Regulation of muscle fat oxidation and storage by ACSL enzymes and effects of acute exercise training in humans

by
Victoria Boechler

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Kinesiology
(Honors Associate)

Presented May 28, 2020
Commencement June 2021

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Victoria Boechler for the degree of Honors Baccalaureate of Science in Kinesiology presented on May 28, 2020. Title: Regulation of muscle fat oxidation and storage by ACSL enzymes and effects of acute exercise training in humans

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Abstract approved:_____

Sean Newsom

The increase in obesity rates in both children and adults in the United States may be a result of impaired or dysregulation of fat metabolism. Long chain acyl-CoA synthetases (ACSLs) may be key regulators of skeletal muscle cell fat metabolism, including fat oxidation and storage. The purpose of the study was to identify what ACSLs are present in human skeletal muscle, the effects of exercise on isoform abundance, and to see if a relationship exists between ACSLs and measures of fat metabolism. Enrolled participants (n=14) performed two metabolic study visits in a randomized, crossover design. During one visit the individuals remained sedentary (CON), while in the second, they performed a single session of moderate-intensity exercise (60 minutes at 65% VO2max). Fat oxidation was measured at rest and during exercise using indirect calorimetry. Muscle biopsies were taken after rest or exercise as well as two hours post exercise. We showed the presence of 4 of the 5 known ACSLs and a significant increase in ACSL 5 with exercise (P= 0.01). We also showed a relationship between ACSL1 and fat oxidation during exercise (P=0.07) and a significant correlation between ACSL6 and measures of triacylglycerol abundance (P= 0.05). We conclude that 4 of the 5 ACSL isoforms are present in human skeletal muscle with ACSL5 significantly affected by acute exercise training. We also conclude that ACSL1 during exercise and ACSL6 are related to measures of fat metabolism.

Key Words: Long Chain Acyl-CoA Synthetases, Lipid Metabolism, ACSL, Obesity.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Victoria Boechler, Author

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Introduction

Skeletal muscle tissue accounts for approximately 40-50% of tissue mass in the average adult, making it a significant determinant of whole-body metabolism, including lipid metabolism. There is growing interest in understanding key regulators of skeletal muscle lipid metabolism, due largely to the surge in obesity prevalence among US adults and children in recent decades (10). Obesity increases the risk of numerous chronic diseases, including insulin resistance and type 2 diabetes (T2D) (8). Much of the increase in risk is attributable to excessive accumulation of lipids within muscle cells (3), that may be secondary to impaired mitochondrial fat oxidation and/or dysregulation of fat storage (2,4). Exercise training interventions can reduce the risk for the development of insulin resistance and T2D, in part, via improvements in skeletal muscle lipid metabolism (e.g., increased fat oxidation and improved fat storage) (18). Nevertheless, what serves to regulate the fate of fatty acids entering the muscle cell (e.g., oxidation, storage) and how such processes may be regulated by exercise training remains poorly understood. The overarching purpose of the current study is to address these key gaps in knowledge.

Fatty acids entering skeletal muscle cells have many potential metabolic fates (1). In general, fatty acids may be oxidized to be used as energy for the cell, stored as triacylglycerols (TAG), or used by the cell to synthesize lipid-molecules, such as the phospholipid bilayer cell membrane (8). For both health and exercise performance, there is interest in understanding regulatory processes that may facilitate fat oxidation and fat storage. Evidence indicates long-chain acyl-CoA synthetases (ACSLs) regulate the partitioning of fatty acids towards oxidation and/or storage (1).

Evidence from model systems (i.e., mice and cultured cells) shows various ACSL isoforms direct fatty acids to either storage or oxidation. Of the five known ACSLs, previous studies have shown that in skeletal muscle, ACSL1 may direct fatty acids to beta oxidation within the mitochondria, whereas ACSL6 may facilitate fat storage (2,3). Studies in mice have shown that the absence of ACSL1 reduced skeletal muscle fat oxidation by 83% in the gastrocnemius muscle and 63% in the soleus muscle (2). In cultured skeletal muscle cells, experimental reduction of ACSL6 led to a decrease in lipid content, whereas overexpression increased lipid storage (4). These findings suggest that ACSL1 may regulate fat oxidation in skeletal muscle cells, where ACSL6 may contribute to an increase in the partitioning of fatty acids toward storage. Nonetheless, there is still a gap in knowledge on the relationship of ACSLs with changes in skeletal muscle lipid metabolism.

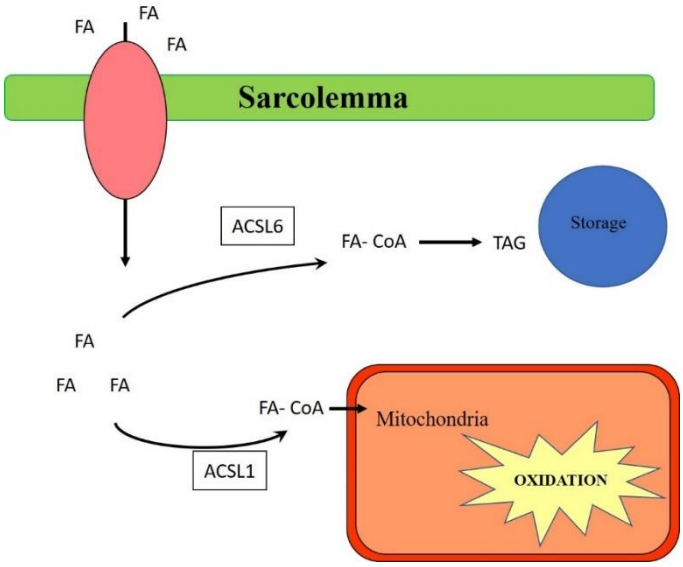

Figure 1: Proposed mechanisms of ACSL partitioning of fatty acids in skeletal muscle. Fatty acids, indicated by "FA" are shown to be entering a skeletal muscle cell via the sarcolemma. As the fatty acid enters, it is either directed towards storage by the presence of ACSL 6 or towards oxidation within the mitochondria in the presence of ACSL 1.

Exercise is a potent stimulus capable of inducing significant changes in skeletal muscle lipid metabolism, including increased fat oxidation and fat storage after exercise (6,11).

However, the mechanisms behind these changes with exercise remain poorly understood. Exercise has been shown to increase fat oxidation in the mitochondria through increased mitochondrial enzymatic activity, which may be attributable to the role of ASCL1 in partitioning towards oxidation (2,3,7). Furthermore, our lab has previously shown that ACSL6 abundance increases following aerobic exercise training (4).

The purpose of this study was to first determine ACSL isoform protein abundance within human skeletal muscle and understand how acute exercise effects ACSL isoform protein abundance. Although there is evidence on how ACSLs affect other tissues in the body, little is still known on the purpose of ACSLs in skeletal muscle. The second purpose of this study was to identify the relationship between ACSL isoform abundance and fat metabolism in humans. The results of our study showed that there is a presence of 4 of the 5 known ACSL isoforms in human skeletal muscle, excluding ACSL 3. We also show that there is a tendency for ACSL 1 and ACSL 6 to be related to measures of fat metabolism.

Methods:

Participants

This study was approved by the Institutional Review Board at Oregon State University (#7605). Study participants were generally free of medical conditions and not taking medications (other than use of oral contraceptives). Participants were between 18-45 years old and had a body mass index (BMI) between 18-25 kg/m^2. Participants did not smoke, had a stable weight (± 2 kg for ≥ 6 months), and were sedentary (< 60 minutes of purposeful exercise each week, for ≥ 6 months). Exclusionary criteria included hyperglycemia (fasting glucose >126ml/dl), hypertension (high blood pressure), cancer, heart disease, pregnancy, hyper- or hypothyroid, or allergy to lidocaine.

Participants were excluded if they were taking any of the following medications: insulin, metformin, thiazolidinediones, statins and chronic non-steroidal anti-inflammatory medications. For metabolic study days, females were studied during the early follicular phase of the menstrual cycle.

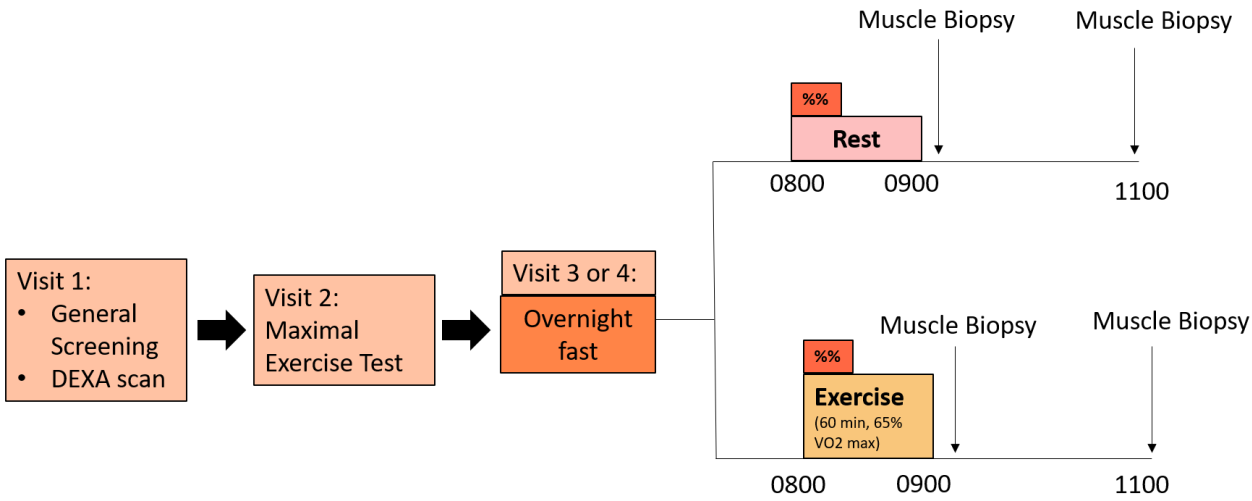

Figure 2: Overview of the study design. Visit one included a general screening and dual-energy x-ray absorptiometry (DEXA) scan. During visit 2 participants underwent a maximal exercise test. Visits 3 and 4 took place through a randomized cross-over design in which during one visit the participant rested while undergoing an indirect calorimetry test (annotated by %%) and then received a muscle biopsy after an hour of rest. A biopsy was also performed at 11:00 after the rest trial, but was not measured to avoid duplicate resting measures. The other of the two visits was characterized by a bout of acute exercise (60 minutes) at 65% of their VO2 max, while simultaneously undergoing an indirect calorimetry test, and two muscle biopsies, one after rest and exercise at 9:15 and another approximately two hours later at 11:00 after exercise.

General Experimental Design

For this study, participants, 10 females and 4 males, were recruited for the study and provided written, informed consent. On the first visit, consented individuals were screened to determine eligibility for the study and given a Dual-energy X-ray absorptiometry (DEXA) scan in order to determine body composition. On the second visit, participants underwent a VO_2 max test which entailed pedaling on a stationary, electronically braked bike for 10-15 minutes while heart rate was monitored through a 12-lead electrocardiogram (EKG). This test occurred at least a week prior to their first metabolic study visit. The VO_2 max test was characterized by a 2-minute warm up at an intensity of 50 W, increasing by 25 W for males and 15 W for females after each minute. The test ended when participant reached fatigue (~8-12 minutes). Participants were asked to report ratings of perceived exertion throughout the test, using the 6-20 Borg's scale. Blood pressure was collected every three minutes during the test. VO_2 max was defined by the peak oxygen consumption that occurred as an average 30-second interval with inability to maintain pedaling cadence of 60 revolutions per minute, reaching about 10% of maximal heart rate predicted by age, and a respiratory exchange ratio that exceeded 1.1. The third and fourth visits were metabolic studies that were assigned through a randomized, cross-over design. During one visit, individuals remained sedentary, while in the other they performed a 60-minute bout of moderate-intensity (65% of VO_2max) exercise. During both the rest and exercise trials, the participants simultaneously received an indirect calorimetry test to measure VO_2 and VCO_2. These measures were taken during the first 20 minutes of the exercise trial and over a course of 30 minutes for the resting trial. Aside from this difference, both metabolic studies followed the same timeline, beginning with the first muscle sample of the vastus lateralis, taken after exercise or rest at the 9:15 hour. Another muscle biopsy was taken for both trials two hours later at 11:00, from the contralateral leg. The 11:00 hour post-rest sample was not used to determine ACSL

abundance in order to eliminate duplicate resting measures. All other biopsies were frozen in liquid nitrogen and stored at -80 C until they were used to measure ACSL abundance and fat storage.

Tissue Analysis/Western Blotting

Collected muscle samples were frozen in liquid nitrogen at -80 C until analysis was performed. Analysis of muscle samples was performed at the same time, to eliminate potential batching effects. Muscle samples were homogenized through a 1:10 buffer, then incubated at 4 C for 20 minutes, and lastly centrifuged for 10 minutes at 10,000 x *g* at 4 C. About 35-40 μg of protein were loaded onto tri-bis gels and run by electrophoresis to separate proteins by weight. The separated protein map was transferred onto nitrocellulose membranes. An internal control sample was run in two lanes of every gel, to normalize band density. A ponceau stain was used to ensure equal amounts of protein transferred to each gel. After, a 5% bovine serum albumin in tris-buffered saline with tween (TBST) was used to block membranes. The membranes were then incubated at 4 C in the primary antibodies. These primary antibodies included ACSL 1 (product no. 4047, Cell Signaling Technology), ACSL 3 (product no. 166374, Santa Cruz), ACSL 4 (product no. PA5-27137, Invitrogen), ACSL 5 (product no. 365478, Santa Cruz), and ACSL 6 (product no. PA5-30465, Invitrogen). All antibodies were diluted 1:1000. After incubation with primary antibody, the membranes were washed using TBST before they were incubating with a secondary antibody. The secondary antibodies that were used were an anti-mouse-800 and anti-rabbit-700. Western blots were imaged using a LI-COR machine. Images were then analyzed for protein content.

Energy Expenditure and Substrate Oxidation

Skeletal muscles are prominent contributors to whole body fat oxidation and thus it was important to measure fat oxidation during both rest and exercise (5). Whole-body fat oxidation was calculated using VO_2 and VCO_2 measures. These data were collected through measures of indirect calorimetry. The indirect calorimetry tests were given while the participants were resting for 30 minutes using a ventilated-hood. During the exercise trial, indirect calorimetry was tested during the first 20 minutes of exercise using a two-way valve mouthpiece. The equations formulated by Frayn were used to estimate whole-body fat oxidation (9).

Skeletal Muscle Lipid Content

Skeletal muscle lipid samples were dissected without any contaminating liquid. In order to determine skeletal muscle lipid content of the vastus lateralis muscle samples, biopsies (~10 mg) were homogenized in 900 µl ddH2O, and an aliquot was taken for protein concentration (Pierce BCA). Then, 750 µl of homogenized sample was transferred to a glass screw cap tube with methanol (MeOH), methyl tert-butyl ether (MTBE), and internal standards. The internal standard cocktail included TAG-d5 (14:0/16:1/14:0) and TAG (17:0/17:0/17:0), among many other reference lipids. Samples were vortexed, rotated for 5 min at room temperature, and centrifuged at 2500 x *g* for 5 min to separate phases. The upper phase containing lipids was transferred to new glass culture tubes. Residual lipids in the lower phase were repeat extracted with additional MTBE. The combined extracts were dried under nitrogen gas and low heat (~30°C). The total lipid extract was transferred to autosampler vials using 2:1 chloroform:methanol and re-dried under nitrogen gas. Lipids were resuspended in 95:5:0.1 hexane:dichloromethane:actic acid for analysis. Triacylglycerols (TAG) were analyzed by a Sciex API 2000 triple quadrupole mass spectrometer. TAG species were separated by reverse phase chromatography (Phenomenex C8,

using Solvent A - Acetonitrile/water (60/40) with 10 mM ammonium acetate, Solvent B – Isopropanol/Acetonitrile (900/100) with 10 mM ammonium acetate. Standard curves were generated with reference standards combined with the same quantity of internal standard cocktail added to samples upon extraction. Concentration was determined by comparing ratios of unknowns to internal standards, and compared to standard curves representing typical lipid species. TAG species were quantified using MultiQuant software (Sciex, Framingham, MA).

Statistical Analysis

The acute effect of exercise on ACSL abundance for the five known ACSLs was assessed using a one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison test to compare post-exercise measures with rest. The correlation between ASCL1 abundance and whole-body fat oxidation at rest and exercise as well as the abundance of ACSL6 and TAG abundance at rest were analyzed using Pearson's correlation. Statistical significance was set to be $P \leq 0.05$. The data are presented as means and standard deviations.

Results

Participant Characteristics

Participant characteristics are presented in Table 1. Participants were younger adults (28 ± 7 years of age) and were relatively lean (BMI 22 ± 2 kg/m^2). All participants were able to complete the 1-hour exercise training session at the target intensity (63 ± 3% VO_2max). Resting skeletal muscle TAG concentration was 31.82 ± 26.15 pmol/ug.

Table 1. Participant characteristics and measures of fat oxidation and fat storage

Variable	Mean ± SD	Range
Age (years)	28 ± 7	19 – 44
BMI (kg/m^2)	22 ± 2	19 – 26
Waist circumference (cm)	77 ± 8	67 – 93
Systolic blood pressure (mmHg)	113 ± 9	99 – 127
Diastolic blood pressure (mmHg)	74 ± 7	64 – 90
Body mass (kg)	61.6 ± 10.3	49.4 – 86.3
Fat free mass (kg)	44.9 ± 8.2	34.3 – 62.9
Fat mass (kg)	16.6 ± 4.4	8.4 – 24.0
Body fat (%)	27.0 ± 5.3	13.0 – 34.4
Absolute VO_2max (L/min)	2.01 ± 0.35	1.44 – 2.82
Relative VO_2max (ml/kg/min)	32.2 ± 4.5	23.6 – 40.0
Basal RER	0.78 ± 0.08	0.70 – 0.85
Exercise RER	0.94 ± 0.04	0.88 – 0.97
Basal fat oxidation (μmol FA/kg/min)	4.37 ± 0.95	2.87 – 6.43
Exercise fat oxidation (μmol FA/kg/min)	8.26 ± 3.65	2.96 – 14.81
Skeletal muscle triacylglycerol (pmol/μg)	31.8 ± 26.2	7.9 – 101.9

Data are presented as mean ± SD (standard deviation). RER, respiratory exchange ratio; FA, fatty acid.

Skeletal muscle ACSL protein abundance

The first purpose of this study was to determine what ACSL isoforms are expressed in human skeletal muscle. Four of the five known ACSLs, isoforms 1, 4, 5 and 6 were detected within the vastus lateralis muscle of humans using western blotting. ACSL3 protein content was not detected. Of the 4 detected ACSLs, there was a significant increase in abundance seen in ACSL 5 (P=0.01 vs. Rest) directly after a bout of acute exercise training (at the 9:15 hour), which returned to basal levels at 2 hours post-exercise. All other detected ACSLs did not have significant changes with acute aerobic exercise. Taken together, 4 of the 5 known ACSL proteins were readily detected in human skeletal muscle and acute exercise did not significantly alter ACSL protein abundance aside from transient changes in ACSL5 abundance.

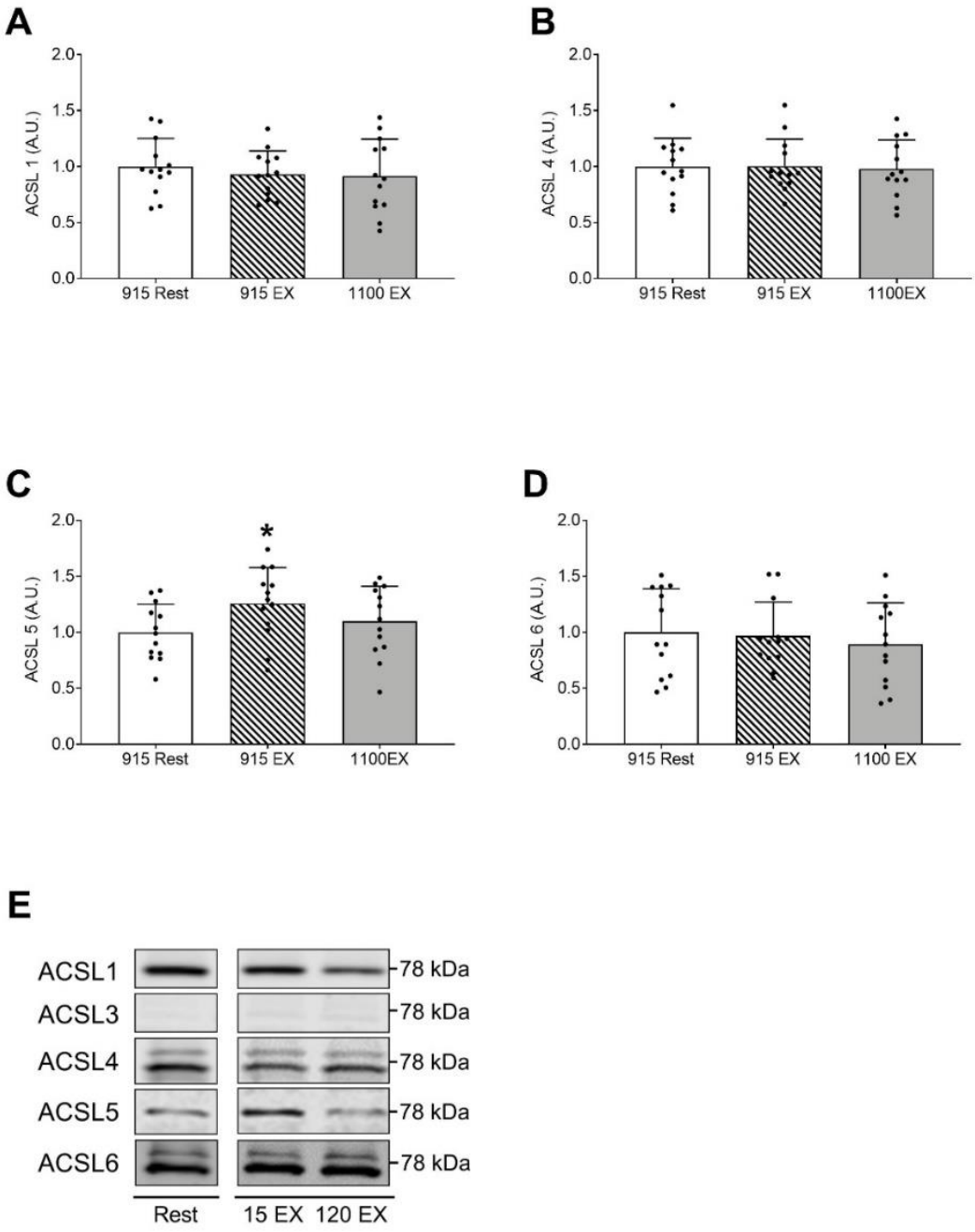

Figure 3: ACSL detected isoform abundance at rest and after exercise. **A.** ACSL 1 protein abundance at rest and after exercise. **B.** ACSL4 protein abundance at rest and after exercise. **C.** ACSL5 protein abundance at rest and after exercise. **D**. ACSL6 protein abundance at rest and after exercise. Statistically significant (annotated by *) increase in protein abundance with exercise was seen with ACSL5 (P=0.01 vs. Rest). Data are represented by means and standard deviation. Number of participants: n=14, including 10 females and 4 males.

Relationship between ACSL 1 and 6 with measures of fat metabolism

The second purpose of this study was to determine the relationship between ACSL abundance and measures of fat oxidation and fat storage. Comparisons were determined prior to the experiment based on evidence presented in literature indicating a role of ACSLs in fat metabolism (2,4). Skeletal muscle ACSL1 was not related to measures of whole-body fat oxidation at rest; however, whole-body fat oxidation measured during acute exercise tended to be related to ACSL1 protein abundance (P=0.07). Additionally, skeletal muscle TAG concentration (i.e., fat storage) was related to ACSL6 protein abundance at rest (P=0.05, r=0.57). These relationships suggest a connection between ACSL6 and fat storage as well as between ACSL1 and whole-body fat oxidation during a bout of acute exercise.

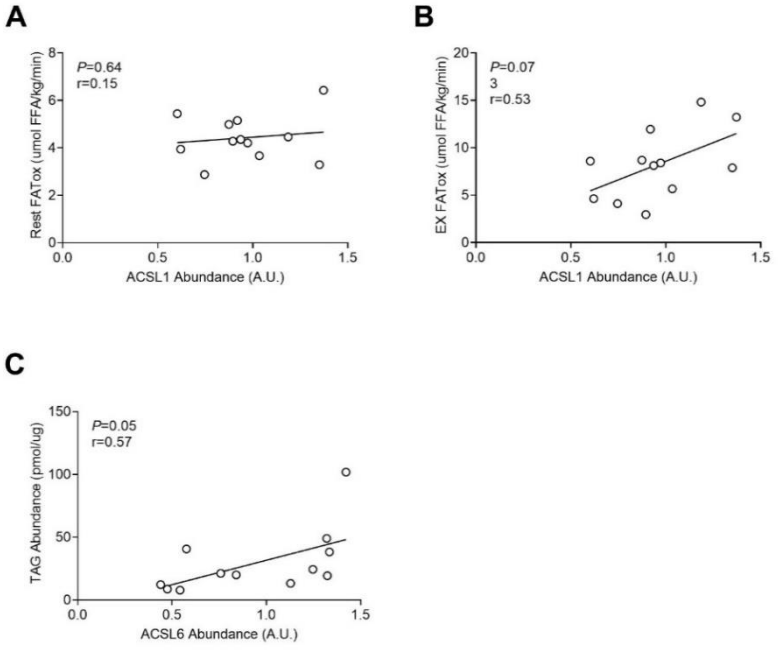

Figure 4: Correlations of ACSL1 and 6 abundance with measures of fat metabolism. **A.** Rest FATox = whole-body fat oxidation at rest. Relationship between ACSL1 protein abundance at rest and fat oxidation at rest (P=0.6). **B.** EX FATox = whole-body fat oxidation during exercise. Relationship between ACSL1 abundance at rest and fat oxidation during exercise (P=0.07). **C.** TAG = Triacylglycerols. Statistically significant relationship between ACSL 6 protein abundance at rest and TAG abundance at rest (P= 0.05).

Discussion:

The purpose of this study was to first determine ACSL isoform protein abundance within human skeletal muscle and understand how acute exercise effects ACSL isoform protein abundance. ACSL abundance was measured at rest and after an acute bout of aerobic exercise training. ASCL protein abundance was correlated with measures of whole-body fat oxidation calculated from indirect calorimetry measures. We were able to detect four of the five ACSLs, with ACSL3 below the limit of detection. We observed transient changes in ACSL5 abundance after exercise, while all other isoforms remained unchanged after exercise. ACSL1 protein abundance tended to be related to whole-body fat oxidation during exercise and ACSL6 protein abundance was related to skeletal muscle TAG abundance at rest. We interpret these data to indicate ACSL1 is related to fat oxidation, while ACSL 6 is related to fat storage in humans.

ACSLs are of interest for their possible regulation of lipid metabolism (2,3). Exercise has the ability to increase lipid oxidation. We show through indirect calorimetry measures that at 65% of VO_2max, there is a significant correlation between lipid metabolism and ACSL1 abundance. Our lab has previously shown the presence of ACSL protein in the skeletal muscle of mice, including isoforms 1, 4, 5, and 6 (4). That study also showed that isoforms 4 and 6 were significantly impacted by exercise interventions. Our study provides new information on ACSL protein abundance in human skeletal muscle and the effects of exercise on isoform abundance. We show that ACSL5 protein abundance is significantly increased by acute exercise in human skeletal muscle, while the other 3 detected isoforms were not. This study also showed that ACSL 6 protein abundance is related to TAG content at rest.

We were able to detect all but 1 ACSL isoform, ACSL3. It is known that ACSL3 is found in the endoplasmic reticulum as well as lipid droplets (15). The manufacture of the ACSL3 antibody showed ACSL3 signaling in C2C12 cells. Thus, ACSL 3 may have been undetectable

in human skeletal muscle due possibly to technical limitations of the antibodies used to detect ACSL3. These data show that ACSL1, 4, 5 and 6 isoforms are detectable in human skeletal muscle and ACSL3 may be detectable without technical limitations.

We anticipated potential changes in ACSL protein abundance after exercise for various reasons. We expected ACSL1 protein abundance to increase with acute aerobic exercise through activation of peroxisome proliferative- activated receptor alpha (PPAR-alpha). PPAR-alpha is a known regulator of ACSL1 transcription and has been shown to increase in response to exercise (13,14). Our results were coherent with previous studies that have shown similar data in skeletal muscle of mice (4,12). This may be due to complex regulation of PPAR-alpha with exercise or suggests that exercise may affect ACSL1 independent of PPAR-alpha. We also expected ACSL6 abundance may increase following exercise due to the link between ACSL 6 and fat storage and evidence indicating increased skeletal muscle lipid content in response to acute exercise (3,11). We did not detect any change in ACSL 6 protein abundance with exercise, which may have been due to testing lipid content only up to 2 hours post exercise.

We hypothesized that skeletal muscle ACSL 1 protein abundance may be related to measures of whole-body fat oxidation based on previous literature showing a 60-85% decrease in skeletal muscle fat oxidation in the absence of ACSL 1 (2). Based on our findings that ACSL1 tends to be correlated with lipid oxidation, ACSL1 has the potential to be a regulator of fat oxidation during a bout of acute exercise (P=0.07). We also hypothesized that skeletal muscle ACSL 6 may be related to TAG content given that myotubes lacking ACSL 6 have diminished fat storage (3). In further support of this hypothesis, we identified a significant relationship between ACSL 6 protein abundance and TAG content, indicating ACSL 6 may facilitate fat storage. This finding is supported by our lab's previous research that shows that intramyocellular

lipid content (IMCL) was positively correlated with ACSL6 protein abundance in the skeletal muscle of mice (4). Taken together, our results indicate ACSL1 and ACSL6 may have an effect on fat metabolism in human skeletal muscle.

Some important considerations of this study are the measures of fat metabolism. We used whole-body fat oxidation measures for this study. While muscle can account for a significant portion of whole-body fat metabolism, these measures are not solely reflective of skeletal muscle fat oxidation; this may have contributed to the lack of a relationship between skeletal muscle ACSL 1 protein abundance and fat-oxidation at rest. It is also important to consider that protein abundance is not a measure of ACSL activity. While evidence does suggest that exercise increases ACSL activity, further research has shown phosphorylation and acylation sites on ACSL 1 (16, 17). Antibodies for these specific sites are unavailable, which makes studying post translational modifications of great interest because of their possible regulation of enzyme activity that could enhance or inhibit ACSL activity (16). This study utilized acute aerobic exercise interventions, which may have contributed to most ACSL isoforms exhibiting no change with exercise. Our lab has previously conducted a study in mice models that used an 8-week aerobic exercise intervention, 50 minutes per day, 5 days a week (4). With this longer-term exercise, changes in ACSL protein abundance was seen in both 4 and 6 isoforms. However, it is important to acknowledge that acute exercise could affect ACSL isoforms differently than long-term exercise due to factors beyond merely length of training. These findings implicate further studies using longer-term aerobic exercise in human skeletal muscle to observe changes in ACSL protein abundance.

In conclusion, this study fills in gaps in knowledge regarding ACSL abundance and regulation through an acute bout of aerobic exercise training in human skeletal muscle. This

study was completed to further the understanding of which ACSLs are present in human skeletal muscle, how they may be altered by acute exercise, and how the different isoforms may relate to fat oxidation and fat storage. We conclude that ACSL 1, 4, 5, and 6 are readily detectable in human skeletal muscle and largely unchanged in the hours following an acute bout of exercise. We also identify positive relationships between whole-body fat oxidation during acute exercise and skeletal muscle ACSL 1 content and skeletal muscle TAG abundance and ACSL 6 content. We interpret such evidence to indicate ACSL 1 and ACSL 6 may be associated with fat metabolism in skeletal muscle and thus remain of critical interest in order to build further understanding of the regulation of lipid metabolism in human skeletal muscle.

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