > 2.5 pouches "

7**. Weight History**

What do you consider a good weight for you? _____ Weight at age 21? _____
Weight since age 21? _____ Weight one year ago? _____
Weight now? _____

8. **Cardio-Respiratory History**

Any heart disease now?.....	yes no
Any heart disease in the past?.....	yes no
Heart murmur?.....	yes no
Occasional chest pains?.....	yes no
Chest pains on exertion?.....	yes no
Fainting?.....	yes no
Daily coughing?.....	yes no
Cough that produces sputum?.....	yes no
High blood pressure?.....	yes no

Shortness of breath --

at rest..... yes no
lying down..... yes no
sleeping at night..... yes no
after 2 flights of stairs..... yes no

9. **Muscular History**

Any muscle injuries or illnesses now?..... yes no
Any muscle injuries in the past?..... yes no
Muscle pain at rest?..... yes no
Muscle pain on exertion?..... yes no

10. **Bone-Joint History**

Any bone or joint (including spinal) injuries or illnesses now?..... yes no
Any bone or joint (including spinal) injuries or illnesses in the past?..... yes no
Ever had painful joints?..... yes no
Ever had swollen joints?..... yes no
Flat feet?..... yes no

11. **Nutritional Survey**

How many times do you usually eat per day? _____

What time of day do you eat your largest meal? _____

How many times per week do you usually eat...

____ Hamburger ____ Sausage ____ Bacon
____ Beef ____ Pork ____ Cheese
____ Shellfish (shrimp, oysters, scallops, clams, etc.)
____ Fish ____Poultry ____ Fried Foods
____ Breads ____ Cereals ____ Vegetables
____ Eggs ____ Desserts ____ Ice Cream
____ Other

How many servings per week do you usually consume?

____ Whole milk ____ Coffee
____ Low-fat milk (2% milk fat) ____ Tea
____ Skim milk (non-fat) ____ Soft drinks
____ Buttermilk ____ Other

12. **Physical Activity Survey**

a. Compared to a year ago, how much regular physical activity do you currently get? (Check One)

____ much less ____ somewhat less ____ about the same
____ somewhat more ____ much more

b. For the last three months, have you been exercising on a regular basis?

____ yes ____ no

c. What type of exercise or physical activity do you currently do or have done regularly in the past three months?

(For example: walking, swimming, weight lifting, gardening, etc.)

_____

_____

_____

d. On the average, how many days per week do you exercise? _____

e. How long do you exercise each time? For how many minutes? _____

f. How hard do you exercise on a scale from 1 to 5: with 1 being easy and 5 being very hard?

____ 1 ____ 2 ____ 3 ____ 4 ____ 5

g. Do you ever check your heart rate (pulse) to determine how hard you are exercising?

____ yes ____ no

h. What aerobic activity or activities would you prefer in a regular exercise program for yourself?

____ Walking and/or running	____ Tennis	____ Bicycling
____ Racquetball	____ Swimming	____ Basketball
____ Aerobic dance	____ Stationary cycling	____ Soccer
____ Stair climbing	____ Rowing	_____Other

13. **Alcohol History**

Do you ever drink alcoholic beverages? Yes ____ No ____

If yes, what is your approximate intake of beverages per week?

Beer _____ Wine _____ Mixed Drinks _____

14. **Sleeping Habits**

Do you ever experience insomnia (trouble sleeping)? Yes ____ No ____

If yes, approximately how often?_____

How many hours of sleep do you usually average per night?_____

15. **Education**

Please indicate the highest level of education completed.

____ Grade School	____ Junior High	____ High School
____ College	____ Graduate	____ Postgraduate

Please indicate degree earned (i.e. B.A., M.S., Ph.D.)_____

16. **Menopausal status (for women)**

Date of most recent menstrual cycle?_____

Have you missed more than one period in the last year? Yes ___ No___ If yes, how many?____

Birth control? Yes ___ No____ If yes, what type? _____How long?_____

Have you had a hysterectomy? Yes _____ No_____ If so, when? _____

Are you post-menopausal? Yes _____ No_____ If so, for how long?_____

If you are on estrogen therapy, for how many years have you been on? _____

17. **Family Physician**
Name: _____
Address:_____
Phone: _____
Should it be necessary, may we send a copy of your results to your physician?

Signature: _____
Date: _____

Appendix D

Food Diary Form

FOOD DIARY

Department of Exercise and Sport Science
East Carolina University
Greenville, NC 27858
252-328-2575

Name_____ DOB:_____

Instructions:

1. Choose 3 days immediately prior to the start of the study to record.

2. Record all foods and beverages (including water) that are consumed.

3. List portion size of all foods and beverages. Be as specific as possible. For example: 2 ounces of chicken breast, ¼ cup of mashed potatoes with milk, 8 ounces of orange juice. Estimate portion size ***after*** cooking.

4. If you are not sure about what the portion size is, give another descriptor such as a deck of cards or the size of a baseball.

5. List brand names of foods if known.

6. Describe how each food is prepared. For example: fried chicken, scrambled eggs, and steamed cabbage.

7. Record any "extras" or condiments used and their amounts. For example: 1 tablespoon of mayonnaise, 1 teaspoon butter, 2 tablespoons Italian salad dressing.

8. List any snacks-foods, beverages and candy consumed in between meals.

9. If a combination food was consumed, such as a casserole, salad, or stew, please list all ingredients and the amount consumed.

10. Please return this to the above address as instructed.

11. Please call at 252-328-2575 if you have any questions.

Appendix E

Menstrual Cycle Recall

MENSTRUAL CYCLE RECALL

Please answer these questions as best you can and return them with any other appropriate paperwork.

Are you taking birth control pills? _____yes _____no
If yes, which kind_____

Are you on another form of birth control? _____yes _____no
If yes, please specify_____

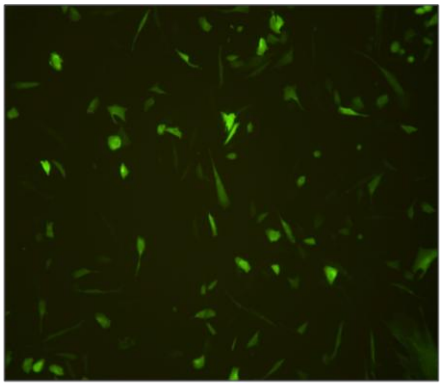
What was the first day of your last menstrual cycle? _____

How many days is your cycle? _____

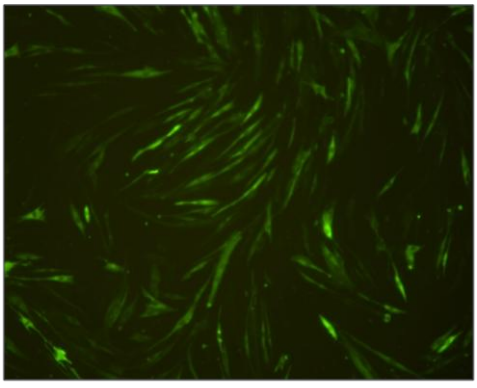
Appendix F

Time Course Green Fluorescent Protein with Transfection

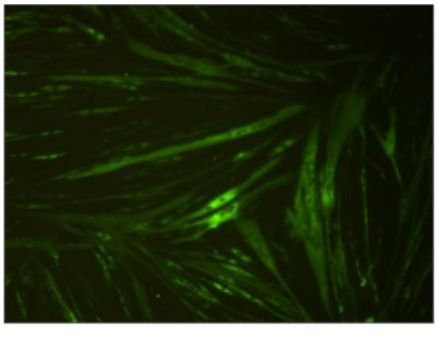
Data courtesy of H.B. Kwak, Ph.D. (personal communication, June 29, 2011)



A. Myoblasts



B. Myotubes (3 days)



C. Myotubes (7 days)

Primary human skeletal myoblasts were transfected by GFP plasmid DNA and showed approximately 50% of transfection efficiency at 24 hours after transfection (**A**). GFP fluorescence showed initiation of differentiation at 48 hours of differentiation by switching growth media by differentiation media (**B**). At day 7 of differentiation period, GFP fluorescence was still expressed strongly, suggesting fully differentiation in primary human skeletal muscle cells (**C**).

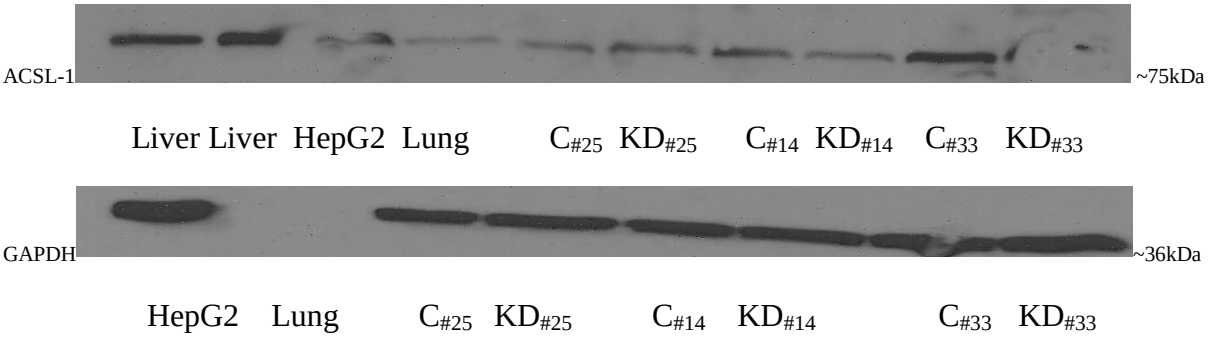
Appendix G

Western Blotting Bands

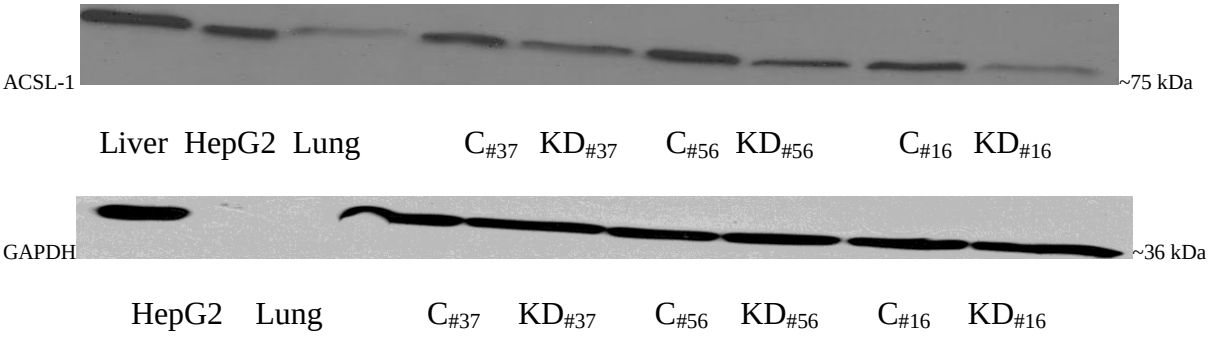
Western blotting bands

Liver samples protein were loaded 1 µg, and 2.5 µg, HepG2, lung, Control (C), and knockdown (KD) samples were loaded 15 µg. A third Western was performed on samples number 33 due to a bubble on the band. Each sample was normalized to liver (1µg).

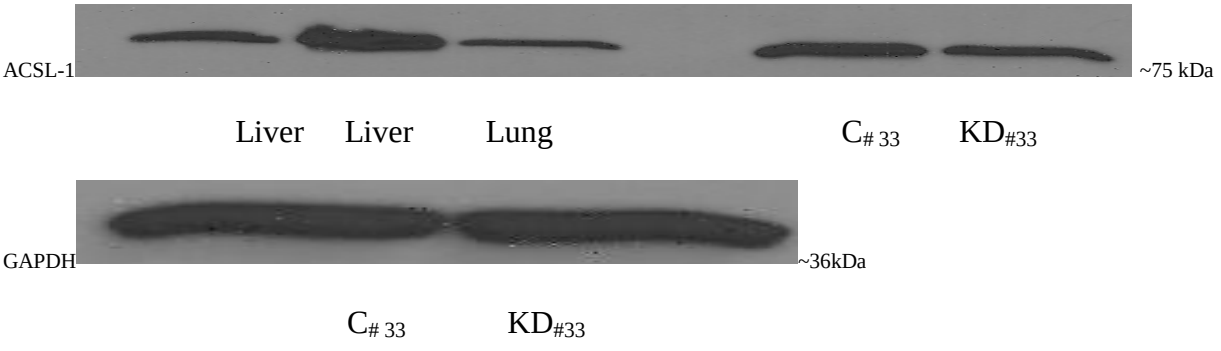
Western #1



Western #2



Western #3

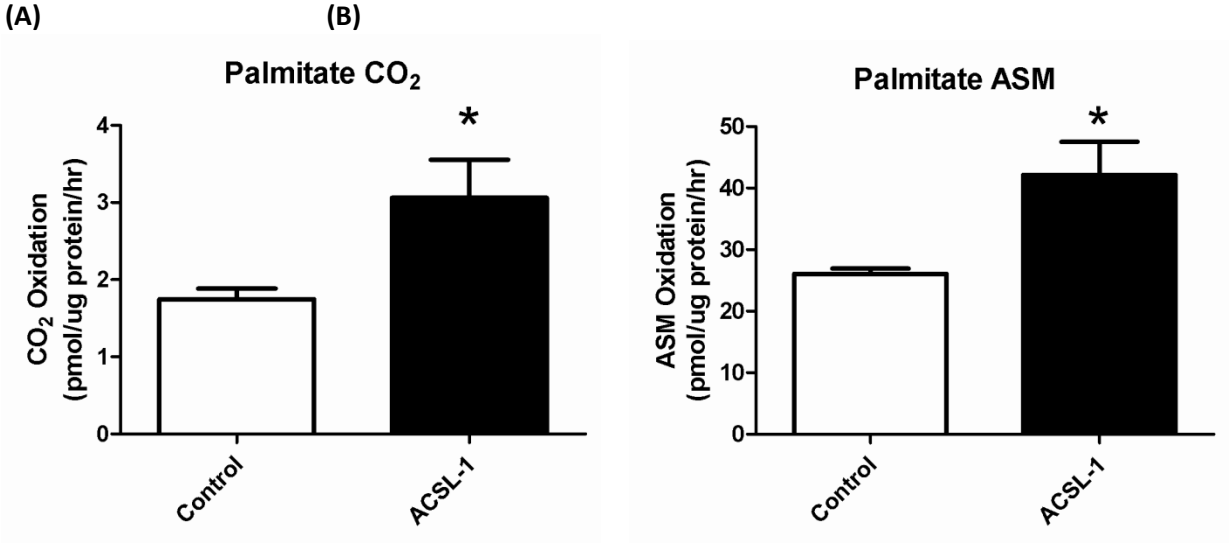


Appendix H

ACSL-1 and ACSL-5 Overexpression Graphs

Data courtesy of H.B. Kwak, P.h.D.(personal communication, June 29, 2011)

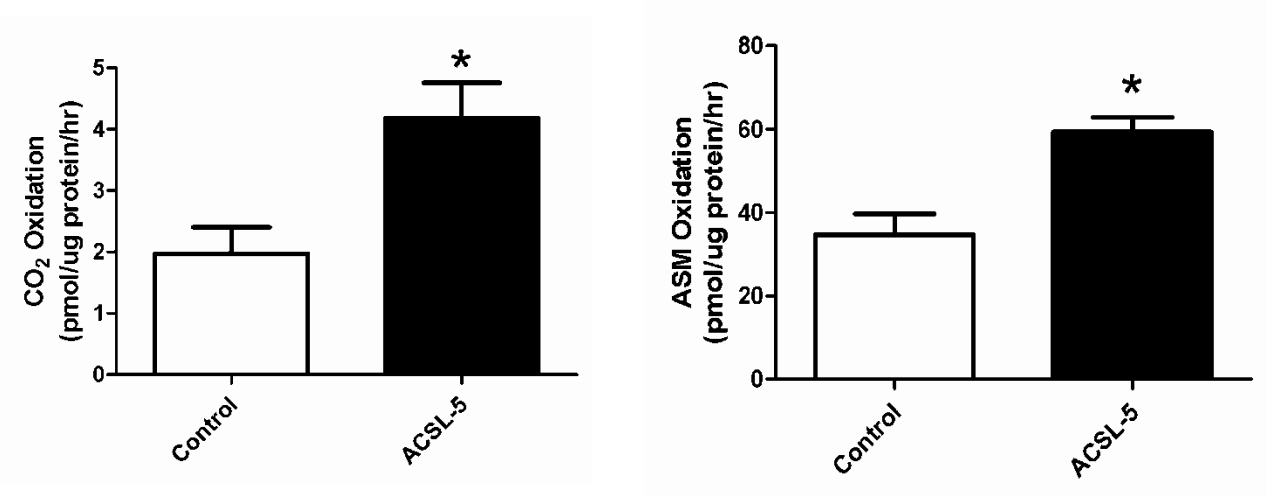
ACSL-1 Overexpression Data



Effect of ACSL-1 overexpression on fatty acid oxidation. Subjects are 6 obese AAW which were biopsied from the vastus lateralis muscle following an overnight fast. Satellite cells were cultured and differentiated into mature myotubes. Oxidation studies were performed according to methods described above. Fatty acid oxidation was measured by ^{14}C-leveled radioactivity of CO_2 (**A**) and ASM (**B**) by liquid scintillation counting (open bar, control cells; closed bar, ACSL-1 transfected cells). Palmitate complete CO_2 oxidation was approximately 82% higher in ACSL-1 overexpression compared with control (**A**). Similarly, palmitate incomplete ASM (acid soluble metabolites; indicator of β-oxidation activity) oxidation significantly increased (+62%) by ACSL-1 overexpression (**B**). Data are presented as mean ± SEM (n=6). * $P<0.05$ versus control. These results provide proof of methods for transfection of human skeletal muscle myotubes with specific ACSL myotubes and indicate that alterations in ACSL-1 content results in predicted changes in fatty acid oxidation. Studies will be repeated with knockdown of ACSL-1 in lean CW.

ACSL-5 Overexpression Data

(A) Palmitate CO_2 **(B) Palmitate ASM**

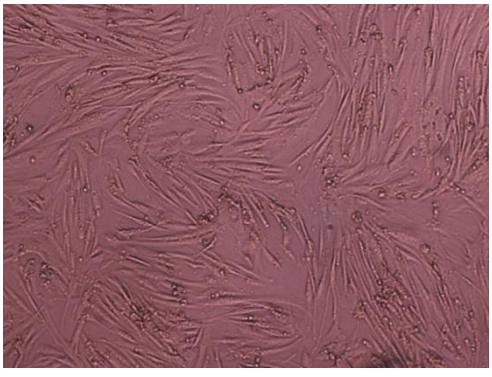


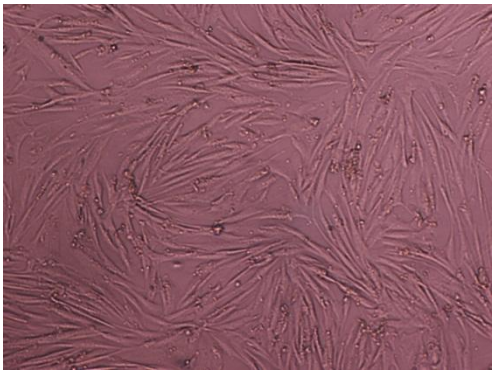
Effect of ACSL-5 overexpression on fatty acid oxidation and total lipid synthesis. Fatty acid oxidation was measured by ^{14}C-labeled radioactivity of CO_2 (**A**) and ASM (**B**) by liquid scintillation counting (open bar, control cells; closed bar, ACSL-5 transfected cells). Palmitate complete CO_2 oxidation was approximately 2 folds higher in ACSL-5 overexpression compared with control (**A**). Similarly, palmitate incomplete ASM oxidation significantly increased by ACSL-5 overexpression (**B**).

Appendix I

ACSL-1 knockdown and control Myoblast and Myotubes

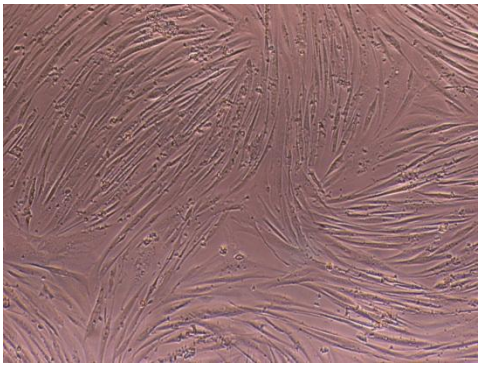
A. Myoblast control **B. Myoblast ACSL-1 KD**

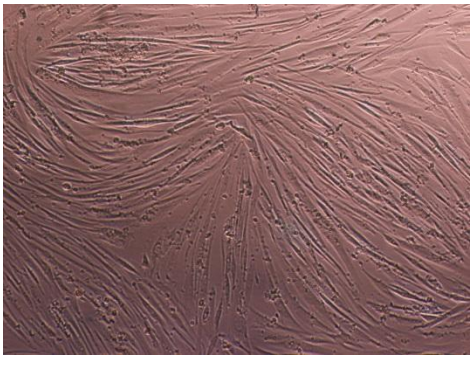




Pictures show myoblast 48 hours post transfection in growth media. A) Myoblast control cells transfected with scramble 2 ug of shRNA. B) Myoblast ACSl-1 KD cells transfected with 2 ug of ACSL-1 shRNA.

C. Myotube Control **D. Myotube ACSL-1 KD**





Pictures show myotubes day 6 in differentiation media. C)Myotube control cells and D) myotube ACSL-1 KD.