AN ABSTRACT OF THE DISSERTATION OF

Harrison D. Stierwalt for the degree of Doctor of Philosophy in Kinesiology presented on May 14, 2020.

Title: Regulation of Skeletal Muscle Insulin Action and Fatty Acid Trafficking with Obesity and Exercise

Abstract approved: _____
Sean A. Newsom Matthew M. Robinson

Altered skeletal muscle fat metabolism is linked to changes in skeletal muscle insulin sensitivity. Whereby the accumulation of bioactive signaling lipids (i.e., diacylglycerols and ceramides) within skeletal muscle are negatively associated with insulin sensitivity and the exercise-induced changes to fatty acid trafficking are related to improved skeletal muscle insulin action. However, the mechanisms that contribute to these observations remain to be completely elucidated. The overall aim of this dissertation was to investigate potential underlying mechanisms that contribute to altered skeletal muscle fat metabolism and/or insulin sensitivity in response to lipid/obesity-induced insulin resistance, exercise training, and a single session of exercise. The primary mechanisms of focus were the role of skeletal muscle Ras-

related C3 botulinum toxin substrate 1 (Rac1) in insulin- and exercise-stimulated glucose regulation and the contribution of long-chain acyl-coenzyme a synthetases (ACSL) to skeletal muscle fat metabolism.

Rac1 is required for normal insulin-stimulated glucose transporter 4 (GLUT4) translocation and evidence suggest it may be negatively regulated by lipids. Therefore, the primary aim of my second chapter was to investigate the potential role for diacylglycerols and ceramides as negative regulators on insulin-stimulated Rac1 activation. Cultured muscle cells were treated overnight with or without fatty acids, one of which is known to induced insulin resistance (i.e., palmitate). Overnight fatty acid treatments were followed by a time course approach to measure insulin-stimulated Rac1 activation (GTP-binding), other components of insulin signaling, and functional output measure of insulin action, GLUT4 translocation. Overnight palmitate treatment resulted accumulation of diacylglycerols and ceramides and almost complete ablation of insulin-stimulated GLUT4 translocation. However, impaired GLUT4 translocation occurred independent of changes to insulin-stimulated Rac1-GTP binding. Phosphorylation of a Rac1 downstream effector protein p21-activated kinase (PAK) was blunted by palmitate treatment as was Akt phosphorylation, which stimulates GLUT4 translocation independent of Rac1. Collectively, we interpret our findings to indicate that palmitate-induced down regulation of PAK1 activation and GLUT4 translocation occur independent to insulin-stimulated Rac1-GTP binding.

Evidence suggest ACSLs may regulate fat oxidation and fat storage within skeletal muscle. Whereby specific skeletal muscle isoform ACSL1 is suggested to contribute to fat oxidation and ACSL6 may play a role in fat storage. However, it is

unknown if ACSLs are regulated by diet-induced obesity and/or aerobic exercise training. The primary aims of my third chapter were to investigate skeletal muscle ACSL isoform protein expression following high fat diet (HFD)-induced obesity and aerobic exercise training, and determine potential roles for ACSL1 and 6 with measures of fat metabolism in mice. In mouse gastrocnemius muscle, protein abundance for 4 of the 5 known ACSL isoforms was detected. HFD-induced obesity resulted in a non-significant increase in ACSL1, significantly greater ASCL6, and no change in ACSL4 or ACSL5. Aerobic exercise training resulted in decreased ACSL4 protein abundance, greater ACSL6, and no changes in ACSL1 or ACSL5. Skeletal muscle ACSL1 protein abundance was not related to measures of whole-body fat oxidation at rest, whereas ACSL6 was positively associated with intramyocellular lipid content. Taken together, we interpret our findings to demonstrate ACSLs undergo isoform specific regulation by diet and exercise and ACSL6 may be a regulator of skeletal muscle fat storage.

Model systems implicate ACSLs as key regulators of skeletal muscle fat oxidation and fat storage; however, such roles remain underexplored in humans. The primary aims of my fourth chapter were to determine the protein expression of ACSL isoforms in skeletal muscle at rest and in response to acute exercise, and identify relationships between skeletal muscle ACSLs and measures of fat metabolism. In vastus lateralis biopsy samples collected from relatively lean sedentary adults, protein abundance for 4 of 5 known ACSL isoforms was detected. ACSL isoforms were largely unaltered by acute exercise aside from a transient increase in ACSL5 15 minutes post-exercise which returned to resting levels by 120 minutes. Skeletal muscle ACSL1 was not related to measures of resting whole-body fat oxidation. ACSL1 did tend to be

positively related to whole-body fat oxidation during exercise, when skeletal muscle a major determinant of whole-body substrate oxidation. Skeletal muscle ACSL6 was positively related to skeletal muscle triacylglycerol concentration suggesting a role in the regulation of fat storage. We interpret our findings to indicate the protein abundance for ACSLs undergo isoform specific regulation by acute exercise and provide further evidence for their role in skeletal muscle fat metabolism in humans.

A single session of exercise improves insulin sensitivity in most adults. Rac1 facilitates GLUT4 translocation and is activated by both insulin and exercise (i.e., mechanical stress). However, it is unknown whether insulin-stimulated Rac1 activation is further enhanced by prior exercise. The primary aims of my fifth chapter were to determine the effects of previous exercise on insulin sensitivity, Rac1 signaling, and other components of insulin signaling in the hours after exercise in relatively lean sedentary adults. A single session of moderate-intensity exercise improved measures of whole-body insulin sensitivity. Observed improvements in insulin sensitivity occurred independent of enhanced insulin-stimulated Rac1 activation, phosphorylation of its downstream effector protein PAK, or insulin-stimulated Akt activation post-exercise. Exercise induces activation of AMPK and its downstream effector protein TBC1D1 and contribute to glucose uptake in the hours after exercise. AMPK-specific activation of TBC1D1 was increased 15 minutes post-exercise and remained elevated at 180 minutes compared with basal measures and the rest trial, which closely coincided with when measures of insulin sensitivity during the steady state of the hyperinsulinemic-euglycemic clamp (i.e., 270-300 minutes post-exercise). Collectively, we interpret our

results to suggest the mechanisms independent of insulin-stimulated Rac1 signaling such as TBC1D1 may contribute to the insulin sensitizing effects of exercise.

This collection of studies indicate that lipid-induced insulin resistance occurs independent to impairments in insulin-stimulated Rac1 activation. Skeletal muscle ACSLs are related to measures of fat metabolism in mice and humans and demonstrate isoform specific regulation by diet-induced obesity and aerobic exercise training in mice, but are largely unaltered by acute exercise in humans. These isoform specific changes in ACSL protein abundance may contribute to the altered skeletal muscle fat metabolism with diet-induced obesity and/or aerobic exercise training. Lastly, the insulin sensitizing effects occur independent to enhanced insulin-stimulated activation of Rac1 post-exercise.

Regulation of Skeletal Muscle Insulin Action and Fatty Acid Trafficking with Obesity and Exercise

by
Harrison D. Stierwalt

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented May 14, 2020
Commencement June 2020

Doctor of Philosophy dissertation of Harrison D. Stierwalt presented on May 14, 2020

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

Harrison D. Stierwalt, Author

ACKNOWLEDGEMENTS

I would like to express the utmost appreciation for Drs. Sean Newsom and Matt Robinson. Their guidance throughout my time here at Oregon State University has helped me go through a tremendous amount of professional and personal growth. I would not be here without their guidance and I truly value them as mentors and friends, thank you. I would like to thank the members of my committee, Drs. Marc Norcross, Chrissa Kioussi, and Urszula Iwaniec for their support, enthusiasm, and for challenging me during my numerous committee meetings. I thank my lab cohort and great friend Sarah Ehrlicher for her help in getting me acquainted with the bench skills I have today and her continued guidance when I need to talk through an idea or troubleshoot a problem. I would also like to thank the newest members of the Translation Metabolism Research Lab, Philip Batterson and Erin McGowan for pushing me to become a better leader. I would like to thank the American College of Sports Medicine regional and national chapters for providing the funds necessary for several of my dissertation chapters. Last but not least, I would like to thank the many undergraduate students for all of their help in my projects and for giving me opportunities to build my leadership.

I would also like to thank all of my friends and family. I would not be here without their constant support in whatever career path I choose. Finally, I would like to thank my partner Kristin Neill and my dog Oliver. I am so happy to have met you here at Oregon State University and you helped push me to get here today and Oliver has provided much needed comic relief in the past year. I am so excited for our next move to Kansas City and for our future together, I love you.

CONTRIBUTION OF AUTHORS

Sean Newsom, Ph.D., Matthew Robinson, Ph.D., and Sarah Ehrlicher, M.S. all contributed to the study design, data acquisition, analysis, interpretation, and manuscript editing for all chapters of my dissertation. Bryan Bergman, Ph.D. contributed to some of the data analysis of chapters two and four. Specific author contribution for the chapters of my dissertation are listed within each respective chapter.

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Chapter 1 – General introduction

Economic burden of type 2 diabetes

The current annual economic burden of diagnosed diabetes is estimated to be over $325 billion dollars in the United States, which does not include costs from more than 7 million undiagnosed individuals or roughly 84 million who demonstrate insulin resistance with prediabetes (American Diabetes Association, 2018). In 2015 the global economic burden of type 2 diabetes was $1.07 trillion and if past trends continue the economic burden is estimated to increase 88% by 2030 (Bommer et al., 2018). Obesity is strongly associated with insulin resistance which is a fundamental aspect of the etiology of type 2 diabetes (DeFronzo & Tripathy, 2009; Rizza, Mandarino, & Gerich, 1981). In the past few decades, obesity has been steadily increasing in the United States. The center for disease control and prevention reported in 2018 that all U.S. territories had at least a 20% prevalence of obesity. Therefore, there is a high demand for therapeutic interventions to prevent or slow the progression of insulin resistance to type 2 diabetes. Insulin resistance is characterized by the impaired ability for insulin to stimulate glucose uptake into skeletal muscle (Rizza et al., 1981). Skeletal muscle accounts for up to 85% of glucose uptake in the post prandial state (DeFronzo & Tripathy, 2009) making it a critical regulator for glucose homeostasis and a primary opportunity for therapeutic intervention.

Mechanisms of insulin-stimulated glucose uptake

The skeletal muscle insulin signaling cascades that contribute to glucose uptake in response to insulin are summarized in Figure 1.1.

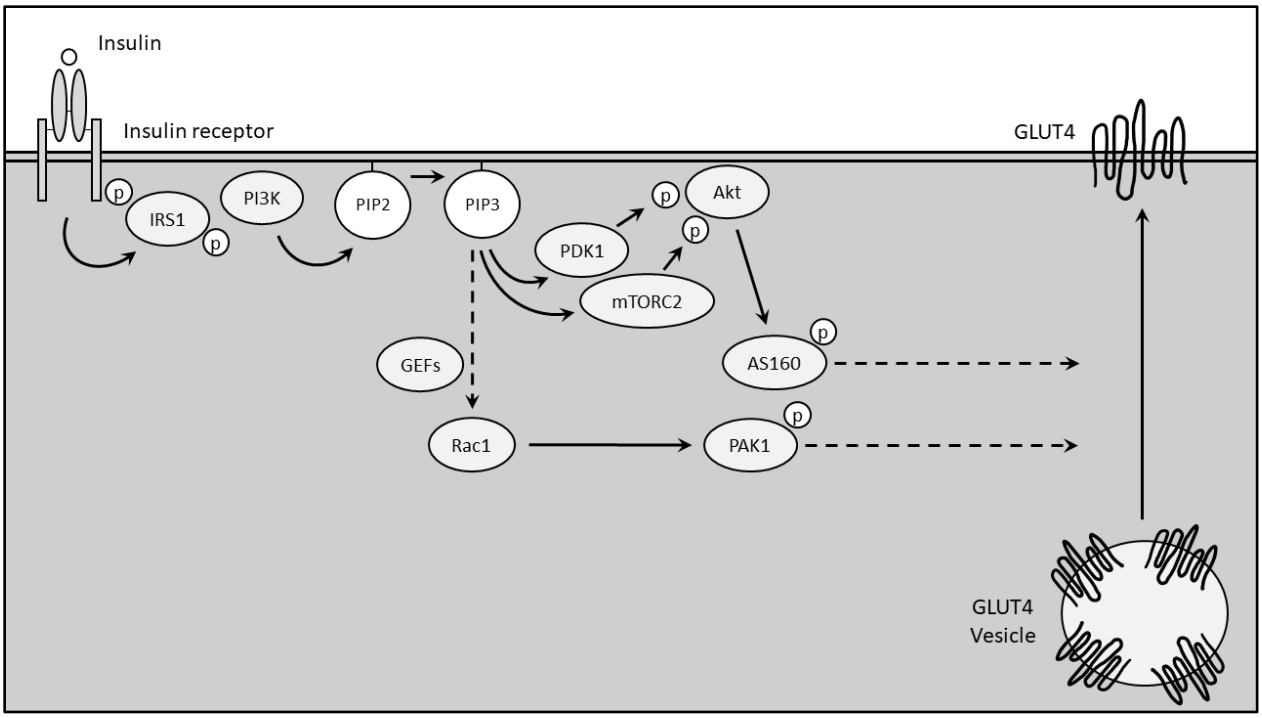

Figure 1.1 Skeletal muscle insulin signaling.

Mechanisms that contribute to insulin-stimulated glucose transporter 4 (GLUT4) translocation within skeletal muscle. Dashed lines represent unknown/abbreviated mechanisms. insulin receptor substrate 1, IRS1; phosphatidylinositol 3-kinase, PI3K; phosphatidylinositol 4,5-biphosphate, PIP2; phosphatidylinositol 3,4,5-triphosphate, PIP3; phosphatidylinositol-dependent kinase 1, PDK1; mTOR complex 2, mTORC2; protein kinase B, Akt; Akt substrate of 160 kDa, AS160; guanine nucleotide exchange factor, GEFs; Ras-related C3 botulinum toxin substrate 1, Rac1; p21-activated kinase, PAK1.

Insulin induces autophosphorylation through the binding of its receptor leading to the recruitment and phosphorylation of insulin receptor substrate 1 (IRS-1). IRS-1 activation recruits phosphatidylinositol 3-kinase (PI3K) resulting in activation which then phosphorylates phosphatidylinositol 4,5-biphosphate (PIP_2) to form phosphatidylinositol 3,4,5-triphosphate (PIP_3) within the cell membrane(Klip, Sun, Chiu, & Foley, 2014). Altering the plasma membrane upregulates at least two signaling cascades which

include serine/threonine kinase Akt and the Rho-family GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) (Klip et al., 2014). Activation of both signaling cascades is required for normal translocation of glucose transporter protein 4 (GLUT4) in response to insulin (Chiu, Jensen, Sylow, Richter, & Klip, 2011; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a).

Insulin signaling events involving Akt and Rac1 ultimately promote GLUT4 translocation (i.e., exocytosis) to the sarcolemmal membrane (Chiu et al., 2011; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). The independence of these signaling cascades have been previously demonstrated in which the downregulation of one (Akt or Rac1) impairs insulin-stimulated glucose uptake without affecting the activation of the other (e.g., inhibition of Akt impairs glucose uptake with no impairments to Rac1 activation or actin filament remodeling) (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). The production of PIP_3 through phosphorylation by PI3K recruits Akt resulting in its activation. Akt is activated through phosphorylation by phosphatidylinositol-dependent kinase 1 (PDK1) and mTOR complex 2 (mTORC2). Activated Akt phosphorylates Akt substrate of 160 kDa (AS160) to facilitate GLUT4 translocation (Klip et al., 2014). In parallel with Akt activation, insulin activates Rac1, a regulator of actin filament remodeling at the sarcolemma (Ueda et al., 2010). Insulin activates guanine nucleotide exchange factors (GEFs) which activate Rac1 through the exchange of GDP for GTP. Activated GTP-bound Rac1 activates p21-activated kinase (PAK) through allosteric binding resulting in its autophosphorylation and increased activity (Lei et al., 2000). PAK then contributes to cortical actin remodeling near the sarcolemmal membrane to facilitate GLUT4 translocation (Sylow,

Jensen, Kleinert, Højlund, et al., 2013a; Tunduguru et al., 2014). In summary, insulin-stimulated glucose uptake involves many “players” required for complete GLUT4 translocation and there is evidence to suggest that obesity-induced impairments to insulin signaling may be due, in part, to impairments in these signaling cascades (Brozinick, Roberts, & Dohm, 2003; Krook, Roth, Jiang, Zierath, & Wallberg-Henriksson, 1998; Sylow, Jensen, Kleinert, Højlund, et al., 2013a).

Aim 1: Identify mechanisms of lipid-induced impairments to skeletal muscle insulin signaling

Obesity is associated with ectopic lipid accumulation and insulin resistance (Goodpaster, He, Watkins, & Kelley, 2001). Historically, obesity-induced insulin resistance was first associated with elevated levels of intramyocellular lipids (IMCL) (Goodpaster et al., 2001). The contribution of skeletal muscle lipids in the etiology of insulin-resistance has progressed over the past few decades from total IMCL content to specific classes, individual species, and the localization of lipids within skeletal muscle (Bergman et al., 2016; Bergman, Hunerdosse, Kerege, Playdon, & Perreault, 2012; Perreault et al., 2018; Szendroedi et al., 2014). Diacylglycerol (DAG) and ceramide are lipids that have been directly implicated in impaired insulin signaling (Chung, Koutsari, Blachnio-Zabielska, Hames, & Jensen, 2017; Perreault et al., 2018; Szendroedi et al.,

2014). Some of the identified and proposed mechanisms of DAG and ceramide induced impairments to skeletal muscle insulin signaling are summarized in Figure 1.2.

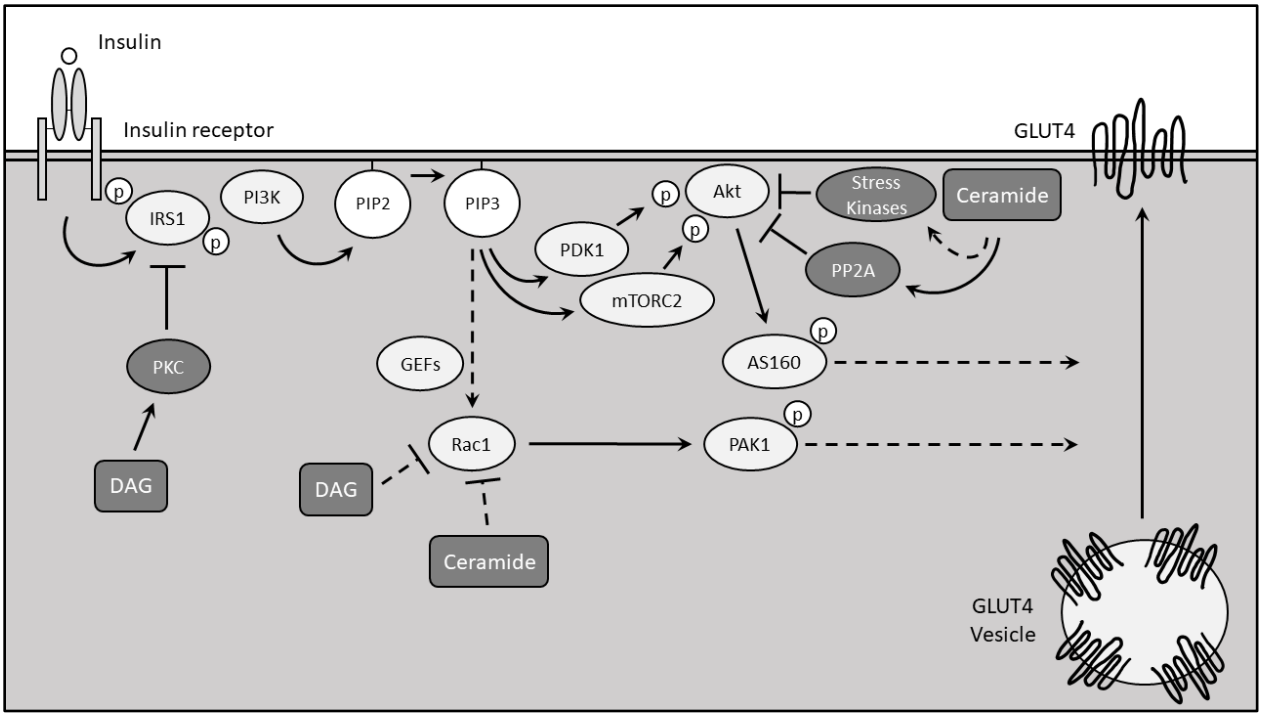

Figure 1.2: Lipid induced downregulation of skeletal muscle insulin signaling.

Known and proposed mechanisms of lipid induced impairments to insulin signaling that ultimately impair glucose transporter 4 (GLUT4) translocation in response to insulin within skeletal muscle. Dashed lines represent unknown/abbreviated mechanisms. insulin receptor substrate 1, IRS1; phosphatidylinositol 3-kinase, PI3K; phosphatidylinositol 4,5-biphosphate, PIP_2; phosphatidylinositol 3,4,5-triphosphate, PIP_3; phosphatidylinositol-dependent kinase 1, PDK1; mTOR complex 2, mTORC2; protein kinase B, Akt; Akt substrate of 160 kDa, AS160; guanine nucleotide exchange factor, GEFs; Ras-related C3 botulinum toxin substrate 1, Rac1; p21-activated kinase, PAK1; diacylglycerol, DAG; protein kinase C, PKC; protein phosphatase 2A, PP2A.

Briefly, skeletal muscle DAG accumulation has been linked to the activation of protein kinase C through allosteric binding of a C1 domain which then contributes to the inhibition of proximal insulin signaling at the insulin receptor and IRS-1 (Szendroedi et al., 2014). Elevated levels of skeletal muscle ceramide have been reported to result in activation of stress kinase signaling (Holland et al., 2011) and protein phosphatase 2A (PP2A) (Stratford, Hoehn, Liu, & Summers, 2004), both of which can impair insulin

signaling at Akt (Holland et al., 2011; Schubert, Scheid, & Duronio, 2000). Rac1 activation may also be impaired with obesity as evidenced through blunted measures of PAK1 activation, a downstream effector of Rac1 (Sylow, Jensen, Kleinert, Højlund, et al., 2013a). Numerous upstream regulators and downstream effectors of Rac1 exhibit C1 binding domains, providing a potential mechanism by which DAG (Colon Gonzalez & Kazanietz, 2006) and/or ceramide (Yin et al., 2009) may alter these proteins and thereby Rac1 signaling. Indeed, there is evidence in neuronal tissue that demonstrate DAG-dependent downregulation of Rac1 activation (H. Wang & Kazanietz, 2006) and incubation of cultured muscle cells in ceramide C2 analog impairs Rac1 GTP-binding (JeBailey et al., 2007). However, the mechanisms of impaired Rac1 signaling with obesity remain poorly understood. We hypothesize that skeletal muscle DAG and ceramide accumulation will be associated with impaired Rac1-GTP binding.

The primary aim of this study is to identify skeletal muscle lipids as potential negative regulators of Rac1 activation. To achieve this aim, insulin-stimulated Rac1 activation will be measured in cultured muscle cells following an overnight incubation with and without fatty acids (e.g., palmitate and oleate), a common model for lipid induced insulin resistance *in vitro* (Coll et al., 2008; Dimopoulos, Watson, Sakamoto, & Hundal, 2006; D. Gao, Griffiths, & Bailey, 2009; Nieuwoudt et al., 2017). We will also measure phosphorylation of PAK (a downstream effector of Rac1) and a functional output of insulin-stimulation via GLUT4 translocation. It is expected that overnight fatty acid treatment will be sufficient to induce significant accumulation of skeletal muscle DAG and ceramide. We further expect that the accumulation of lipids will be associated with blunted insulin-stimulated GLUT4 translocation which is, in part, due to impaired

activation of Rac1 and its downstream effector PAK. Such findings will be interpreted as evidence that elevated skeletal muscle DAG and ceramide act as negative regulators for insulin-stimulated Rac1 activation.

Aim 2: Identify the effects of diet-induced obesity and exercise training on skeletal muscle long chain acyl-CoA synthetases

Both obesity and aerobic exercise training have been reported to alter skeletal muscle lipid accumulation and fat oxidation (Dasari, Newsom, Ehrlicher, Stierwalt, & Robinson, 2018; Goodpaster et al., 2001; Kawanishi et al., 2018; Newsom et al., 2017). Briefly, our lab has demonstrated greater fat oxidation with diet induced obesity in mice despite becoming insulin resistant (Newsom et al., 2017), whereas others have demonstrated greater lipid accumulation with obesity (Itani, Ruderman, Schmieder, & Boden, 2002; Kawanishi et al., 2018; Ritter, Jelenik, & Roden, 2015). Aerobic exercise training known to improve insulin sensitivity has also been reported to result in greater skeletal muscle lipid accumulation (Goodpaster et al., 2001) and mitochondrial oxidation (Holloszy, 1967; 1973). However, the mechanisms involved in the trafficking of fatty acids whether toward skeletal muscle lipid accumulation and/or fat oxidation are not well understood. Long chain acyl-CoA synthetases (ACSL) activate fatty acids to form acyl-CoA prior to further metabolic processes such as oxidation or storage (Grevengoed, Klett, & Coleman, 2014; Mashek, Li, & Coleman, 2007). Recent findings demonstrate that 5 known isoforms of ACSLs are differentially expressed in various metabolic tissues and respond differently to extended fasting suggesting that function may vary by isoform

and/or tissue (Mashek, Li, & Coleman, 2006). In mice, skeletal muscle specific knockdown of ACSL1 resulted in a 60-80% reduction of fat oxidation at rest and exercise intolerance compared with wild type animals suggesting this isoform may be a critical regulator of fat oxidation (Li et al., 2015). Evidence in mice and humans suggests ACSL6 may direct fatty acids toward storage (Teodoro et al., 2017). ACSL6 mRNA expression is greater in the hours following a high-fat meal in both mice and humans (Teodoro et al., 2017). Knockdown of ACSL6 in cultured myotubes resulted in decreased lipid droplet size and triacylglycerol content whereas overexpression resulted in greater phospholipid accumulation and lower fat oxidation (Teodoro et al., 2017). Taken together, these findings indicate ACSL1 and ACSL6 may have distinct roles in skeletal muscle fat oxidation and fat storage, respectively, which are summarized in Figure 1.3.

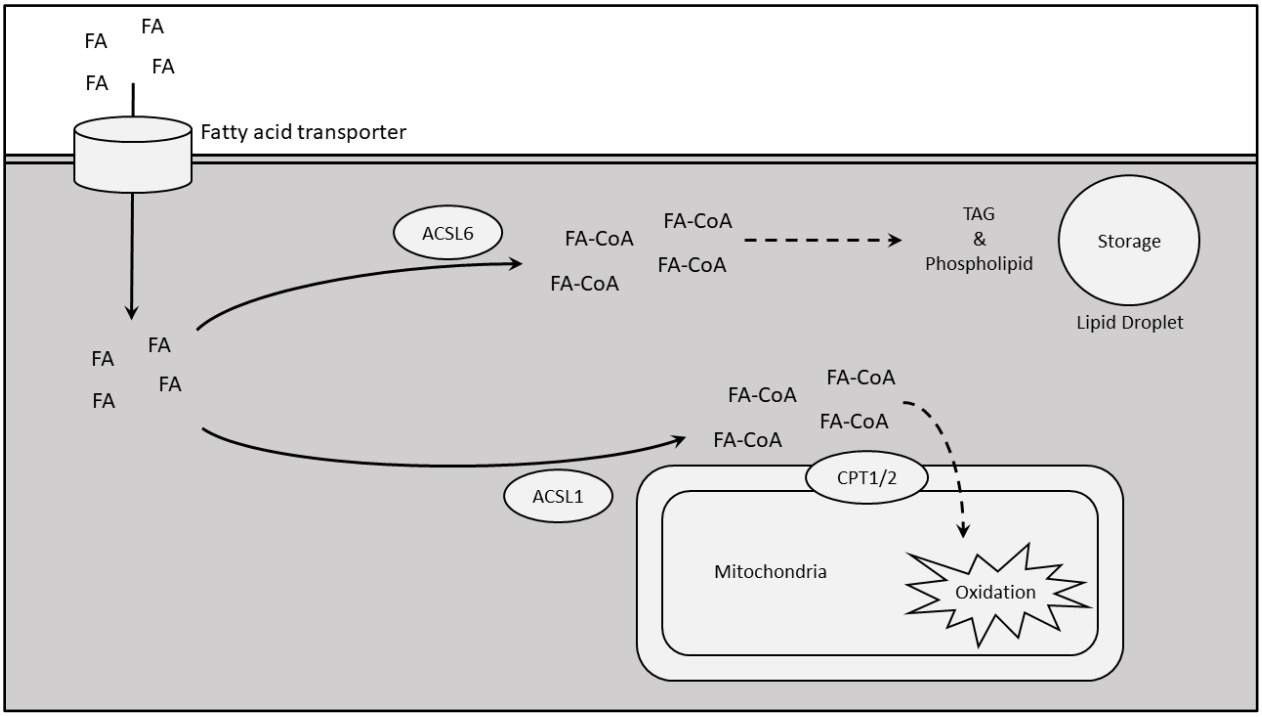

Figure 1.3: Skeletal muscle fatty acid trafficking.

Proposed mechanisms of long chain acyl-CoA synthetase (ACSL) fatty acid (FA) trafficking toward storage or oxidation. Dashed lines represent unknown/abbreviated mechanisms. fatty acyl-CoA, FA-CoA; triacylglycerol, TAG; carnitine palmitoyl transferase, CPT.

Although evidence demonstrates altered skeletal muscle fat oxidation and accumulation with obesity and/or aerobic exercise training, the extent such interventions influence skeletal muscle ACSL isoforms is unknown.

The primary aim of this study is to identify the effects of high fat diet induced obesity and aerobic exercise training on ACSL isoforms and determine potential relationships between ACSL1 and fat oxidation and ACSL6 and fat storage. Following a 4-week diet invention (low fat diet vs. high fat diet), C57BL/6J mice either began exercise training or remained sedentary for 8 additional weeks. During week 10, indirect calorimetry was measured using metabolic cages. At week 12 mice were anesthetized, and tissues were harvested following a 4-hour fast. To achieve this aim IMCL content and protein abundance of skeletal muscle ACSL isoforms will be measured in addition to whole-body fat oxidation. It is expected that aerobic exercise training will result in elevated fat oxidation and IMCL content, both of which will be even greater with high fat diet induced obesity. We further expect that these changes in fat oxidation and IMCL content will be positively associated with greater protein abundance of skeletal muscle ACSL1 and 6. Such findings will be interpreted as evidence that skeletal muscle ACSL1 and 6 are regulators of fatty acid trafficking toward oxidation and storage, respectively.

Aim 3: Identify mechanisms of exercise-induced improvements in skeletal muscle insulin sensitivity and altered fat metabolism

Exercise can improve insulin sensitivity, even from a single bout of low- to moderate-intensity exercise (Newsom, Everett, Hinko, & Horowitz, 2013; Newsom et al., 2010; Schenk & Horowitz, 2007). However, the exercise-induced mechanisms that improve the ability for insulin to stimulate glucose uptake are not completely understood. Identifying such mechanisms may provide promising therapeutic targets for the benefit of human health. Previous findings have demonstrated improvements in insulin-stimulated glucose uptake post-exercise can occur in the absence of changes to proximal insulin signaling (i.e., IRS1 and/or Akt activation) (Funai, Schweitzer, Castorena, Kanzaki, & Cartee, 2010; Goodyear, Giorgino, Balon, Condorelli, & Smith, 1995; Wojtaszewski et al., 2000). These findings provide evidence to suggest mechanisms independent of the canonical insulin signaling cascade may contribute to enhanced insulin sensitivity observed post-exercise. Rac1 is a novel regulator of GLUT4 translocation activated by both muscle contraction (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b) and insulin (Chiu et al., 2011; JeBailey et al., 2007; Sylow, Jensen, Kleinert, Højlund, et al., 2013a) through independent mechanisms. However, it is currently unknown whether these independent mechanisms for Rac1 activation may be additive, resulting in enhanced insulin-stimulated Rac1 activation post-exercise. We hypothesize that these two independent mechanisms of Rac1 activation may provide stepwise increases in activation via GTP-binding contributing to enhanced insulin-stimulated glucose uptake post-exercise.

Altered trafficking of fatty acids following exercise may also contribute to post-exercise improvements in insulin sensitivity. For example, a single session of exercise enhanced insulin sensitivity following overnight lipid infusion compared with rest (Schenk & Horowitz, 2007), an intervention known to induce insulin resistance (Bachmann et al., 2001). Improved insulin sensitivity post-exercise was also accompanied by greater intramuscular triacylglycerol synthesis (IMTG) and lower accumulation of DAG and ceramide compared with sedentary rest (Schenk & Horowitz, 2007). The partitioning of fatty acids toward oxidation (Kimber, Heigenhauser, Spriet, & Dyck, 2003; Votruba, Atkinson, & Schoeller, 2003) and intramuscular triacylglycerol (IMTG) storage (Décombaz et al., 2001; Ikeda et al., 2002; Schenk & Horowitz, 2007) have been evidenced to be greater following a single bout of exercise. Greater fatty acid partitioning toward oxidation or storage may limit the accumulation of skeletal muscle DAG and/or ceramide and thereby limiting impairments to insulin signaling. However, the mechanisms that contribute to increased trafficking of fatty acids toward oxidation or storage post-exercise remain to be completely understood. As described in aim 2, recent evidence suggests that ACSL isoforms 1 and 6 may act as mechanisms that direct fatty acids toward oxidation and storage but their response to a single bout of exercise in humans is unknown (Li et al., 2015; Teodoro et al., 2017). We hypothesize that ACSL 1 and 6 are upregulated following a single session of exercise and thereby contribute to increased fatty acid trafficking toward oxidation and/or storage, respectively.

The primary aim of this study is to identify potential mechanisms of exercise-induced improvements in insulin sensitivity and altered fat metabolism. In order to

achieve this aim, we will measure insulin sensitivity via hyperinsulinemic-euglycemic clamp, Rac1 activation and total protein abundance of ACSL isoforms following an acute bout of moderate-intensity exercise (65% VO_2max) compared with sedentary rest in humans. It is expected that a single bout of moderate-intensity exercise will be sufficient to enhance insulin sensitivity compared with rest. We further expect that exercise will enhance insulin-stimulated Rac1-GTP binding and upregulate protein abundance of ACSL 1 and 6. These findings will be interpreted as evidence that enhanced Rac1 activation and/or upregulation of ACSL-dependent fatty acid trafficking contribute to improved insulin sensitivity in the hours after a single session of exercise.

Significance of proposed studies

Identifying the maladaptive mechanisms that contribute to insulin resistance may provide insight into targets that could be used for therapeutic intervention against the rising prevalence of obesity. There is evidence that skeletal muscle lipid accumulation, commonly observed with obesity, contributes to impairments in insulin signaling; however, mechanisms have yet to be completely elucidated. Exercise training and even a single bout of exercise are capable of reversing the detriments of insulin resistance. Further elucidating the mechanisms that contribute to improved insulin sensitivity post-exercise may identify targets for therapeutic intervention and there is evidence to suggest that altered fatty acid trafficking and lipid accumulation within skeletal muscle may impact our sensitivity to insulin through altered insulin signaling. It is expected that

the proposed experiments will identify mechanisms of development and reversal of skeletal muscle insulin resistance.

Chapter 2 – Insulin-stimulated Rac1-GTP binding is not impaired by palmitate treatment in L6 myotubes

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Physiological Reports

30 Farringdon Ln, Farringdon, London EC1R 3AX, United Kingdom

Volume 6, Issue 24, December 2018

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Title (50-word max): 12
Abstract (300 max): 243
Word count: 3227

Keywords: Rac1, PAK, skeletal muscle, insulin signaling

Abstract

Ras-related C3 botulinum toxin substrate 1 (Rac1) is required for normal insulin-stimulated glucose transport in skeletal muscle and evidence indicates Rac1 may be negatively regulated by lipids. We investigated if insulin-stimulated activation of Rac1 (i.e., Rac1-GTP binding) is impaired by accumulation of diacylglycerols (DAG) and ceramides in cultured muscle cells. Treating L6 myotubes with 100 nM insulin resulted in increased Rac1-GTP binding that was rapid (occurring within 2 minutes), relatively modest (+38 ± 19% vs. basal, $P<0.001$), and short-lived, returning to near-basal levels within 15 minutes of continuous treatment. Incubating L6 myotubes overnight in 500 µM palmitate increased accumulation of DAG and ceramides ($P<0.05$ vs. no fatty acid control). Despite significant accumulation of lipids, insulin-stimulated Rac1-GTP binding was not impaired during palmitate treatment ($P=0.39$ vs. no fatty acid control). Nevertheless, phosphorylation of Rac1 effector protein p21-activated kinase (PAK) was attenuated in response to palmitate treatment ($P=0.02$ vs. no fatty acid control). Palmitate treatment also increased inhibitory phosphorylation of insulin receptor substrate-1 and attenuated insulin-stimulated phosphorylation of Akt at both Thr308 and Ser473 (all $P<0.05$ vs. no fatty acid control). Such signaling impairments resulted in near complete inhibition of insulin-stimulated translocation of glucose transporter protein 4 (GLUT4; $P=0.10$ vs. basal during palmitate treatment). In summary, our finding suggest that Rac1 may not undergo negative regulation by DAG or ceramides. We instead provide evidence that attenuated PAK phosphorylation and impaired GLUT4 translocation during palmitate-induced insulin resistance can occur independent of defects in insulin-stimulated Rac1-GTP binding.

Introduction

Obesity is strongly linked with the development of impaired insulin-stimulated glucose uptake in skeletal muscle, which is a risk factor for type 2 diabetes (DeFronzo, 1988). Skeletal muscle accounts for up to 80% of glucose uptake in the postprandial state (DeFronzo & Tripathy, 2009) and thus is a significant regulator of systemic glucose metabolism. It is therefore critical to understand mechanisms underlying skeletal muscle insulin resistance with obesity, including the role for lipids as regulators of skeletal muscle glucose uptake (Bajaj et al., 2005; Frangioudakis & Cooney, 2008; Schenk, Harber, Shrivastava, Burant, & Horowitz, 2009).

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a Rho-family GTPase that is required for normal insulin-stimulated glucose transport in skeletal muscle (Chiu et al., 2011; Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). GTP-bound Rac1 induces reorganization of cortical actin-filaments near the sarcolemmal membrane which then facilitate the translocation of glucose transporter vesicles to the cell surface (JeBailey et al., 2007; Ueda et al., 2010; Ueda, Kataoka, & Satoh, 2008). Insulin-stimulated “release” of GLUT4 vesicles from intracellular compartments requires activation of protein kinase B (Akt) (Eguez et al., 2005; Larance et al., 2005). Evidence indicates both Rac1 and Akt are activated downstream of phosphatidylinositol 3-kinase (PI3K) (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Activation of both Akt and Rac1 is required for normal insulin-stimulated glucose transport, but these pathways may otherwise be independent (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). For example, over-expression of a dominant negative Akt impairs

insulin-stimulated GLUT4 translocation without affecting Rac1 signaling or actin filament reorganization (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Similarly, inducible knockout of skeletal muscle Rac1 attenuates insulin-stimulated GLUT4 translocation without impairing Akt phosphorylation (Raun et al., 2018; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Taken together, Rac1 has emerged as a critical regulator of skeletal muscle glucose uptake.

Skeletal muscle Rac1 signaling was recently shown to be impaired in models of insulin resistance characterized by excess availability of lipids, including obese humans, high fat-fed mice and during acute infusion of lipids (i.e., intralipid) (Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). These studies report Rac1 signaling using phosphorylation of a downstream target, p21 activated kinase (PAK). Direct measures of Rac1 activation (i.e., GTP-binding) are therefore needed to confirm impairment to Rac1 in skeletal muscle insulin resistance. Further, regulatory mechanisms responsible for impaired activation of Rac1 signaling during lipid-induced skeletal muscle insulin resistance remain unclear. An intriguing possibility is that Rac1 may be negatively regulated by the accumulation of intramyocellular lipids. Both diacylglycerols (DAG) and ceramides are implicated in the development of obesity-related insulin resistance (Chung et al., 2017; Perreault et al., 2018; Powell, Turban, Gray, Hajduch, & Hundal, 2004; Ritter et al., 2015), and can alter protein function via allosteric binding of C1 domains (Szendroedi et al., 2014; Yin et al., 2009). C1 domains are common among proteins that regulate Rac1 and other Rho-family GTPases (Colon Gonzalez & Kazanietz, 2006) and may serve as a mechanism for DAG and ceramides to regulate Rac1. Indeed, DAG is known to

negatively regulate Rac1 in non-skeletal muscle tissues (Sosa, Lewin, Choi, Blumberg, & Kazanietz, 2009; H. Wang & Kazanietz, 2006). However, to what extent Rac1-GTP binding may be negatively regulated by accumulation of lipids in skeletal muscle cells remains unclear.

The purpose of this study was to identify a role for lipids as negative regulators of Rac1 in muscle cells. We hypothesized that accumulation of DAG and ceramides in L6 muscle cells would impair insulin-stimulated Rac1-GTP binding, leading to attenuated activation of PAK and decreased GLUT4 translocation. In contrast to our hypothesis, insulin-stimulated Rac1-GTP binding was not impaired in palmitate treated cells despite significant accumulation of DAG and ceramides. Nevertheless, PAK phosphorylation and GLUT4 translocation were attenuated during treatment with palmitate, suggesting that these impairments may occur independent of defects in insulin-stimulated Rac1-GTP binding.

Methods

Cell Culture

L6 myoblasts (CRL-1458; American Type Culture Collection) were grown in high glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (AbAm) in a 5% CO2 humidified atmosphere at 37°C. Cells at ~80-90% confluence were differentiated into myotubes by switching to low glucose (1g/L) DMEM supplemented with 2% horse serum and 1%

AbAm for 5-7 days. Fatty acid treatments were prepared in ethanol and added to 37°C differentiation media supplemented with 2% bovine serum albumin (BSA) and 1 mM carnitine, at a final concentration of 500 µM with 0.5% ethanol. A no fatty acid control treatment consisted of differentiation media supplemented with 2% BSA, 1 mM carnitine and 0.5% ethanol. Myotubes were incubated for 22 hours with palmitate (PALM), oleate (OLEA), or no fatty acid control (CON) treatments, with the final 4 hours of incubation involving serum starvation (i.e., treatment media devoid of horse serum). Insulin treatment consisted of 100 nM insulin for durations ranging from 2-15 minutes, as described for individual experiments. CN04, a pharmacological activator of Rac1 (Cytoskeleton Inc.), was used according to manufacturer recommendations at a concentration of 1 µg/ml for 4 hours. Culture media and supplements were purchased from Gibco and Millipore-Sigma. Details regarding the number of experiments for each analytical method are provided below.

Lipid Analysis

L6 myotubes grown in 10 cm plates were harvested in ddH_2O and transferred to a glass screw cap tube with methanol (MeOH), methyl tert-butyl ether (MTBE) and internal standards. Samples were then vortexed, rotated for 5 minutes at room temperature, and centrifuged at 2,500 x *g* for 5 minutes to separate phases. The upper phase containing lipids was transferred to new glass culture tubes. The lower phase was repeat-extracted with an additional MTBE, MeOH, and acidified H_2O. The combined extracts were dried under nitrogen gas and low heat (~37°C). The total lipid

extract was transferred to autosampler vials using 2:1 chloroform:methanol, then re-dried under nitrogen gas. Lipids were resuspended in 95:5:0.1 hexane:dichloromethane:actic acid for analysis. DAG and ceramide species were analyzed using an Agilent 1100 high performance liquid chromatographer (HPLC) connected to a Sciex API2000 triple quadrupole mass spectrometer (LC-MS/MS). The mass spectrometer was used in multiple reaction monitoring mode. 1,3- and 1,2-DAG isomers were separated chromatographically using a Hilic 2.1 µM, 2.1×100 mm column with a two-stage gradient of mobile phase from 2%B to 64%B (A=isooctane; B=705:20:75, isooctane:acetonitrile:isopropanol). Post column addition of 30 mM ammonium acetate in 95:5 isopropyl alcohol:water was used to promote formation of ammonium adducts of the neutral lipid species in the electrospray ion source of the mass spectrometer. Standard curves were generated with reference standards combined with the same quantity of internal standard cocktail added to samples upon extraction. The concentration of each molecular species in the samples was determined by comparing area ratio obtained by dividing the peak area of analyte by the peak area of its internal standard to standard curves. Lipid species were quantified using MultiQuant software (Sciex). Lipid analysis reflect 2 independent experiments each consisting of n=3-4 for a total sample size of n=7 for all conditions.

Rac1 Activation

Rac1-GTP binding was measured via immunosorbent assay according to manufacturer recommendations (BK128, Cytoskeleton Inc.). Myotubes grown in 6-well

plates were harvested in lysis buffer plus protease inhibitors. Homogenates and lysates were centrifuged for 1 minute at 10,000 × *g* and flash frozen in liquid nitrogen. Sample protein concentrations were equalized and loaded into wells coated with Rac1-GTP binding domain. GTP-bound Rac1 was determined following a colorimetric reaction using antibodies towards Rac1 linked with horseradish peroxidase activity. Rac1 activation analyses during basal, 2, 5, and 10 minutes of insulin stimulation reflect 3 independent experiments each consisting of n=3 for a total sample size of n=9. Insulin-stimulated Rac1 activation at 15 minutes and during CN04 treatment were each assessed during 1 experiment with a sample size of n=3.

GLUT4 Translocation

L6 myotubes with stable transfection of *myc*-tagged GLUT4 (L6-GLUT4*myc*; Kerafast Inc.) were used to determine insulin-stimulated GLUT4 translocation as previously described (Q. Wang, Khayat, Kishi, Ebina, & Klip, 1998). Briefly, L6-GLUT4*myc* myoblasts were grown in minimum essential medium alpha (αMEM) supplemented with 10% FBS and 1% AbAm and differentiated by switching to αMEM supplemented with 2% FBS and 1% AbAm. Fatty acid and control treatments were prepared and executed as described above (see *Cell Culture*) using αMEM media. Latrunculin B (428020, Millipore-Sigma), a pharmacological inhibitor of actin filament remodeling, was prepared in DMSO at provided at a final concentration of 5 µM for 1 hour (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Myotubes were stimulated with 100 nM insulin for 20 minutes. For detection of cell

surface GLUT4*myc*, myotubes were rinsed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS, blocked with 5% goat serum in PBS and incubated with anti-c-myc polyclonal antibody (C3956, Millipore-Sigma). Myotubes were extensively rinsed with PBS before incubation in horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (111-035-114, Jackson ImmunoResearch). Myotubes were rinsed with PBS before o-phenylenediamine reagent (P5412, Millipore-Sigma) was added to each well. The colorimetric reaction was stopped by the addition of 3M HCl. Supernatant was collected and the optical absorbance was measured at 492 nm. GLUT4 translocation analyses reflect 3 independent experiments each consisting of n=3 for a total sample size of n=9 for fatty acid and no fatty acid control conditions. Latrunculin B treatment included 1 experiment for basal conditions and 2 independent experiments during insulin-stimulated conditions, each consisting of n=3 for a total sample size of n=3 and n=6, respectively.

Western Blotting

Western blot analysis was performed on cell lysates as previously described (Newsom et al., 2017). L6 myotubes grown in 6-well plates were harvested in lysis buffer plus protease inhibitors and centrifuged at 10,000 $\times$ *g* for 10 minutes at 4°C with the supernatant stored at -80°C until analysis. Approximately 30 µg protein was separated on bis-tris gels then transferred to nitrocellulose membranes. Each gel was loaded with the same internal control sample in 2 lanes, the average density of both lanes was used to normalize band density between gels. Ponceau staining of

membranes was used to verify equal loading and transfer of protein. Membranes were blocked in 5% bovine serum albumin in tris-buffered saline with tween (TBST) and incubated in primary antibodies at 4°C. Membranes were washed in TBST and incubated in secondary antibody diluted in blocking buffer at room temperature. Images were generated using infrared detection (LI-COR Odyssey). Primary antibodies used included Rac1 (ARC03; Cytoskeleton Inc.) and Akt (2920), pAkt Ser473 (9271), pAkt Thr308 (9275), pPAK1/2 Thr423/Thr402 (2601), PAK1 (2602), pIRS1 Ser1101 (2385), IRS1 (3194), pPDK1 Ser241 (3438), and PDK1 (3062) from Cell Signaling Technology. Secondary antibodies used included anti-rabbit-700 (926-68071) and anti-mouse-800 (926-32212) from LI-COR. Insulin signaling measures via western blotting reflect 3 independent experiments each consisting of n=3 for a total sample size of n=9 for all conditions.

Statistical Analysis

Initial characterization of Rac1-GTP binding was analyzed by one-way ANOVA, with Dunnett's post-hoc analysis comparing insulin-stimulated time points and CN04 treatment to basal. Intracellular lipid abundance was analyzed by one-way ANOVA, with Dunnett's post-hoc analysis comparing fatty acid treatments to the no fatty acid control condition. Insulin signaling outcomes were analyzed by two-way *time x treatment* ANOVA. A main effect of insulin was evaluated by comparing 2, 5, and 10 minutes of insulin-stimulation with basal using Dunnett's analysis. Main effects of fatty acid treatments were evaluated by comparing palmitate and oleate with the no fatty acid

control condition using Dunnett's analysis. Cell-surface GLUT4*myc* during basal and insulin-stimulated conditions was analyzed via unpaired two-tailed students t-test. Statistical significance was set as $P<0.05$. Statistical analysis was performed using Prism version 6 (GraphPad Software). In accordance with the published recommendations (Curran-Everett, 2008), data are presented as mean + standard deviation.

Results

Insulin-stimulated Rac1 activation is short-lived in L6 myotubes

To address our primary aim, we first sought to characterize insulin-stimulated Rac1-GTP binding in L6 myotubes. Incubating myotubes in 100 nM insulin resulted in a rapid increase in GTP binding, occurring within 2 minutes; yet the increase was transient, returning to near-basal levels within 15 minutes of continuous insulin stimulation (Figure 2.1). Further, the insulin-stimulated increase in Rac1-GTP binding was relatively modest (+38 ± 19% vs. basal) compared with pharmacological activation of Rac1 using CN04 (+191 ± 11% vs. basal, Figure 2.1). Together these data indicate

insulin-stimulated Rac1 activation in L6 myotubes is rapid, short-lived and relatively modest.

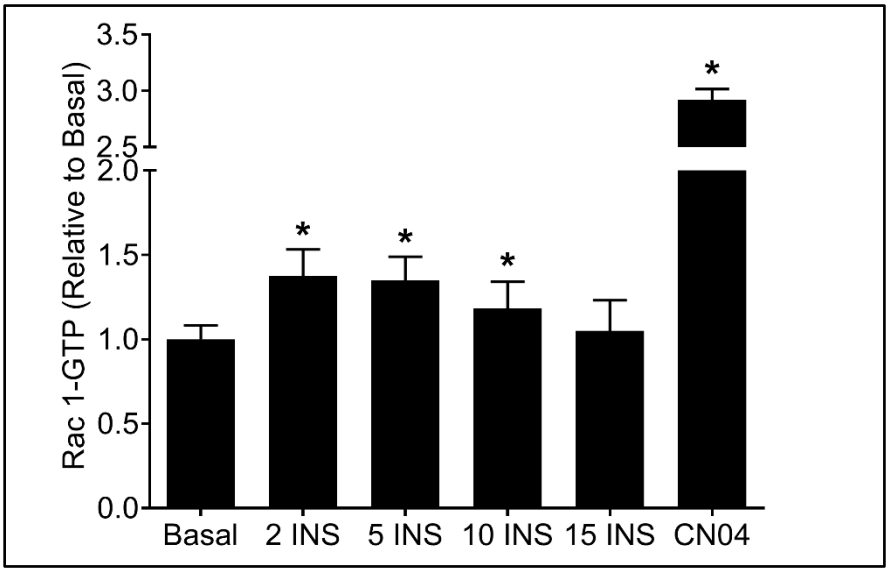

Figure 2.1: Insulin-stimulated Rac1-GTP binding in L6 myotubes.

Rac1-GTP binding was assessed via immunosorbent assay in L6 myotubes under basal serum-free conditions and 100 nM insulin (INS) for 2, 5, 10 and 15 minutes. For comparison, L6 myotubes were incubated for 4 hours with CN04, a pharmacological treatment to maximize Rac1-GTP binding (Lerm, Pop, Fritz, Aktories, & Schmidt, 2002). Rac1-GTP binding was analyzed by one-way ANOVA with Dunnett's post-hoc analysis comparing each time point and CN04. Rac1-GTP content during basal, 2, 5, and 10 minutes of insulin stimulation reflects 3 independent experiments each consisting of n=3 for a total sample size of n=9. Insulin-stimulated Rac1-GTP binding at 15 minutes and during CN04 treatment each reflect 1 experiment with a sample size of n=3. Data are presented as mean + standard deviation. *P<0.05 vs. Basal.

DAG and ceramides accumulate during palmitate treatment in L6 myotubes

We next determined the effect of overnight lipid treatments on accumulation of DAG and ceramides. Both total DAG and ceramide abundance were significantly increased following overnight incubation with palmitate compared with a no fatty acid control condition (Figure 2.2A and 2.3A). Such accumulation of lipids was largely attributable to increased abundance of 1,2- and 1,3-DAG containing C16:0 moieties (Figure 2.2B and 2.2C), as well as increased C16:0, C18:0 and C22:0 ceramide

abundance (Figure 2.3B). Treating myotubes with oleate resulted in no change in total DAG abundance and reduced total ceramide abundance compared with the no fatty acid control condition (Figure 2.2 and 2.3).

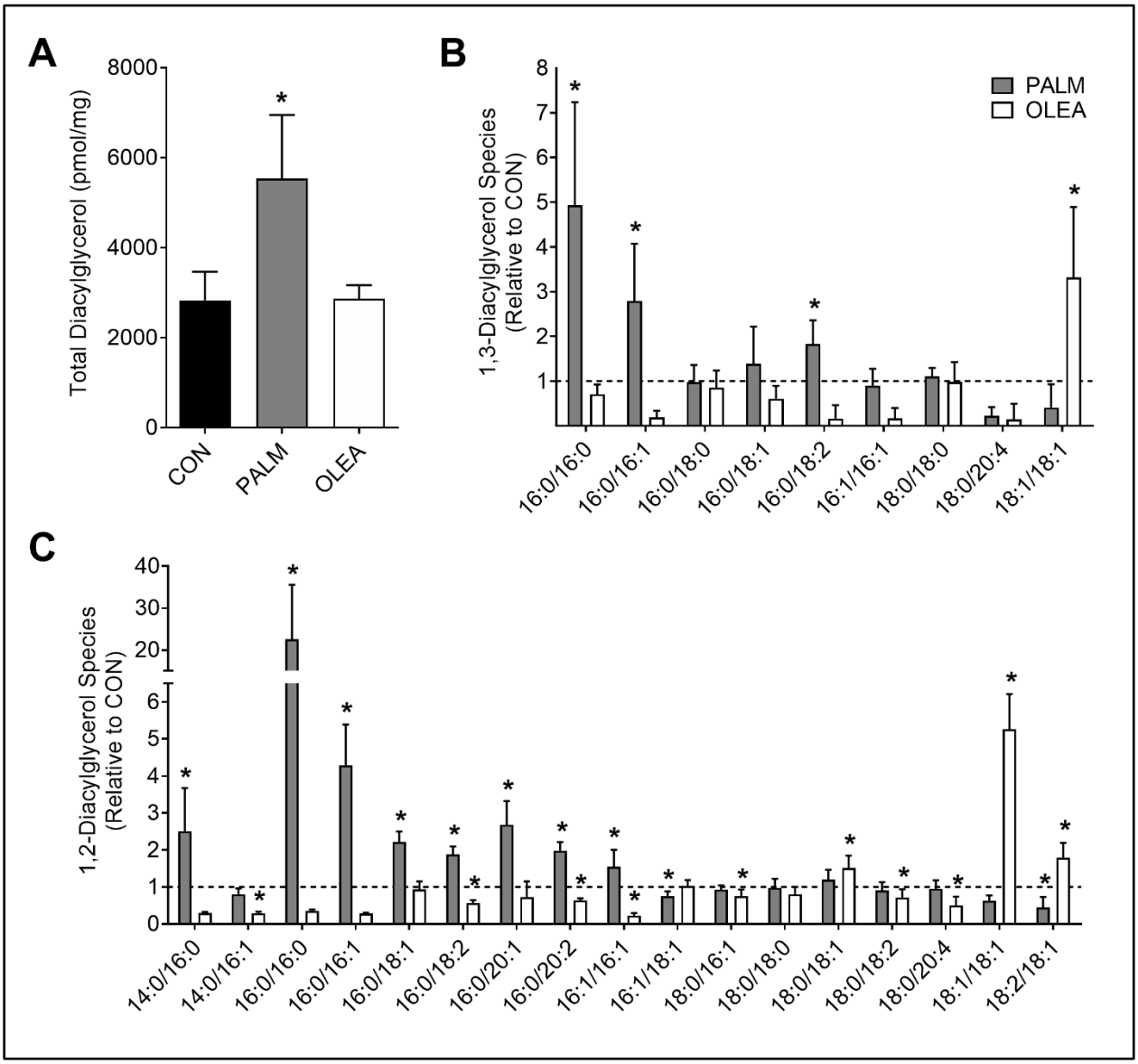

Figure 2.2: Diacylglycerol abundance following overnight fatty acid treatment in L6 myotubes.

Intracellular lipids were assessed in L6 myotubes via targeted liquid chromatography tandem mass spectrometry during no fatty acid control (CON), palmitate (PALM, 500 µM) or oleate (OLEA, 500 µM) treated conditions. **A)** Total intracellular diacylglycerol abundance, **B)** 1,3-diacylglycerol and **C)** 1,2-diacylglycerol species abundance. Diacylglycerol species abundance is presented as PALM and OLEA relative to CON, with the dashed line representing the mean value for CON. Intracellular lipid abundance was analyzed by one-way ANOVA with Dunnett's post-hoc analysis comparing fatty acid treatments to no fatty acid control. Lipid analysis reflects 2 independent experiments each consisting of n=3-4 for a total sample size of n=7 for all conditions. Data are presented as mean + standard deviation. *P<0.05 vs. CON.

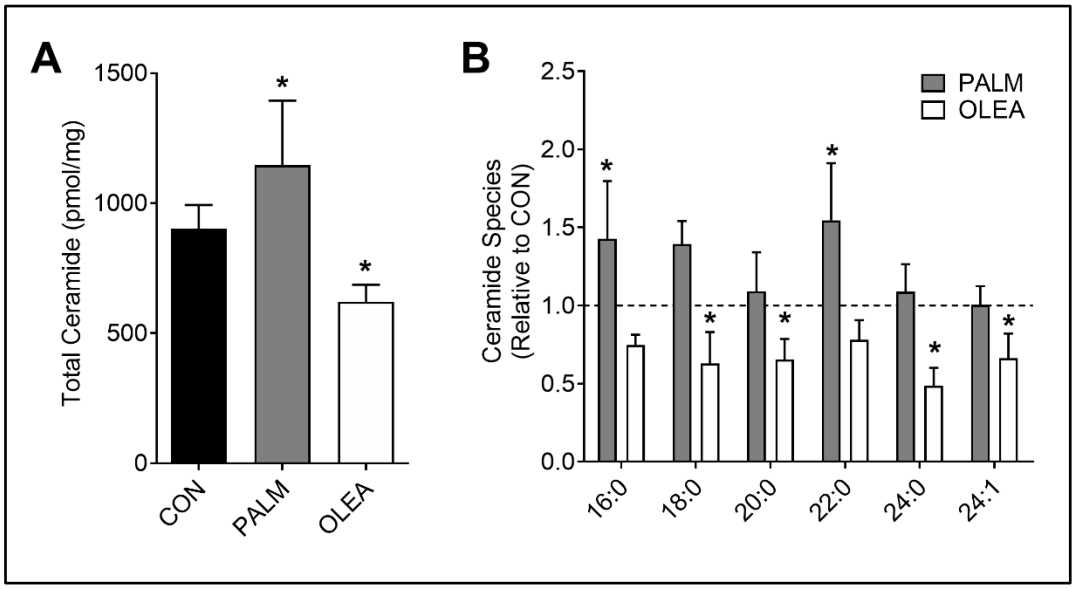

Figure 2.3: Ceramide abundance following overnight fatty acid treatment in L6 myotubes.

Intracellular lipids were assessed in L6 myotubes via targeted liquid chromatography tandem mass spectrometry during no fatty acid control (CON), palmitate (PALM, 500 µM) or oleate (OLEA, 500 µM) treated conditions. A) Total intracellular ceramide abundance and B) ceramide species abundance. Ceramide species abundance is presented as PALM and OLEA relative to CON, with the dashed line representing the mean value for CON. Intracellular lipid abundance was analyzed by one-way ANOVA with Dunnett's post-hoc analysis comparing fatty acid treatments to no fatty acid control. Lipid analysis reflects 2 independent experiments each consisting of n=3-4 for a total sample size of n=7 for all conditions. Data are presented as mean + standard deviation. *P<0.05 vs. CON.

Palmitate does not impair insulin-stimulated Rac1-GTP binding in L6 myotubes

We next determined insulin-stimulated Rac1 activation following overnight fatty acid treatment. Despite robust DAG and ceramide accumulation during palmitate treatment, insulin-stimulated Rac1-GTP binding was not altered compared with no fatty acid control or oleate treatment (Figure 2.4A). Palmitate treatment did, however, increase inhibitory phosphorylation of IRS1 at Ser1101 (*P*=0.02, PALM vs. CON; data not shown) and attenuate insulin-stimulated phosphorylation of Akt at both Thr308 and Ser473 (Figure 2.4C and D). In agreement with other models of lipid-induced insulin

resistance (Sylow, Jensen, Kleinert, Højlund, et al., 2013a), palmitate treatment also blunted insulin-stimulated PAK phosphorylation compared with no fatty acid control (Figure 2.4B). Overnight lipid treatment had no effect on total protein abundance of Rac1, Akt, or PAK1 (Figure 2.4). Together these data indicate insulin-stimulated Rac1-GTP binding is not impaired by the accumulation of DAG and ceramides, yet PAK phosphorylation was attenuated during treatment with palmitate.

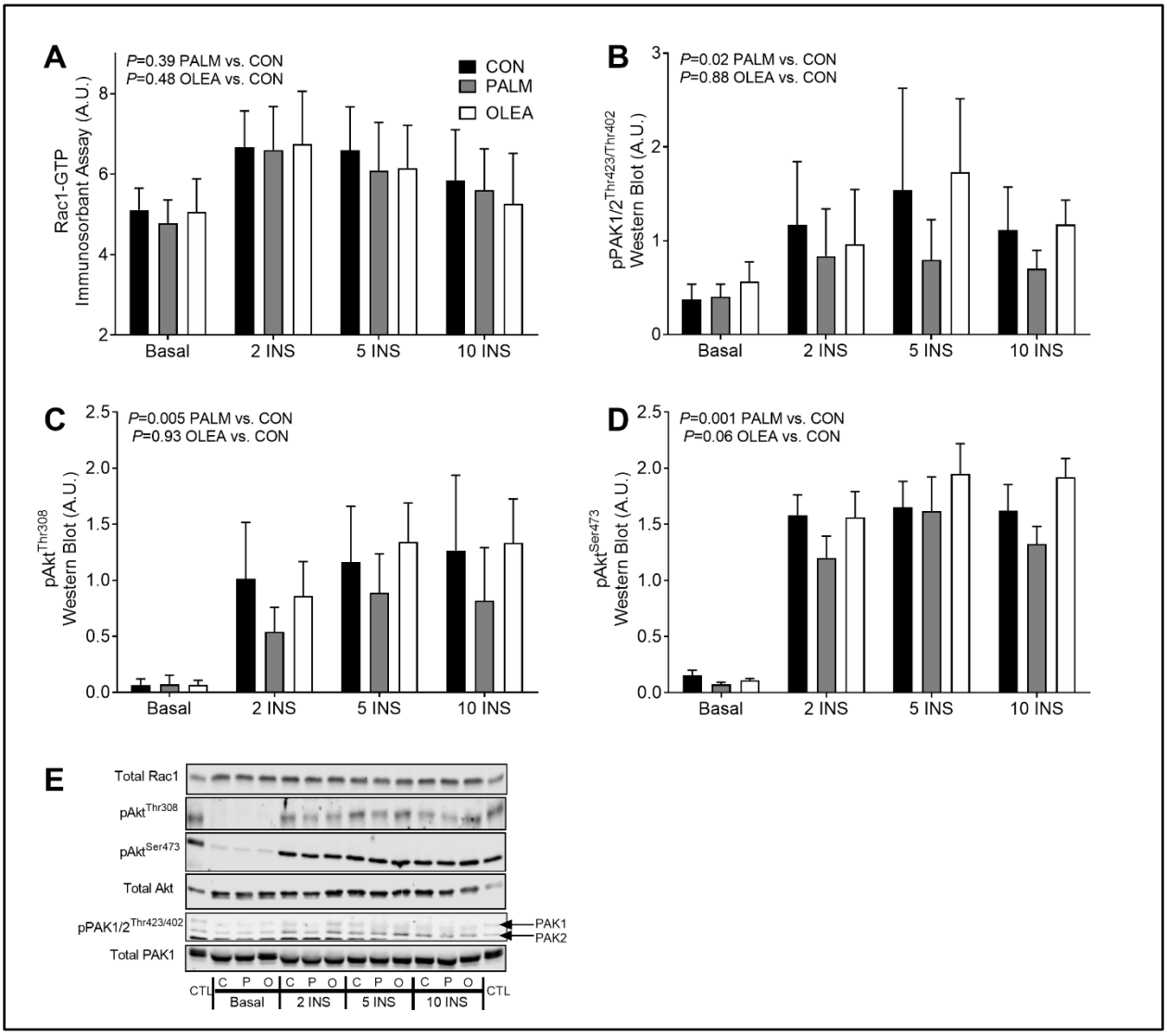

Figure 2.4: Insulin signaling following overnight fatty acid treatment in L6 myotubes.

Insulin signaling in L6 myotubes following overnight treatment in no fatty acid control (CON), palmitate (PALM, 500 µM) or oleate (OLEA, 500 µM), under basal serum-free or 100 nM insulin for 2, 5 or 10 minutes. A) Rac1-GTP binding was assessed via immunosorbent assay. Western blotting was used to assess B) phosphorylation of PAK 1 and PAK2 (pPAK1/2 Thr423/Thr402), and C-D) phosphorylation of Akt at Thr308 (pAkt Thr308) and Ser473 (pAkt Ser473). E) Representative western blot images. Insulin signaling outcomes were analyzed by two-way time x treatment ANOVA. A main effect of insulin was evaluated by comparing 2, 5, and 10 minutes of insulin-stimulation with basal using Dunnett's analysis. Main effects of fatty acid treatments were evaluated by comparing palmitate and oleate with the no fatty acid control condition using Dunnett's analysis. Total protein abundance of insulin signaling proteins was not affected by insulin or fatty acid treatments. Main effect P-values for time and treatment, respectively, are provided for total Rac1 (P=0.33, P=0.31), total Akt, (P=0.76, P=0.17) and total PAK1, (P=0.39, P=0.95). Insulin signaling analysis reflects 3 independent experiments each consisting of n=3 for a total sample size of n=9 for all conditions. Data are presented as mean + standard deviation.

Insulin-stimulated GLUT4 translocation is blunted by palmitate in L6 myotubes

The significance of palmitate-induced lipid accumulation and related impairments in insulin signaling was assessed via GLUT4 translocation. Insulin stimulation increased content of GLUT4 detected at the cell surface during the no fatty acid control condition (P<0.001 vs. basal), with no adverse effect of oleate treatment (P<0.001 vs. basal, Figure 2.5). Conversely, insulin-stimulated GLUT4 translocation was blunted during treatment with either palmitate (P=0.10 vs. basal) or latrunculin B (P=0.70, Figure 2.5), an inhibitor of actin polymerization (Spector, Shochet, Blasberger, & Kashman, 1989). These data indicate that palmitate-induced lipid accumulation and related impairments in insulin signaling effectively prevent insulin-stimulated GLUT4 translocation in L6

myotubes and further demonstrate the need for a functional actin cytoskeleton during insulin-stimulated GLUT4 translocation.

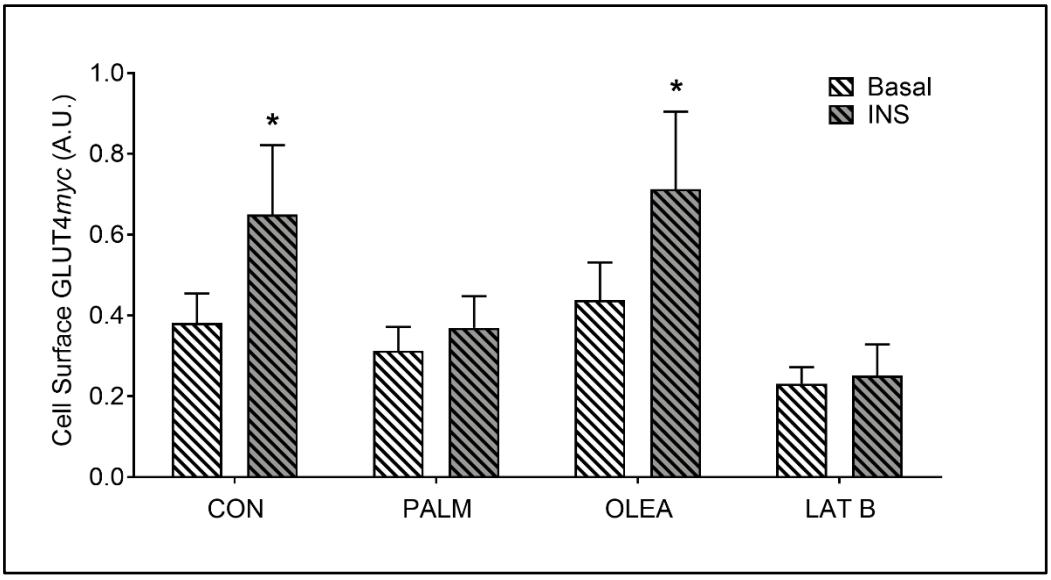

Figure 2.5: Insulin-stimulated GLUT4 translocation following overnight fatty acid treatment in L6 myotubes.

Glucose transporter protein 4 (GLUT4) translocation was assessed via cell surface detection of *myc* in L6 myotubes with stable expression of *myc*-tagged GLUT4 following overnight treatment in no fatty acid control (CON), palmitate (PALM, 500 µM), oleate (OLEA, 500 µM) during basal serum-free or 100 nM insulin for 20 minutes. Latrunculin B (LAT B, 5 µM) was administered during the last hour of serum starvation in the LAT B condition. Cell-surface GLUT4*myc* during basal and insulin-stimulated (INS) conditions was analyzed via unpaired two-tailed students t-test. GLUT4 translocation analysis reflects 3 independent experiments each consisting of n=3 for a total sample size of n=9 for all conditions. Data are presented as mean + standard deviation. *P<0.05 vs. Basal.

Discussion

The purpose of this study was to identify a role for lipids as negative regulators of Rac1 in muscle cells. We hypothesized that palmitate-induced accumulation of DAG and ceramides would impair insulin-stimulated Rac1-GTP binding. In contrast to our hypothesis, activation of Rac1 was not impaired by overnight palmitate treatment, in spite of significant accumulation of DAG and ceramides. Nevertheless, phosphorylation of PAK, a downstream target of Rac1 signaling, was impaired. Deleterious effects of

palmitate were also observed for IRS1 and Akt, resulting in almost complete inhibition of insulin-stimulated translocation of GLUT4. Together these findings suggest that lipid-induced impairments in myocellular insulin action may be independent of defects in insulin-stimulated Rac1-GTP binding.

It is important to understand interactions between intramuscular lipid accumulation and impaired translocation of GLUT4. Rac1 has emerged as a critical regulatory point of skeletal muscle insulin signaling based on pharmacological inhibition studies and knockout models demonstrating impairment in insulin action (Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Nevertheless, direct measures of Rac1 activation during models of lipid-induced insulin resistance were needed. Here we report no impairment in Rac1-GTP binding despite significant accumulation of DAG and ceramides, inhibitory signals for upstream regulators of Rac1 (e.g., IRS1) and attenuated activation of downstream targets (e.g., PAK). Our findings advance the field by demonstrating diminished activation of PAK and attenuated GLUT4 translocation can occur without impairment to Rac1-GTP binding. These findings also show that Rac1-GTP binding is preserved despite evidence for inhibition of proximal insulin signaling. Regulation of insulin-stimulated Rac1-GTP binding is not fully resolved but involves PI3K-dependent activation of guanine nucleotide exchange factors (GEFs) (Chiu et al., 2011; Das et al., 2000; Rosenfeldt, Vázquez-Prado, & Gutkind, 2004; Tybulewicz, 2005). Additional evidence suggests that Rac1 can also undergo regulation that is PI3K-independent (Lambert et al., 2002). How stimulation of Rac1-GTP binding can be maintained during

insulin resistance and if other impairment to Rac1 activation is present during insulin resistance, including altered localization, remain important questions of interest.

Our findings agree with previous reports demonstrating impaired phosphorylation of PAK in models of insulin resistance. Indeed, insulin-stimulated phospho-PAK was attenuated in obese humans and those with type 2 diabetes, high fat-fed and ob/ob mice, and during acute infusion of lipids in otherwise lean, healthy adults (Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). While these findings implicated defects in Rac1 signaling, our current data suggest that such impairment in activation of PAK may be independent of dysregulated Rac1. This possibility is further supported by evidence that inducible knockout of Rac1 in skeletal muscle exacerbates high fat diet-induced insulin resistance in mice (Raun et al., 2018). These findings indirectly suggest that Rac1 remains responsive to insulin in the high fat fed mice, otherwise knocking it out would presumably result in no further impairment in insulin action. Insulin-stimulated Rac1-GTP binding was not reported during conditions of insulin resistance in these previously published models (Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a).

Activation of PAK is a multi-step process that requires both GTP-bound Rac1 to bind the autoregulatory region and phosphorylation at Thr423 in the kinase activation loop (Zenke, King, Bohl, & Bokoch, 1999). In the non-activated state PAK exists as a homodimer, whereby binding of GTP-bound Rac1 disrupts this dimerization causing conformational change to rearrange the kinase activation loop to a catalytically

competent state (Lei et al., 2000). Previous evidence suggests that an exogenous kinase such as phosphoinositide-dependent kinase 1 (PDK1) may be required for phosphorylation of PAK at Thr423, as autophosphorylation at this residue does not readily occur (C. C. King et al., 2000). In the current study phosphorylation of PDK1 at Ser241, which is essential for PDK1 activation (Casamayor, Morrice, & Alessi, 1999), was not impaired during treatment with palmitate (data not shown). However, Akt phosphorylation at Thr308 was attenuated and is a known PDK1 phospho-site (D Sarbassov, Guertin, Ali, & Sabatini, 2005). Previous reports in C2C12 myotubes have demonstrated inhibitory phosphorylation of PDK1 during incubation with high concentrations of palmitate (C. Wang et al., 2008). These findings suggest impaired PDK1 activity may contribute to attenuated PAK phosphorylation during skeletal muscle insulin resistance.

The current findings did not support our hypothesis that accumulation of DAG and ceramides negatively regulate Rac1. This was unexpected given evidence from other model systems demonstrating negative regulation of Rac1-GTP binding by DAG (Sosa et al., 2009; H. Wang & Kazanietz, 2006). Some proteins important for regulation of Rac1 in these other cell types have low (or no) expression in skeletal muscle (e.g., β2-chimaerin), which may explain this discrepancy. We also acknowledge that cultured muscle cells differ in protein expression and structure from fully developed skeletal muscle *in vivo* (Ravenscroft et al., 2007; Tondeleir, Vandamme, Vandekerckhove, Ampe, & Lambrechts, 2009). Nevertheless, a C2-ceramide analogue was previously shown to impair insulin-stimulated Rac1-GTP in L6-GLUT4*myc* myotubes (JeBailey et al., 2007). The mechanism for this effect was unknown, but was not recapitulated in the

current study during palmitate-induced accumulation of C16:0, C18:0 and C22:0 ceramides. We used targeted lipidomics to identify molecular species involved with impairments to insulin signaling. The position and species of acyl chains affect signaling properties and thus regulatory functions of these lipids (Ritter et al., 2015). Further many of the DAG and ceramides that accumulated during palmitate treatment have been linked to insulin resistance (Chung et al., 2017; Szendroedi et al., 2014). Our results cannot exclude the possibility that Rac1 may be negatively regulated by species of DAG and ceramides other than those which accumulated during palmitate treatment, but do agree with numerous reports showing that incubating muscle cells in oleate does not impair insulin action (Coll et al., 2008; D. Gao et al., 2009; Peng et al., 2011).

In conclusion, insulin-stimulated Rac1-GTP binding was not impaired in L6 myotubes during palmitate-induced accumulation of DAG and ceramide. Nevertheless, PAK phosphorylation and GLUT4 translocation were attenuated during treatment with palmitate, suggesting that these impairments can occur independent of defects in insulin-stimulated Rac1-GTP binding. Mechanisms responsible for attenuated PAK phosphorylation remain unresolved, but may involve impaired activation of upstream kinases such as PDK1. Our findings further support the critical need for functional actin remodeling for skeletal muscle insulin action and the potential role for effectors of Rac1, such as PAK, in the development of insulin resistance.

Acknowledgements

We thank Kathleen Harrison, Ph.D. for her assistance with lipidomic measures, as well as Amira Klip, Ph.D. and Philip Bilan, Ph.D. for their assistance with L6-GLUT4*myc* cells. We also thank Emily Burney and Bergen Sather at Oregon State University for their skilled assistance and contributions on this project.

Disclosures

The authors declare no conflict of interest.

Grants

This project was supported by several awards from the National Institutes of Health: S.A.N. and the project were supported by KL2TR002370 as part of the Oregon Clinical & Translational Research Institute CTSA award UL1TR002369, B.C.B was supported by R01DK111559 and M.M.R. was supported by K01DK103829. H.D.S and S.E.E. were supported by fellowships provided by the College of Public Health and Human Sciences at Oregon State University.

Author contributions

H.D.S., S.E.E., and S.A.N. designed and conducted the experiments. H.D.S. compiled the data and drafted the manuscript. All authors contributed to the interpretation of the data. H.D.S., B.C.B., M.M.R., and S.A.N. contributed to manuscript revisions and all authors approved the final version of the manuscript. S.A.N. is the guarantor of this work and, as such, takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Chapter 3 – Diet and exercise training influence skeletal muscle long-chain acyl-CoA synthetases

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Medicine & Science in Sport & Exercise

401 West Michigan Street, Indianapolis, IN 46202-3233

Volume 52, Issue 3, March 2020

Diet and exercise training influence skeletal muscle long-chain acyl-CoA synthetases

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Title (85 characters including spaces): 84
Abstract (275-word limit): 271
Word count: 3814

Keywords: Long-chain acyl-CoA synthetase, lipid metabolism, skeletal muscle, obesity, aerobic exercise training

Abstract

Introduction: Long-chain acyl-CoA synthetases (ACSLs) are implicated as regulators of oxidation and storage of fatty acids within skeletal muscle; however, to what extent diet and exercise alter skeletal muscle ACSLs remains poorly understood. **Purpose:** To determine effects of diet and exercise training on skeletal muscle ACSLs and examine relationships between ACSL1 and ACSL6 and fat oxidation and fat storage, respectively. **Methods:** Male C57BL/6J mice consumed a 60% high-fat diet (HFD) for 12 weeks to induce obesity compared with low-fat diet (LFD). At week 4, mice began aerobic exercise (EX-Tr) or remained sedentary (SED) for 8 weeks. At week 12, protein abundance of 5 known ACSL isoforms and mRNA expression for ACSL1 and ACSL6 were measured in gastrocnemius muscle, as was skeletal muscle lipid content. Fat oxidation was measured using metabolic cage indirect calorimetry at week 10. **Results:** Of 5 known ACSL isoforms, 4 were detected at the protein level. HFD resulted in greater, yet non-significant, ACSL1 protein abundance (+18%, P=0.13 vs. LFD), greater ACSL6 (+107%, P<0.01 vs. LFD), and no difference in ACSL4 or ACSL5. Exercise training resulted in greater ACSL6 protein abundance in LFD mice (P=0.05 LFD EX-Tr vs. SED) while ACSL4 was lower following exercise training compared with sedentary, regardless of diet. Under fasted conditions, skeletal muscle ACSL1 protein abundance was not related to measures of whole-body fat oxidation. Conversely, skeletal muscle ACSL6 protein abundance was positively correlated with intramyocellular lipid content (P<0.01, r^2=0.22). **Conclusion:** We present evidence that ACSL isoforms 1, 4 and 6 may undergo regulation by HFD and/or exercise training. We further conclude increased

skeletal muscle ACSL6 may facilitate increased intramyocellular fat storage during HFD-induced obesity.

Introduction

There is significant interest in understanding regulation of fat oxidation and fat storage in skeletal muscle, including diverse contexts such as exercise performance and the prevention of metabolic disease (Kiens, 2006). Both obesity and aerobic exercise training influence skeletal muscle lipid metabolism, including oxidation and storage of fat (Goodpaster et al., 2001; Newsom et al., 2017; Perreault et al., 2018; Spina et al., 1996). However, how fatty acids are partitioned toward oxidation or storage within skeletal muscle and to what extent such regulatory factors may be influenced by obesity and aerobic exercise training remain largely unanswered questions of interest.

Fatty acids entering skeletal muscle are activated by long-chain acyl-CoA synthetases (ACSLs) to form long-chain acyl-CoA prior to undergoing oxidation or being used for synthesis of triacylglycerols (i.e., storage) (Coleman, Lewin, & Muoio, 2000; Watt & Hoy, 2012). There are 5 known ACSL isoforms that vary in expression, function, and localization among tissues (Adeva-Andany, Carneiro-Freire, Seco-Filgueira, Fernández-Fernández, & Mouriño-Bayolo, 2018; Grevengoed et al., 2014; Watt & Hoy, 2012). Transcript levels suggest that ACSL isoforms 1, 3, and 6 may be expressed in higher abundance than isoforms 4 and 5 in skeletal muscle (Mashek et al., 2006). However, comprehensive investigation into protein abundance of known ACSL isoforms in skeletal muscle, including to what extent skeletal muscle ACSLs may be altered by diet and exercise interventions, is currently lacking. Such investigations are warranted in light of mounting evidence indicating various ACSL isoforms may serve distinct, critical roles in determining the fate of fatty acids within skeletal muscle.

Mice with skeletal muscle specific knockout of ACSL1 exhibit metabolic inflexibility, attenuated fat oxidation at rest, and exercise intolerance compared with wild type animals (Li et al., 2015). Exercising the knockout mice resulted in accumulation of long-chain acyl-CoA in skeletal muscle and hypoglycemia at half the distance covered compared with wild type mice, suggesting compensatory reliance on glucose oxidation due to impaired fat oxidation (Li et al., 2015). Prolonged fasting (i.e., 48 hours) has also been shown to increase skeletal muscle mRNA expression and protein abundance of ACSL1 (Mashek et al., 2006), which coincides with increased rates of fat oxidation, indicating a potential role for ACSL1 to facilitate oxidation of fatty acids. Equally compelling evidence suggests skeletal muscle ACSL6 facilitates storage of fatty acids (Teodoro et al., 2017). ACSL6 mRNA expression is upregulated in response to a high fat meal (i.e., when fatty acid storage is elevated) and downregulated in response to a prolonged fast (i.e., when fat storage is minimal) (Teodoro et al., 2017). Knockdown of ACSL6 in cultured muscle cells attenuated lipid storage, as evidenced by decreased lipid droplet size and reduced content of triacylglycerols. Conversely, overexpression of ACSL6 increased accumulation of phospholipids and decreased fat oxidation (Teodoro et al., 2017). Taken together, these findings indicate ACSL1 and ACSL6 may have distinct roles in skeletal muscle fat oxidation and fat storage, respectively.

Despite marked changes in skeletal muscle lipid metabolism in response to high fat diet induced obesity or aerobic exercise training (Dasari et al., 2018; Holloszy, 1967; Newsom et al., 2017; Spina et al., 1996), to what extent such interventions influence skeletal muscle ACSL isoforms remains unknown. Therefore, the first purpose of this study was to identify the effects of diet-induced obesity and aerobic exercise training on

skeletal muscle ACSL isoforms. The second purpose was to investigate relationships between skeletal muscle ACSL1 and ACSL6 and the oxidation or storage of lipids. Herein we demonstrate 4 of the 5 known ACSL isoforms were readily detected in skeletal muscle at the protein level, with isoform-specific differences in response to diet and exercise intervention. Additionally, we present evidence that diet-induced changes in skeletal muscle ACSL6 protein are related to intramyocellular lipid content.

Methods

Study Design

This study was approved by the Animal Care and Use Committee at Oregon State University (#4788). Twelve-week-old male C57BL/6J mice (Jackson Laboratories) were housed 3-5 per cage under 12-hour light-dark cycle at 22°C conditions with free access to food and water. As depicted in Figure 3.1, mice consumed either a low-fat diet (D12450J; Research Diets) or high-fat diet (D12492; Research Diets) for 12 weeks.

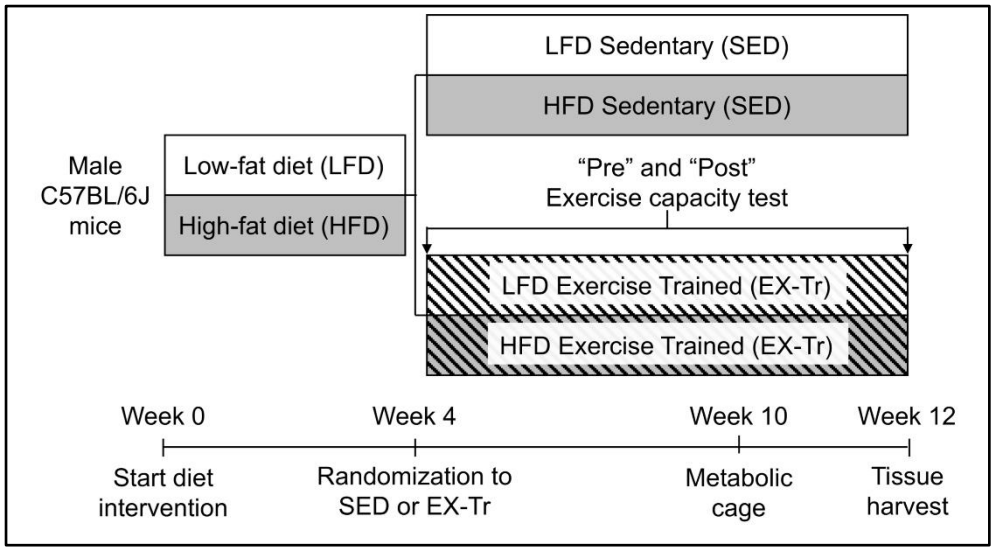

Figure 3.1: Study Design.

C57Bl/6J mice consumed either low fat diet or high fat diet for 12 weeks. At week 4, mice either remained sedentary or began graded aerobic exercise training performed on a motorized treadmill for 50 min/day 5 days/week. Graded exercise tests were performed before and after aerobic exercise training to assess exercise capacity. Body composition and in-cage metabolic assessment were performed during week 10 to phenotype mice and determine substrate oxidation, respectively. At week 12, mice were anesthetized after a 4-h fast, 36 h after their last bout of exercise or remaining sedentary (n = 10 mice per condition)

The percentage of total kilocalories from fat/carbohydrate/protein for LFD was 10/70/20 and for HFD was 60/20/20. At week 4 of the diet intervention, mice either remained sedentary (SED) or began graded aerobic exercise training (EX-Tr). Aerobic exercise training was performed on a motorized treadmill for 50 minutes per day, 5 days per week. Mice were acclimated to the motorized treadmill (Panlab, Harvard Apparatus, Holliston, MA, USA) for one week with low speed (0-12m/min) for 15 minutes. Exercise then gradually progressed in intensity and duration from 10m/min at 0% incline for 30 minutes to 17m/min for 50 minutes at 10% incline which comprised the last 12 days of exercise training. A graded exercise test was performed after acclimation and at the end of the training period to assess exercise capacity. The belt speed started at 6m/min and 0% incline for 5 minutes then increased by 3m/min and 5% incline every 2 minutes until 18m/min and 15% incline was reached. Speed was then increased by 1-2 m/min every

minute until exhaustion. Mice were removed from the treadmill when they refused to run despite sitting on the shock grid continuously for 5 seconds. In-cage metabolic assessment (Promethion, Sable Systems Int.) was performed at week 10 along with body composition measures using dual-energy x-ray absorptiometry (GE Lunar, PIXImus2). Following an acclimation period of 24 hours, in-cage substrate oxidation during fed and fasted conditions were assessed in 12-hour cycles and following a 3-5 hour fast, respectively, using indirect calorimetry (see *Fat Oxidation*, below, for calculations). At week 12, mice were anesthetized by sodium pentobarbital overdose following a 4 hour fast, 36 hours after their last bout of exercise or remaining sedentary. Tissues were collected, flash frozen in liquid nitrogen, and stored at -80°C until further analysis (n=10 mice/condition). Importantly, tissue analysis was completed using mixed gastrocnemius muscle (isolated independent from soleus and plantaris muscles) as an ideal representative muscle due to its mixed fiber type (Augusto, Padovani, & Campos, 2004), previously reported ACSL mRNA expression (Mashek et al., 2007), and recruitment during treadmill exercise training (Davidson, Burnett, & Hoffman-Goetz, 2006).

Western Blotting

Gastrocnemius muscle (~30 mg) was homogenized as described previously (Newsom et al., 2017). Homogenates rotated at 4ºC for 20 minutes and then centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatant was stored at -80°C until further analysis. Approximately 35 µg protein was separated on bis-tris gels and transferred to

nitrocellulose membranes. Each gel was loaded with the same internal control sample in 2 lanes, the average density of both lanes was used to normalize band density between gels. Ponceau staining of membranes was used to verify equal loading and transfer of protein. Membranes were blocked in 5% bovine serum albumin in tris-buffered saline with tween (TBST) and incubated in primary antibodies at 4°C. Following primary incubation, membranes were washed in TBST and incubated in secondary antibody diluted in blocking buffer at room temperature. Images were generated using infrared detection (LI-COR Odyssey). Primary antibodies used included ACSL1 (product no. 4047, Cell Signaling Technology), ACSL3 (product no. 166374, Santa Cruz), ACSL4 (product no. PA5-27137, Invitrogen), ACSL5 (product no. 365478, Santa Cruz) and ACSL6 (product no. PA5-30465, Invitrogen) diluted 1:1000. Primary antibodies for ACSL isoforms 1, 4, and 5 have been previously verified by knockdown and/or overexpression models (Kwak et al., 2019; Li et al., 2006). Primary antibodies for ACSL3 and 6 have not been verified by knockdown or overexpression; however, internal testing by the manufacturer verified expression of ACSL3 in mouse muscle cell line (C2C12) and ACSL6 in mouse heart lysate. The secondary antibodies used were anti-rabbit-700 (product no. 926-68071) and anti-mouse-800 (product no. 926-32212) from LICOR diluted 1:10,000.

Quantitative Polymerase Chain Reaction (qPCR)

Sequence information for target genes are listed in Table 3.1. Total mRNA was measured as described previously (Dasari et al., 2018). Briefly, mRNA was extracted

from gastrocnemius muscle (~20 mg) to determine transcriptional activation of skeletal muscle ACSL isoforms 1 and 6. mRNA concentration and contamination was determined by spectrophotometry (NanoDrop, ThermoFisher Scientific), with 1 µg mRNA reversed transcribed to cDNA. qPCR was performed in triplicate in 384 well clear plates using Sybr® Green reagents (ThermoFisher Scientific), ~20 ng cDNA and 100 nM primers in 20 µl reaction volumes. Thermocycler conditions were 10 minutes at 60°C, 40 cycles of 15 seconds denaturing (95°C) and 60 seconds annealing/extension (60°C) followed by a melt curve. Relative quantification was performed using a 7-point standard curve that spanned 3 log dilutions. Amplification efficiencies were similar between target and reference genes, which were analyzed on separate plates along with no template controls. Target genes were normalized by dividing the average of two reference genes (HSP90 and Cyclophilin) analyzed on separate plates. Melt curves revealed single peaks for each primer set.

Table 3.1. Primer sequence information

Name	Gene Name	Reference Sequence	Sequence
Long-chain acyl-coenzyme a synthetase 1	ACSL1	NM_007981.3	F: GGATTCAGGTGTCAAATAATGG R: GAGAGTTCAGCTTTGTTCAC
Long-chain acyl-coenzyme a synthetase 6	ACSL6	NC_000077.6	Purchased from Bio-Rad Assay ID: qMmuCED0044795
Heat shock protein 90ab1	Hsp90	NM_008302	F: CATCATGGACAGCTGTGACG R: AGTTCTCCTTGTCCTCAGCC
Cyclophilin A	Ppia	NM_008907.1	F: CTTCTTGCTGGTCTTGCCATTCCT R: GGATGGCAAGCATGTGGTCTTTG

F, forward; R, reverse

Fat Oxidation

Previous evidence demonstrates a 48 hour fast alters skeletal muscle ACSL1 mRNA expression and protein abundance (Li et al., 2015). We therefore measured substrate oxidation following 3-5 hour fast to closely mimic the conditions in which skeletal muscle was harvested and ACSL protein abundance and mRNA expression were measured (i.e., following a 4-hour fast). Rates of whole-body fat oxidation were

calculated from in-cage VO_2 and VCO_2 measures using the equations of Frayn (Frayn, 1983) and normalized to body weight. Importantly our measures of fat oxidation are whole-body, however skeletal muscle comprises ~40% of total body mass and previous reports indicate skeletal muscle is a principal determinant of energy expenditure (Zurlo, Larson, Bogardus, & Ravussin, 1990).

Intramyocellular Lipid (IMCL)

IMCL was measured in ~20 mg of gastrocnemius muscle as previously described with minor modifications (Frayn & Maycock, 1980; Newsom et al., 2010). Skeletal muscle was homogenized 2 x 30 seconds in 3 mL of 2:1 chloroform:methanol, with 2.7 mL of homogenate transferred to clean 13mm glass culture tubes. Following an overnight extraction at 4ºC, homogenates were centrifuged for at 3,000 x g for 15 minutes to separate phases and then 1.2 mL of the lower organic phase was dried under N2 gas. Lipids were resuspended and saponified in 4% ethanol-KOH solution at 75°C for 25 minutes and fatty acids were pelleted by the addition of MgSO4 (0.15 M) and centrifugation at 3,000 x g for 15 minutes. Glycerol concentration was determined in the supernatant using colorimetric Infinity Triglyceride Reagent (ThermoFisher Scientific). Total IMCL was calculated from glycerol concentration as:

$$IMCL\ (mg/g) = \frac{(glycerol\ (mg/ml)\ x\ 1.25\ ml)\ x\ \left((3/2.7)\ x\ (2/1.2)\right)}{tissue\ wet\ weight\ (g)}$$

Total glycerol was calculated by multiplying the concentration by the final volume and accounting for lipid-containing volumes left behind during transfer steps. Total glycerol was then normalized to tissue wet weight to yield total mg IMCL content per g of tissue.

Statistical Analysis

The effect of aerobic exercise training on exercise capacity was analyzed by paired, two-tailed Student's t-tests. Independent effects of diet and exercise training on outcome variables were analyzed by unpaired, two-tailed Student's t-tests. More specifically, the effect of diet was evaluated by comparing HFD vs. LFD in the sedentary condition. Effects of exercise training were analyzed within each diet condition by comparing exercise trained mice with sedentary mice. This approach was employed to limit statistical analysis to comparisons of interest. Relationships between ACSL1 and ACSL6 and fat oxidation and fat storage, respectively, were analyzed by Pearson's correlational analysis. Statistical significance was set as $P \leq 0.05$. Statistical analysis and generation of figures were performed using Prism version 6 (GraphPad Software). Data are presented as mean and standard deviation, with individual data points shown, in accordance with recommendations for the field (Curran-Everett, 2008).

Results

Effects of HFD and aerobic exercise training on animal characteristics

Animal characteristics are provided in Table 3.2. Sedentary mice consuming HFD had 67% greater total body mass compared with LFD mice (P<0.01), which was largely attributable to greater fat mass and modest yet significantly greater lean mass (both P<0.01, HFD vs. LFD). Aerobic exercise training resulted in lower total body mass during HFD (P<0.01, HFD Ex-Tr vs. HFD SED), due to lower fat mass (P=0.03) and no difference in lean mass (P=0.73). In LFD mice, aerobic exercise training did not alter body mass or composition. In both LFD and HFD mice, aerobic exercise training increased time to exhaustion during a maximal exercise test (P<0.01 Pre EX-Tr vs. Post EX-Tr in both diet conditions), providing evidence of a robust and effective training stimulus.

Table 3.2. Mouse body composition and exercise tolerance

	LFD		HFD	
	SED	EX-Tr	SED	EX-Tr
Total mass (g)	24.0 ± 2.2	24.9 ± 2.0	40.2 ± 2.5*	35.6 ± 2.4#
Lean mass (g)	20.0 ± 1.7	20.7 ± 1.6	24.0 ± 1.7*	22.4 ± 1.7
Fat mass(g)	4.0 ± 0.9	4.1 ± 0.7	16.2 ± 1.6*	13.2 ± 2.6#
	LFD		HFD	
	Pre EX-Tr	Post EX-Tr	Pre EX-Tr	Post EX-Tr
Time to exhaustion (min)	13.1 ± 1.2	15.8 ± 1.6†	10.2 ± 2.0*	13.0 ± 2.1†

Data are presented as mean ± standard deviation. *P<0.05 for HFD vs. LFD. #P<0.05 for EX-Tr vs. SED. †P<0.05 for Post EX-Tr vs. Pre EX-Tr.

Effects of HFD and aerobic exercise training on skeletal muscle ACSL isoform protein abundance

We successfully detected 4 of the 5 ACSL isoforms in gastrocnemius muscle via western blotting, including ACSL isoforms 1, 4, 5 and 6 (Figure 3.2); ACSL3 protein abundance was below limits of detection (data not shown). HFD resulted in greater

abundance of ACSL6, a tendency for greater abundance of ACSL1, and no effect on protein abundance of isoforms 4 and 5 (Figure 3.2). Aerobic exercise training also resulted in greater abundance of ACSL6 in LFD mice (P=0.05, LFD EX-Tr vs. LFD SED, Figure 3.2D). Regardless of diet, exercise training resulted in modest yet significantly lower abundance of ACSL4 protein (P<0.05, Ex-Tr vs. SED in both diet conditions) with no effect on ACSL isoforms 1 and 5 (Figure 3.2). Together these data indicate diet and exercise serve as isoform-specific regulators of skeletal muscle ACSL protein abundance. Because ACSL1 and 6 have been implicated as key determinants of fat oxidation and fat storage (Li et al., 2006; Teodoro et al., 2017), we further investigated their regulation and relationship to measures of fat oxidation and IMCL content.

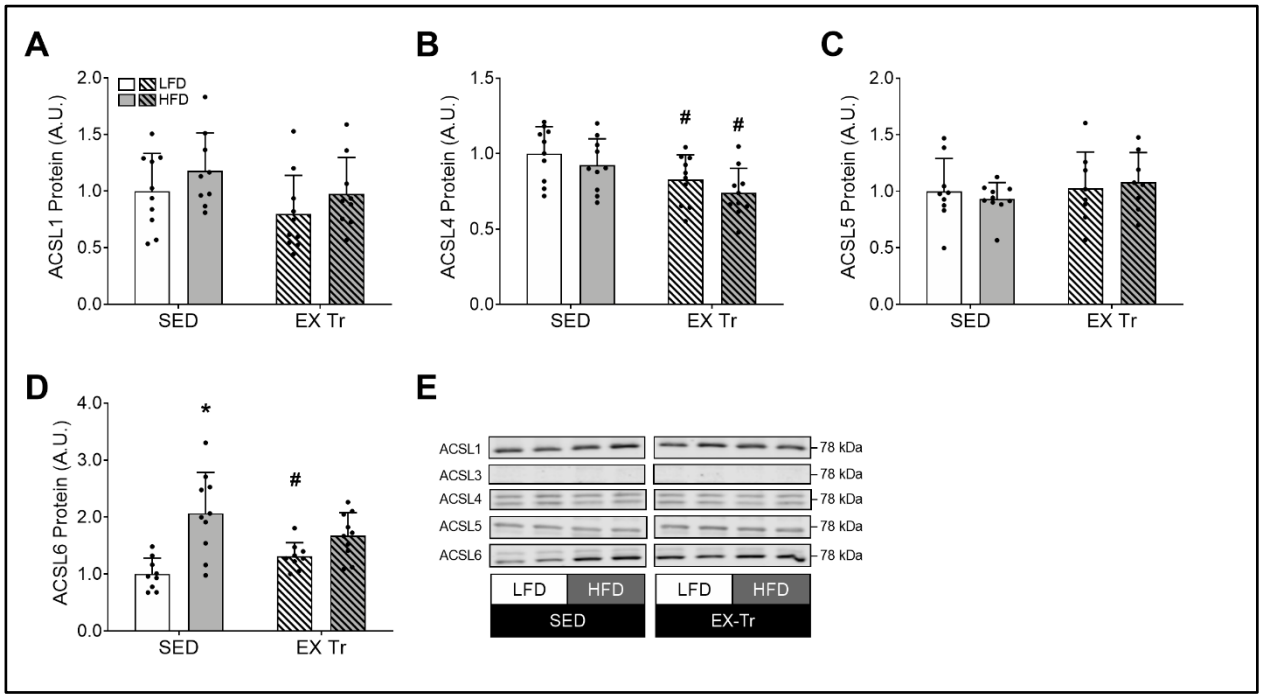

Figure 3.2: Skeletal muscle ACSL isoform protein abundance following diet-induced obesity and aerobic exercise training.

ACSL isoform protein abundance in gastrocnemius muscle following either a 12-week low-fat (LFD) or high-fat diet (HFD) in sedentary (SED) and aerobic exercise trained (EX-Tr) mice. Total protein abundance for **A)** ACSL1, **B)** ACSL4, **C)** ACSL5, **D)** ACSL6, **E)** with representative western blot images shown. Each representative image was spliced from the same blot to remove groups not used in this study. The effect of diet was analyzed by unpaired students t-test comparing HFD to LFD among sedentary mice. The effect of exercise training was analyzed by unpaired students t-test comparing EX-Tr to SED within each diet condition. Data are presented as mean and standard deviation with individual data points shown. *$P \leq 0.05$ vs. LFD, #$P \leq 0.05$ vs. SED. n=10 for all conditions.

Fasting whole-body fat oxidation is not related to skeletal muscle ACSL1 protein abundance

We further explored possible regulation of skeletal muscle ACSL1 by diet and exercise training via measurement of mRNA expression. ACSL1 mRNA expression was not different as a function of diet; however, exercise training did result in a modest yet significantly greater expression of skeletal muscle ACSL1 mRNA in HFD mice (Figure 3.3A). We next investigated the relationship between skeletal muscle ACSL1 protein

abundance and *in vivo* rates fat oxidation. Importantly, fasting is known to increase ACSL1 mRNA and protein abundance (Mashek et al., 2006). For this reason, ACSL1 protein abundance was related to rates of fat oxidation measured following a 3-5 hour fast to closely mimic physiologic conditions during which tissues were harvested (i.e., to control for any potential effect of fasting on ACSL protein abundance). Following a 4-hour fast, rates of whole-body fat oxidation were similar among all conditions (Figure 3.3B), likely reflecting the increased fasting-induced reliance upon fat oxidation. Under such conditions, rates of whole-body fat oxidation were not related to skeletal muscle ACSL1 protein abundance (Figure 3.3C).

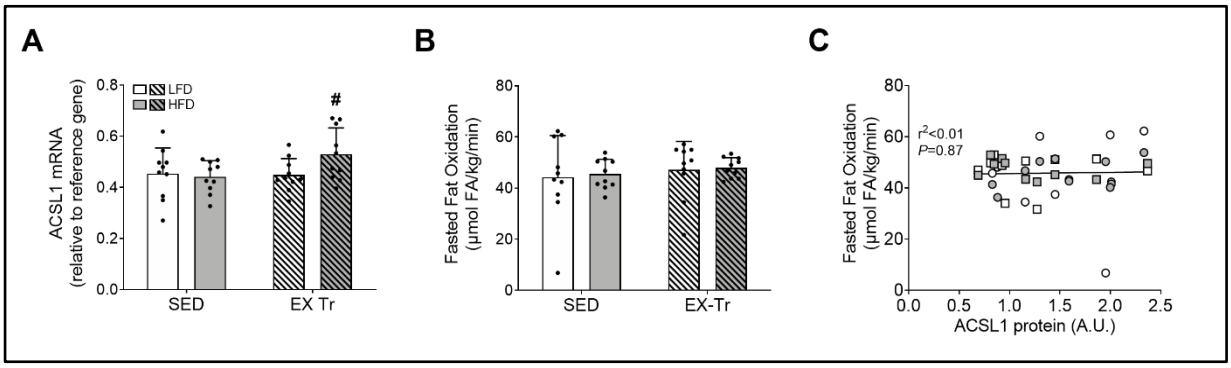

Figure 3.3: Skeletal muscle ACSL1 mRNA expression and relationship to whole-body fat oxidation.

A) ACSL1 mRNA expression following either a 12-week low-fat (LFD) or high-fat diet (HFD) in sedentary (SED) and aerobic exercise trained (EX-Tr) mice. **B)** Whole-body fat oxidation calculated from VO_2 and VCO_2 and normalized to body weight following a 4-hour fast during the light cycle to closely mimic physiologic conditions during which tissues were harvested. The effect of diet was analyzed by unpaired students t-test comparing HFD to LFD among sedentary mice. The effect of exercise training was analyzed by unpaired students t-test comparing EX-Tr to SED within each diet condition. Data are presented as mean and standard deviation with individual data points shown. **C)** Correlation analysis measuring the relationship of skeletal muscle ACSL1 protein abundance and whole-body fat oxidation following a 4 hour fast. LFD SED, white circle; HFD SED, gray circle; LFD EX-Tr, white square; HFD EX-Tr, gray square. Data are presented as individual data points. **P*≤0.05 vs. LFD, #*P*≤0.05 vs. SED. n=10 for all conditions.

Intramyocellular lipid content is positively related to skeletal muscle ACSL6 protein abundance

We measured skeletal muscle ACSL6 mRNA expression to investigate potential diet and/or exercise training-related regulation at the transcript level. ACSL6 mRNA expression tended to be lower in HFD compared with LFD mice (P= 0.06, Figure 3.4A), perhaps reflecting increased translation to support markedly greater ACSL6 protein abundance. Exercise training resulted in a small yet significantly greater skeletal muscle ACSL6 mRNA expression within HFD (Figure 3.4A). We next investigated the relationship between ACSL6 protein abundance and intramyocellular lipid content (IMCL). IMCL content was significantly greater in HFD compared with LFD in SED mice (P<0.01, Figure 3.4B). Exercise training did not alter IMCL content regardless of diet (Figure 3.4B); however, intramyocellular lipid was positively associated with greater ACSL6 protein abundance among all mice (P<0.01, r^2=0.22, Figure 3.4C), indicating ACSL6 may serve to facilitate lipid storage in skeletal muscle.

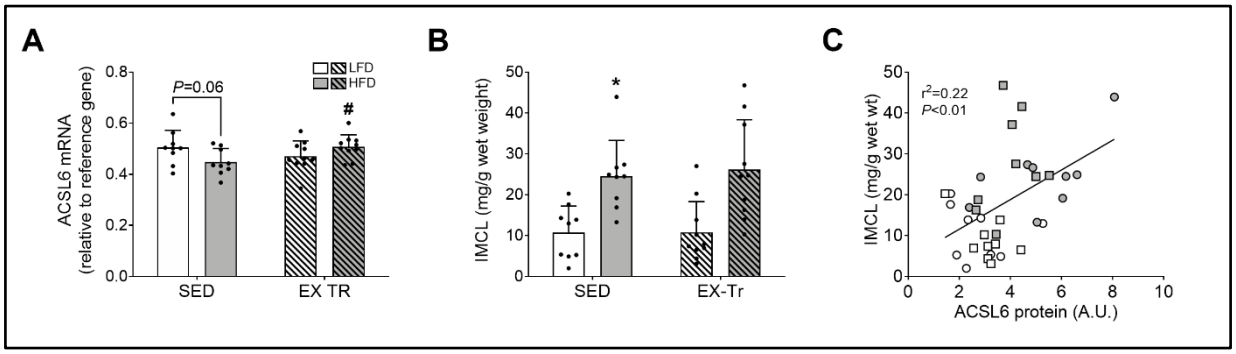

Figure 3.4: Skeletal muscle ACSL6 mRNA expression and relationship to IMCL content.

A) ACSL6 mRNA expression following either a 12-week low-fat (LFD) or high-fat diet (HFD) in sedentary (SED) and aerobic exercise trained (EX-Tr) mice. **B)** Intramyocellular lipid (IMCL) content measured in gastrocnemius muscle. The effect of diet was analyzed by unpaired students t-test comparing HFD to LFD among sedentary mice. The effect of exercise training was analyzed by unpaired students t-test comparing EX-Tr to SED within each diet condition. Data are presented as mean and standard deviation with individual data points shown. **C)** Correlation analysis measuring the relationship of skeletal muscle ACSL6 protein abundance and IMCL content. LFD SED, white circle; HFD SED, gray circle; LFD EX-Tr, white square; HFD EX-Tr, gray square. Data are presented as individual data points. *$P \leq 0.05$ vs. LFD, #$P \leq 0.05$ vs. SED. n=10 for all conditions.

Discussion

The purpose of this study was to identify the independent roles of HFD-induced obesity and aerobic exercise training on skeletal muscle ACSLs and to examine relationships of ACSL1 and ACSL6 to oxidation and storage of lipids, respectively. We readily detected skeletal muscle protein abundance for ACSL isoforms 1, 4, 5, and 6 in gastrocnemius muscle of mice, whereas ACSL3 was below limits of detection. Skeletal muscle ACSL6 protein abundance was significantly greater in high-fat compared with low-fat fed mice, and ACSL4 protein abundance was lower following exercise training compared with remaining sedentary, regardless of diet. ACSL1 protein abundance tended to be greater with HFD, whereas ACSL5 was minimally influenced by either intervention. Skeletal muscle ACSL1 protein abundance was not related to measures of

whole-body fat oxidation following a 4 hour fast; however, skeletal muscle ACSL6 protein abundance was positively related to intramyocellular lipid content, indicating ACSL6 may facilitate accumulation of lipids in skeletal muscle.

There is growing interest in ACSLs for their potentially critical roles as regulators of lipid metabolism. ACSLs activate fatty acids to form fatty acyl-CoA prior to undergoing further metabolism (Coleman et al., 2000; Watt & Hoy, 2012), with isoform-specific function likely determined by subcellular localization and substrate specificity. For example, in hepatocytes, ACSL1 localizes to both the endoplasmic reticulum (ER) and to the mitochondria whereas ACSL3 is localized to the ER and lipid droplets (Fujimoto et al., 2007). Emerging evidence indicates ACSL4 may have specificity for polyunsaturated fatty acids (Kang et al., 1997). Combined with varying levels of expression, ACSLs may therefore help to determine the metabolic fate of intracellular fatty acids. Nonetheless, this information is largely lacking for skeletal muscle, highlighting the need physiological characterizations studies such as this. We have demonstrated isoform-specific effects of diet and exercise training interventions to alter ACSL protein content, which may contribute to changes in skeletal muscle lipid metabolism. Unexpectedly, ACSL3 was below the level of detection in our samples. ACSL3 mRNA is expressed, albeit at relatively low levels, in skeletal muscle of rodents (Mashek et al., 2006). We also detected ACSL3 protein in C2C12 and L6 myotubes, using the same antibody employed here (data not shown). It is thus possible, if not likely, that ACSL3 is expressed in skeletal muscle and was not detected here due to technical limitation.

We anticipated both HFD and exercise training may result in greater ACSL1 given that peroxisome proliferator-activated receptor alpha (PPARα) is a known regulator of ACSL1 (Martin, Schoonjans, Lefebvre, Staels, & Auwerx, 1997; Schoonjans et al., 1995), and can be increased in response to both HFD (Patsouris, Reddy, Müller, & Kersten, 2006) and exercise training (Cresci, Wright, Spratt, Briggs, & Kelly, 1996; Horowitz, Leone, Feng, Kelly, & Klein, 2000). However, we observed no significant differences in ACSL1 protein abundance with HFD or exercise training (Figure 2A). Our findings are consistent with a previous report demonstrating minimal impact of PPARα deletion on known PPARα targets within skeletal muscle of mice (Muoio et al., 2002), suggesting more complex and potentially PPARα-independent regulation of ACSL1 in skeletal muscle. We also anticipated ACSL1 protein abundance may be related to fat oxidation given previous reports using knockout models demonstrating ACSL1 is critical for normal skeletal muscle fat oxidation (Li et al., 2015). However, we observed no relationship between skeletal muscle ACSL1 protein abundance and whole-body fat oxidation measured during the fasted state (Figure 3.3C). One possible explanation for this finding is that whole-body measures of fat oxidation are, of course, not specific to skeletal muscle. However, skeletal muscle represents a significant proportion of whole-body resting energy expenditure and therefore fat oxidation (Zurlo et al., 1990). Perhaps more important is that during the fasting state there is increased reliance upon fatty acids as a source of fuel, as evidenced by the similarly elevated rates of fat oxidation measured among all of our experimental conditions (Figure 3.3B). Increased delivery of fatty acid to skeletal muscle (Romijn et al., 1993) and lowered hormonal stimulus (i.e., insulin) to stimulate oxidation of fat during the fasting state (Kelley, Goodpaster, Wing, &

Simoneau, 1999) may therefore limit the role of ACSL1 as a critical determinant of the rate of fat oxidation. Further studies are needed to clarify the functional role of ACSL1 as a regulator of skeletal muscle fat oxidation during the physiologic transition from fed to fasting states (Mashek et al., 2006), as novel and perhaps targetable regulators of skeletal muscle fat oxidation may have significant therapeutic potential.

Our model of high fat feeding is well-characterized by obesity, hyperinsulinemia and increased skeletal muscle lipid content (Krssak et al., 1999; Pan et al., 1997; Perseghin et al., 1999). In light of previous findings demonstrating insulin-induced expression of ACSL6 (Durgan et al., 2006), we anticipated ACSL6 may be upregulated by high fat feeding. Indeed, HFD resulted in significantly greater ACSL6 protein abundance when compared with LFD (Figure 3.2E). We also found a positive relationship between skeletal muscle ACSL6 protein abundance and lipid content (Figure 3.4C). We interpret these findings as support for the role for ACSL6 in facilitating diet-induced lipid storage within skeletal muscle. Our findings are in agreement with those of Teodoro et al. demonstrating ACSL6 is linked to fat storage *in vitro* (Teodoro et al., 2017). Importantly, aerobic exercise training can increase skeletal muscle lipid content, particularly triacylglycerols (Goodpaster et al., 2001). We therefore anticipated exercise training might also increase skeletal muscle ACSL6 protein abundance and IMCL content. In agreement, exercise training resulted in greater ACSL6 protein compared with remaining sedentary among low fat fed mice (Figure 3.2E); however, IMCL content was unchanged following exercise training (Figure 3.4B). This may be due, in part, to increased rates of fat oxidation measured in LFD mice after exercise training in the fed state (data not shown), which may have lowered the fatty

acid available for lipid storage. Furthermore, our measure of IMCL content may be limited in sensitivity to detect small but perhaps important differences in lipid content. Collectively, we interpret our findings to indicate a role for ACSL6 in accumulation of skeletal muscle lipid in response to obesity and perhaps also in response to aerobic exercise training.

The significance of exercise training-related changes in skeletal muscle ACSL4 is unclear. ACSL4 is highly expressed in the adrenal gland, ovary, testis, and brain, where it demonstrates preference for polyunsaturated fatty acids (Kang et al., 1997). Some evidence suggests this isoform may facilitate synthesis of polyunsaturated phospholipids (Mikhail Y Golovko et al., 2006). For example, overexpression of ACSL4 in human arterial smooth muscle cells results in greater incorporation of arachidonic acid into phospholipids and triacylglycerols (Golej et al., 2011). However, because ACSL isoform function varies by tissue, manipulation of ACSL4 protein abundance in skeletal muscle may be necessary in order to determine its role and the significance of its downregulation with exercise training. For instance, manipulation of ACSL5 by overexpression within skeletal muscle *in vitro* resulted in greater measures of fat oxidation (Kwak et al., 2019). These findings suggest ACSL5 may direct fatty acids toward oxidation; however, we observed no change in total protein abundance in response to HFD or aerobic exercise training.

An important consideration is that our measures of ACSL isoform protein abundance may not reflect ACSL activity. Exercise has been shown to increase total skeletal muscle ACSL activity measured *ex vivo* (Cortright et al., 2006), though the

specific contribution of individual ACSL isoforms is unknown. Furthermore, proteomic analysis has revealed numerous phosphorylation and acetylation sites on ACSL1 (Frahm, Li, Grevengoed, & Coleman, 2011); however, specific antibodies are unavailable and the functional consequence of such post translational modifications remain largely unknown (Frahm et al., 2011). Therefore, the function and regulation of post translational modification among ACSL isoforms in response to diet-induced obesity and aerobic exercise training remain areas of interest. Another important consideration is the extent to which ACSL expression and regulation may differ by fiber type and therefore among skeletal muscles. A previous report demonstrated similar levels of mRNA expression of ACSL isoforms between gastrocnemius and soleus muscles (Mashek et al., 2006); though, mRNA expression is not necessarily indicative of protein abundance Because of known differences in skeletal muscle lipid metabolism as a function of fiber type (Essén, Jansson, Henriksson, Taylor, & Saltin, 1975; He, Watkins, & Kelley, 2001), muscle-specific regulation of ACSLs remains another area of interest for future directions.

In conclusion, protein abundance of ACSL isoforms 1, 4, 5, and 6 were readily detected in mouse gastrocnemius muscle. High fat feeding and exercise training resulted in isoform-specific effects on ACSLs whereby ACSL6 was greater in response HFD-induced obesity and following exercise training in low-fat fed mice, and ACSL4 protein abundance was lower in response to exercise training regardless of diet. Furthermore, we identified skeletal muscle ACSL6 protein abundance as being positively related to intramuscular lipid content, indicating potential contribution to

regulation of lipid storage. ACSLs remain of interest as potentially critical determinants of skeletal muscle lipid metabolism.

Acknowledgements

We thank Donald Jump, Ph.D. at Oregon State University for his generous primer donations for polymerase chain reaction analysis. The results of the present investigation do not constitute endorsement by the American College of Sports Medicine. We declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Disclosures

The authors have no conflict of interest to declare.

Grants

This project was partly supported by the American College of Sports Medicine Northwest Regional Student Research Grant awarded to H.D.S. The mouse project was supported by DK103829 from the National Institutes of Health awarded to M.M.R. S.A.N. is supported by KL2TR002370 as part of the Oregon Clinical & Translational Research Institute Clinical Translational Science Award UL1TR002371 from the

National Institutes of Health. H.D.S. and S.E.E. are supported by fellowships from Oregon State University.

Author Contributions

H.D.S., S.E.E., M.M.R. and S.A.N. performed experiments. H.D.S. performed the analysis and drafted the manuscript. S.E.E., M.M.R. and S.A.N. edited the manuscript. S.A.N. is the guarantor of this work and, as such, takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Chapter 4 – Skeletal muscle ACSL isoforms relate to measures of fat metabolism in humans

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Medicine & Science in Sport & Exercise

401 West Michigan Street, Indianapolis, IN 46202-3233

Under review

Skeletal muscle ACSL isoforms relate to measures of fat metabolism in humans

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Title (85 characters including spaces): 76
Abstract (275-word limit): 274
Word count: 4135

Abstract

Introduction: Evidence from model systems implicates long-chain acyl-CoA synthetases (ACSLs) as key regulators of skeletal muscle fat oxidation and fat storage; however, such roles remain underexplored in humans. **Purpose:** We sought to determine protein expression of ACSL isoforms in skeletal muscle at rest and in response to acute exercise, and identify relationships between skeletal muscle ACSLs and measures of fat metabolism in humans. **Methods:** Sedentary adults (n=14 [4M/10F], BMI 22.2±2.1 kg/m^2, VO_2max 32.2±4.5 ml/kg/min) completed two study visits. Trials were identical other than completing 1 hour of cycling exercise (65% VO_2max) or remaining sedentary. Vastus lateralis biopsies were obtained 15-minutes post-exercise (or rest) and 2-hours post-exercise to determine ACSL protein abundance. Whole-body fat oxidation was assessed at rest and during exercise using indirect calorimetry. Skeletal muscle triacylglycerol (TAG) was measured via lipidomic analysis. **Results:** We detected protein expression for 4 of the 5 known ACSL isoforms in human skeletal muscle. ACSL protein abundances were largely unaltered in the hours following exercise aside from a transient increase in ACSL5 15-minutes post-exercise (*P*=0.01 vs. Rest). Skeletal muscle ACSL1 protein abundance tended to be positively related with whole-body fat oxidation during exercise (*P*=0.07, r=0.53), when skeletal muscle accounts for the majority of energy expenditure. No such relationship between ACSL1 and fat oxidation was observed at rest. Skeletal muscle ACSL6 protein abundance was positively associated with muscle TAG content at rest (*P*=0.05, r=0.57). **Conclusion:** Most ACSL protein isoforms can be detected in human skeletal muscle, with minimal changes in abundance following acute exercise. Our findings agree with those from

model systems implicating ACSL1 and ACSL6 as possible determinants of fat oxidation and fat storage, respectively, within skeletal muscle.

Introduction

Understanding the regulation of skeletal muscle fat oxidation and fat storage remains an area of high interest in the context of exercise performance and disease prevention (Kiens, 2006). Acute exercise is known to influence skeletal muscle fat metabolism, including both fat oxidation and fat storage (Décombaz et al., 2001; Ikeda et al., 2002; Kimber et al., 2003; Schenk & Horowitz, 2007; Votruba et al., 2003). Mounting evidence from model systems indicates different long chain acyl-coenzyme A synthetase isoforms (ACSLs) may play an integral role in the regulation of fat oxidation and fat storage within skeletal muscle, but this remains underexplored in humans (Jung & Bu, 2020; Li et al., 2015; Mashek et al., 2006; Stierwalt, Ehrlicher, Robinson, & Newsom, 2020; Teodoro et al., 2017). Identifying the protein abundance and potential roles of different ACSL isoforms within human skeletal muscle at rest and in response to acute exercise may provide novel insight into the regulation of skeletal muscle fat metabolism in humans.

ACSLs modify fatty acids entering muscle cells by catalyzing the formation of long-chain acyl-CoA, thereby enabling further metabolic processing. For example, such “activation” of fatty acids by ACSLs is required before oxidation or storage can occur (Digel, Ehehalt, Stremmel, & Fuellekrug, 2009; Ellis, Frahm, Li, & Coleman, 2010). There are 5 known ACSL isoforms that vary in expression, function, and localization among tissues (Mashek et al., 2006; 2004), with growing interest in the isoform-specific roles in metabolically active tissues such as skeletal muscle (Adeva-Andany et al., 2018; Watt & Hoy, 2012). Genetically modified mouse and cell models have elucidated potential functions of some ACSL isoforms in skeletal muscle (Jung & Bu, 2020; Kwak

et al., 2019; Li et al., 2015; Teodoro et al., 2017). Skeletal muscle-specific knock out of ACSL1 in mice resulted in attenuated fat oxidation at rest and exercise intolerance compared with wild type animals (Li et al., 2015). Overexpression of ACSL5 in cultured human muscle cells increased complete oxidation of palmitate, as well as basal and maximal uncoupled mitochondrial respiration (Kwak et al., 2019). Such findings suggest ACSL1 and ACSL5 may facilitate skeletal muscle fat oxidation. Conversely, knockdown of ACSL6 in cultured human and rat muscle cells attenuated lipid storage, as evidenced by decreased lipid droplet size and lower triacylglycerol (TAG) content (Jung & Bu, 2020; Teodoro et al., 2017). Overexpression of ACSL6 increased phospholipid accumulation and decreased fat oxidation (Teodoro et al., 2017). These findings demonstrate the potential role for ACSL6 in skeletal muscle fat storage. Taken together, genetic modification models suggest distinct roles for different ACSL isoforms in skeletal muscle fat metabolism.

We recently investigated roles of skeletal muscle ACSL isoforms in regulation of fat metabolism using wild type C57BL/6J mice, including physiologic interventions such as high fat diet (HFD)-induced obesity and aerobic exercise training (Stierwalt et al., 2020). We demonstrated skeletal muscle ACSL1 protein abundance was increased in response to the HFD intervention (Stierwalt et al., 2020), which aligns with other adaptive responses we have reported in this model to increase capacity for fat oxidation within skeletal muscle (Dasari et al., 2018; Newsom et al., 2017). We also reported skeletal muscle ACSL6 protein abundance was greater following both HFD and aerobic exercise training, and was positively associated with skeletal muscle fat storage

(Stierwalt et al., 2020). To what extent ACSLs may play similar regulatory roles in human skeletal muscle remains largely unexplored.

The overall objective of this study was to provide much needed insight into skeletal muscle ACSLs in humans. Our first aim was to determine protein expression of known ACSL isoforms in human skeletal muscle and identify effects of acute exercise on skeletal muscle ACSL protein abundance. Our second aim was to identify relationships between skeletal muscle ACSL isoforms and measures of fat metabolism. Herein we demonstrate detection of protein expression for 4 of 5 known ACSL isoforms at rest and after exercise, and provide evidence indicating ACSL isoforms 1 and 6 may play integral roles in human skeletal muscle fat metabolism.

Methods

Participants

Participants were generally healthy and free of any major medical conditions. Inclusion criteria included 18-45 years old and body mass index (BMI) between 18-26 kg/m^2. All participants were nonsmokers, weight stable (± 2 kg) for ≥ 6 months, and sedentary (less than 60 minutes of purposeful exercise per week) ≥ 6 months. Exclusionary criteria consisted of structured physical activity (>60 minutes per week), changes in body weight (>2 kg in previous 6 months), hyperglycemia (fasting glucose >126 mg/dl), hypertension, cancer, heart disease, pregnancy, un-treated hypo- or hyperthyroid, and allergy to lidocaine. Exclusionary medications included insulin, metformin, thiazolidinediones, statins, chronic non-steroidal anti-inflammatory

medications, hypertensive treatments and hyperlipidemic treatments. Regular use of oral contraceptive medications was permitted. Females were studied during early follicular phase of menstrual cycle for each metabolic study day.

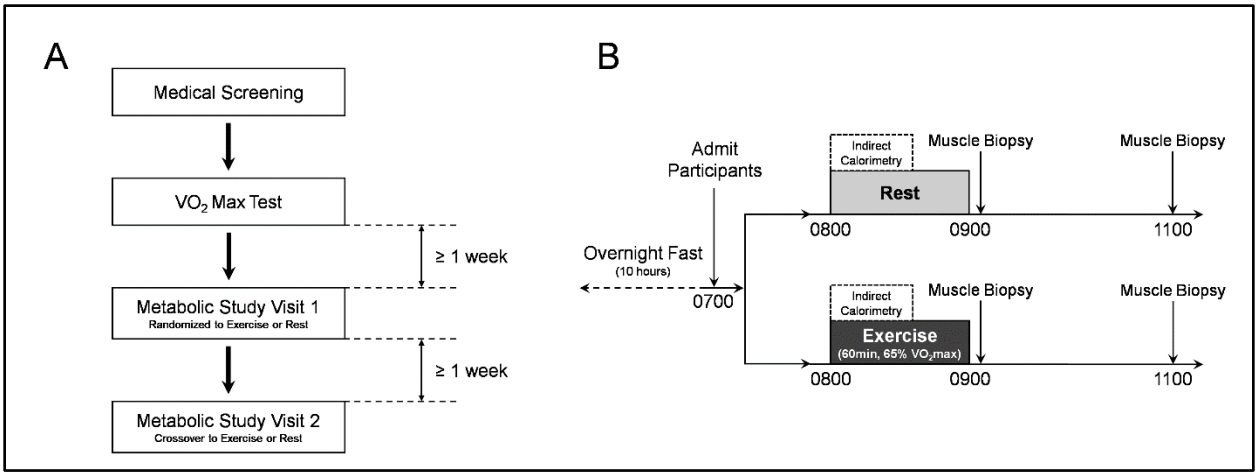

Figure 4.1: Study Design.

A) The overall study design was 4 study visits which included a medical screening, VO_2max testing, and two metabolic study visits separated by at least 1 week. Participants completed metabolic study visits in a randomized crossover design. **B)** Metabolic study visits were identical other than completing 1 h of moderate intensity cycling exercise (65% VO_2max) or remaining sedentary. Muscle biopsy samples obtained at 1100 h during the rest trial were not analyzed for this study to eliminate duplication of resting measures.

Overall Study Design

The overall study design is summarized in Figure 4.1A. The study protocol was approved by the Institutional Review Board at Oregon State University (IRB #7605) and registered at clinicaltrials.gov (#NCT02987491). The current evaluation of ACSL isoforms was performed using a sub-set of vastus lateralis muscle biopsies collected during metabolic study days that investigated glucose metabolism following acute exercise. All study procedures were performed at the Samaritan Athletic Medical Center on the campus of Oregon State University. The four study visits were for medical

screening, maximal aerobic capacity (VO_2max) and two metabolic study days. During the medical screening visit, a member of the study team explained all procedures to the participant prior to obtaining written consent. A fasting blood draw was collected for clinical blood panel (analyzed at Samaritan Regional Medical Center). Anthropometrics were collected including height, weight, waist circumference, and body composition was assessed by dual energy x-ray absorptiometry (Hologic). Females were not pregnant as verified using point-of-care urine testing. Eligible participants returned for a graded exercise test on a cycle ergometer with 12-lead electrocardiogram. Participants then completed study visits three and four, which consisted of two separate metabolic study visits (i.e., one no-exercise “rest” trial and one exercise trial), in a randomized crossover design separated by ≥ 1 week.

VO_2max Test

Upon arrival, participants were fitted with a 12-lead electrocardiogram (Nasiff Associates, Inc., Central Square, NY). The VO_2max test was performed on an electronically braked stationary cycle ergometer (Lode Corival) with indirect calorimeter (Parvo Medics, Sandy, UT) at least 1 week prior to the first metabolic study visit. The protocol consisted of a 2-minute warm up at 50 W intensity then increased 25 W each minute for males and increased 15 W each minute for females until volitional fatigue (~8-12 minutes). Participants were verbally encouraged throughout the test. Ratings of perceived exertion (6-20 on Borg scale) and blood pressure were collected every 3 minutes. VO_2max was defined as peak oxygen consumption during 30-second averaging achieved with 1) failure to maintain pedaling cadence of at least 60

revolutions per minute, 2) reaching approximately 10% of age-predicted maximal heart rate, and 3) respiratory exchange ration greater than 1.1.

Experimental Protocol

The experimental protocol for metabolic study visits is presented in Figure 4.1B. Each participant completed two separate metabolic study visits separated by at least 1 week in randomized order. Participants recorded a 24-hour diet log the day prior to their first metabolic study visit which they were then asked to repeat prior to their second metabolic study visit. Participants were admitted to the Samaritan Athletic Medicine Center at 0700 h following an overnight fast. Between 0800 and 0900 hours, participants either rested in bed or completed a single session of exercise on a stationary cycle ergometer (Lode Corival) at 65% of their pre-determined VO_2max. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured at 0800 h by indirect calorimetry (Parvo Medics TrueOne 2400) during both trials for calculation of whole-body substrate oxidation and to verify exercise intensity. Resting indirect calorimetry was measured while participants lay in a quiet room with low lights for 30 minutes using a ventilated-hood, whereas exercise indirect calorimetry was measured during the first 20 minutes of moderate-intensity exercise using a two-way valve mouthpiece. Vastus lateralis skeletal muscle biopsies were obtained at 0915 h (15-minutes post rest and exercise) and 1100 h (120-minutes post-exercise) during both trials. A local anesthetic (2% lidocaine) was used to numb the biopsy site before a 6-8 mm incision was made through the skin and fascia. The muscle biopsy sample was obtained using a 5 mm Bergström needle with light suction, with the 0915 and 1100 h

biopsies obtained from contralateral legs. For this study, only the 1100 h muscle biopsy sample from the exercise trial was used to measure ACSL protein abundance 120 minutes post-exercise to preserve human muscle sample by eliminating duplication of resting measures. Muscle biopsy samples were dissected free of adipose and connective tissue, blotted dry, frozen in liquid nitrogen, and stored at –80˚C until further analysis.

Whole-body Fat Oxidation

Rates of whole-body fat oxidation were calculated from VO_2 and VCO_2 measures using the equations of Frayn and normalized to body weight (Frayn, 1983). At minimum, the first 5 minutes of data acquisition were not used for calculations to allow for equilibration. The average values for VO_2 and VCO_2 measures were obtained over the course of ~10 minutes once steady state was achieved. Exercise intensity was adjusted via cycle ergometer resistance to achieve the desired 65% of VO_2max. We recognize measures of whole-body fat oxidation are not specific to skeletal muscle. However, skeletal muscle comprises ~40% of total body mass and is known to be a principal determinant of energy expenditure at rest (Zurlo et al., 1990). During moderate-intensity exercise, whole-body substrate oxidation is largely representative of metabolism within skeletal muscle (Romijn et al., 1993; van Loon, Greenhaff, Constantin-Teodosiu, Saris, & Wagenmakers, 2001).

Skeletal Muscle Fat Storage

Skeletal muscle lipids were extracted and analyzed as previously reported by our group (Dasari et al., 2018; Stierwalt, Ehrlicher, Bergman, Robinson, & Newsom, 2018). Vastus lateralis muscle 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).

Skeletal Muscle ACSL Abundance

Vastus lateralis muscle biopsy samples (~30 mg) were homogenized using glass-glass homogenizers as described previously by our group (Stierwalt et al., 2018; 2020). In brief, samples were homogenized 1:10 wt/vol in lysis buffer with protease inhibitors (20 mM Tris HCL, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1x Sigma Protease Inhibitor Cocktail no. P8340). Homogenates rotated at 4ºC for 20 minutes and centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatant was stored at -80°C until further analysis. Approximately 30 µg protein was separated on bis-tris gels and transferred to nitrocellulose membranes. Each gel was loaded with the same internal control sample in 2 lanes, the average density of both lanes was used to normalize band density between gels. Ponceau staining of membranes was used to verify equal loading and transfer of protein. Membranes were blocked in 5% bovine serum albumin (BSA) in tris-buffered saline with tween (TBST) and incubated in primary antibodies at 4°C. Following primary incubation, membranes were washed in TBST and incubated in secondary antibody diluted in blocking buffer at room temperature. Images were generated using infrared detection (LI-COR Odyssey). Primary antibodies used included ACSL1 (product no. 4047, Cell Signaling Technology), ACSL3 (product no. 166374, Santa Cruz), ACSL4 (product no. PA5-27137, Invitrogen), ACSL5 (product no. 365478, Santa Cruz) and ACSL6 (product no. PA5-30465, Invitrogen). All primary antibodies were diluted 1:1000 in blocking buffer (5% BSA in TBST). Primary antibodies for ACSL isoforms 1, 4, and 5 have been previously verified by knockdown and/or overexpression models. Internal testing by the manufacturer verified primary antibodies

for ACSL6 by measuring relative expression in various tissues to ensure that the antibody binds to the antigen stated. Primary antibodies for ACSL3 have not been verified by knockdown or overexpression; however, internal testing by the manufacturer verified expression of ACSL3 in mouse muscle cell line (C2C12). The secondary antibodies used were anti-rabbit-700 (product no. 926-68071) and anti-mouse-800 (product no. 926-32212) from LICOR diluted 1:10,000.

Statistical analysis

This study was a repeated cross-over design. The effect of acute moderate-intensity exercise on skeletal muscle ACSL protein abundance was analyzed by repeated measure one-way analysis of variance (ANOVA) model with Dunnett's post-hoc analysis comparing post exercise time-points with rest. Although our study was not powered to investigate differences between males and females, we investigated potential sex-based differences in ACSL isoform protein abundance by unpaired two-tailed student's t-tests. Relationships between skeletal muscle ACSL isoform protein abundances (measured during the rest trial) and measures of fat oxidation and fat storage were analyzed by Pearson's correlational analysis. Statistical significance was set as $P \leq 0.05$. Statistical analysis and generation of figures was performed using Prism version 8 (GraphPad Software) and R Studio version 3.3.1 (R Studio, Inc). Data are presented as mean and standard deviation, with individual data points shown, in accordance with recommendations for the field (Curran-Everett, 2008).

Results

Participant characteristics and exercise intensity

A total of 14 sedentary women and men (female/male: 10/4) completed the study. A summary of participant characteristics, substrate oxidation at rest and during exercise, and skeletal muscle fat storage at rest are presented in Table 4.1. Study participants were younger adults (28 ± 7 years of age) and (body mass index 22 ± 2). All participants successfully completed the 1-hour moderate-intensity exercise session as planned (mean intensity 63 ± 3% of VO_2max).

Table 4.1. 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 at rest and after exercise

We successfully measured 4 of the 5 ACSL isoforms in human vastus lateralis muscle samples via western blotting, including ACSL isoforms 1, 4, 5, and 6, with

notable variance among individuals (Figure 4.2). ACSL3 was below limits of detection (data not shown). We also identified the effects of acute exercise on skeletal muscle ACSL protein abundance 15- and 120-minutes post-exercise. ACSL5 protein abundance was significantly increased 15-minutes post-exercise (+26% vs. Rest, *P*=0.01, Figure 4.2C) but returned to basal levels by 120-minutes (Figure 2C). ACSL isoforms 1, 4 and 6 were not significantly altered 15- or 120-minutes post-exercise (Figure 4.2).

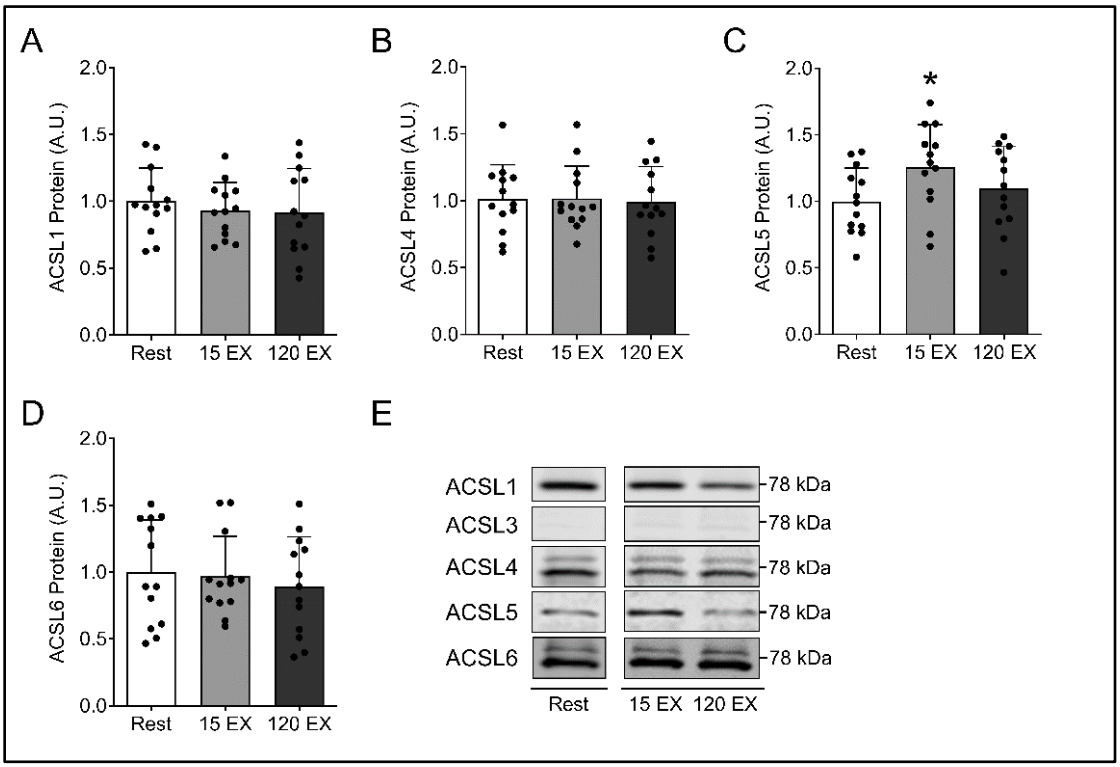

Figure 4.2: Skeletal muscle ACSL isoform protein abundance at rest and following acute exercise.

ACSL isoform protein abundance in vastus lateralis muscle biopsy samples at rest, 15 minutes after acute exercise (15 EX), and 120 minutes after acute exercise (120 EX). Total protein abundance for **A)** ACSL1, **B)** ACSL4, **C)** ACSL5, and **D)** ACSL6, with **E)** representative western blot images. Each representative image was spliced from the same blot/image for the purpose of highlighting samples reported in this study, with no alterations to the images. Full blot and ponceau images for each representative image were made available during the review process. The effects of acute exercise on skeletal muscle ACSL protein abundance were analyzed by repeated measures one-way analysis of variance models, with Dunnett's post-hoc analysis comparing post exercise time-points to rest. Data are presented as mean and standard deviation with individual data points shown. *$P \leq 0.05$ vs. Rest. n=14 (female/male: 10/4).

Although our study was not designed to identify differences between males and females, we investigated the potential for sex-based differences of skeletal muscle ACSL protein abundance at rest. Female participants tended to have greater total protein abundance for all measured ACSL isoforms compared with males, with sex-based differences in ACSL1 and ACSL6 achieving statistical significance ($P \leq 0.01$ for females vs. males, Figure 4.3). There were no sex-based differences on the acute effects of exercise on skeletal muscle ACSL isoform protein abundance (data not shown). Collectively, we demonstrate 4 of the 5 known ACSL isoforms are readily detectable in human skeletal muscle, with only transient changes in ASCL5 protein abundance following acute exercise and the potential for sex-based differences in skeletal muscle ACSL protein abundance.

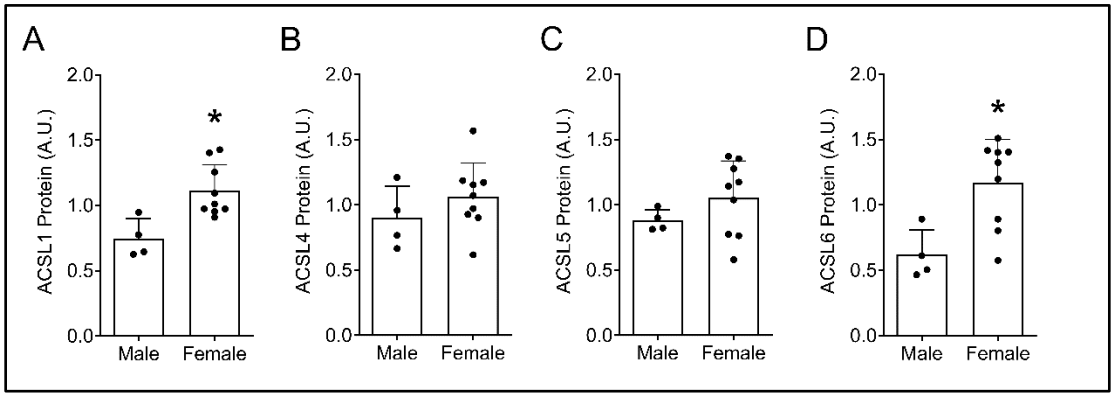

Figure 4.3: Resting skeletal muscle ACSL isoform protein abundance in males and females.

Sex-based differences in ACSLs were evaluated by comparing resting measures of ACSL protein abundances between female and male participants (using resting ACSL data presented in Figure 2). Sex-based evaluation of resting skeletal muscle ACSL abundance includes: **A)** ACSL1, **B)** ACSL4, **C)** ACSL5, and **D)** ACSL6. The effect of sex on skeletal muscle ACSL protein abundance was analyzed by unpaired two-tailed student's t-tests. Data are presented as mean and standard deviation with individual data points shown. *$P \leq 0.05$ vs. Rest. n=14 (female/male: 10/4).

Relationships between skeletal muscle ACSLs and fat metabolism

We next investigated relationships between skeletal muscle ACSL isoforms and measures of fat metabolism in humans. Previous evidence in model systems demonstrates ACSL1 and ACSL5 may direct fatty acids toward oxidation whereas ACSL6 may be important for fatty acid storage; less is known regarding the role of other ACSL isoforms in skeletal muscle fat metabolism. We therefore performed unbiased correlation analysis between ACSL protein abundances and measures of fat oxidation and skeletal muscle TAG concentration (Figure 4.4A). Whole-body fat oxidation at rest was not related to skeletal muscle ACSL1 protein abundance (P=0.64, r=0.15, Figure 4.4B). During exercise, however, when skeletal muscle metabolism constitutes the vast majority of total substrate oxidation (Romijn et al., 1993; van Loon et al., 2001), whole-body fat oxidation tended to positively relate to ACSL1 protein abundance (P=0.07, r=0.53, Figure 4.4C). ACSL5 protein abundance was not related to measures of whole-body fat oxidation at rest or during exercise (P=0.44 and P=0.39, Figure 4.4D and 4.4E, respectively). Resting skeletal muscle TAG concentration (i.e., fat storage) was positively related to ACSL6 protein abundance (P=0.05, r=0.57, Figure 4.4F). Skeletal muscle TAG content was also positively related to skeletal muscle ACSL1 protein abundance (P=0.01, r=0.67). Taken together, these findings suggest ACSL1 and/or ACSL6 may be important for fat oxidation during exercise and skeletal muscle fat storage.

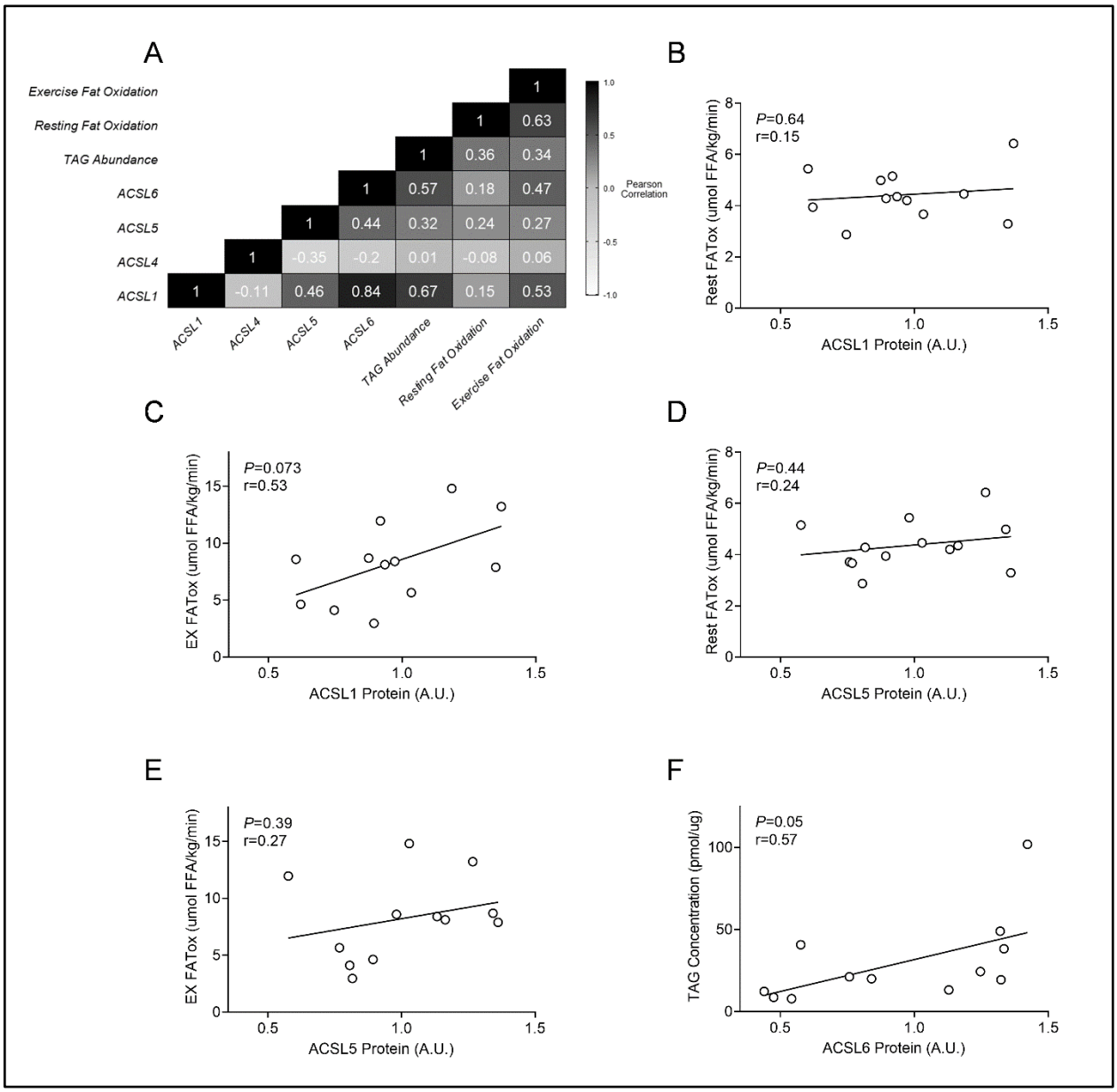

Figure 4.4: Relationships between skeletal muscle ACSL protein abundance and measures of fat metabolism.

A) Unbiased correlation analysis to identify potential relationships between ACSLs and measures of fat oxidation and storage. **B-F)** Relationships of interest determined *a priori* on the basis of other reported findings. The pre-determined relationships of interest included **B)** skeletal muscle ACSL1 protein abundance and whole-body fat oxidation at rest (Rest FATox), **C)** skeletal muscle ACSL1 protein abundance and whole-body fat oxidation during moderate-intensity exercise (EX FATox), **D)** skeletal muscle ACSL5 protein abundance and Rest FATox, **E)** skeletal muscle ACSL5 protein abundance and EX FATox, and **F)** skeletal muscle ACSL6 protein abundance and skeletal muscle triacylglycerol (TAG) concentration. n=14 (female/male: 10/4).

Discussion

The primary aims of this study were to measure known ACSL isoforms in human skeletal muscle, determine the effects of acute exercise on skeletal muscle ACSL protein abundance, and identify relationships between ACSL isoforms and measures of fat metabolism. Protein abundance for ACSL isoforms 1, 4, 5, and 6 was readily detected in human vastus lateralis muscle, whereas ACSL3 was below limits of detection. Even among a relatively homogeneous population of sedentary lean adults, there was notable variability in skeletal muscle ACSL abundance among participants, including strong evidence for possible sex-based differences in ACSL abundance. Skeletal muscle ACSL protein abundance was largely unchanged in the hours after acute moderate-intensity exercise, the one exception being a modest increase in ACSL5 measured 15 minutes post-exercise that returned to resting levels by 120 minutes post-exercise. Skeletal muscle ACSL1 protein abundance tended to be positively related to whole-body fat oxidation during exercise, whereas ACSL6 protein abundance was positively related to resting measures of fat storage (i.e., TAG content). We interpret these findings to indicate skeletal muscle ACSL1 and ACSL6 may be key determinants of skeletal muscle fat oxidation and fat storage, respectively.

ACSLs have emerged as potential critical regulators in fat metabolism; however, the protein expression and roles of ACSL isoforms in human skeletal muscle are currently underexplored. The five known ACSL isoforms exhibit differences in substrate preference and intracellular location (Grevengoed et al., 2014; Mashek et al., 2006; 2007), suggesting distinct functions for each isoform. Isoform localization within skeletal muscle remains to be fully elucidated, though evidence suggests skeletal muscle

ACSL1 and ACSL5 may localize to mitochondria whereas ACSL6 may localize to sarcoplasmic reticulum (Kwak et al., 2019; Li et al., 2015; Teodoro et al., 2017). Additionally, ACSL isoform mRNA expression varies by tissue in rodents, further suggesting isoform-specific roles (Mashek et al., 2006). For example, skeletal muscle mRNA content for ACSL isoforms 1, 3, and 6 are more abundant than isoforms 4 and 5 (Mashek et al., 2006). We recently published evidence of protein expression for ACSL isoforms 1, 4, 5, and 6 in mouse skeletal muscle, demonstrating mRNA may not be indicative of protein abundance (Stierwalt et al., 2020). We add to this literature by demonstrating similar detection of ACSL isoforms 1, 4, 5, and 6 in human skeletal muscle. Protein abundance of ACSL3 was below limits of detection in our samples. This is consistent with our previous findings in mice, yet unexpected on the basis of high reported mRNA expression in rodent skeletal muscle (Mashek et al., 2006). Our findings also demonstrate potential sex-based differences in skeletal muscle ACSL isoform protein expression between male and female participants. Skeletal muscle ACSL1 and ACSL6 were greater in females compared with male participants which may, in part, contribute to sex-dependent differences in skeletal muscle fat metabolism (Lundsgaard & Kiens, 2014). Our study was not designed to investigate such sex-based differences, yet we see this as an important area for continued investigation. Another consideration is the extent by which ACSL expression may vary by muscle fiber type and therefore among skeletal muscles. Skeletal muscle lipid metabolism differs by fiber type (Essén et al., 1975; He et al., 2001), and thus muscle-specific expression of ACSLs remains as an area of interest for future directions.

Acute exercise has been shown to increase rates of fat oxidation (Kimber et al., 2003; Votruba et al., 2003) and intramuscular triacylglycerol synthesis (i.e., fat storage) (Décombaz et al., 2001; Ikeda et al., 2002; Schenk & Horowitz, 2007). Due to the potential role of ACSLs facilitating oxidation and storage of fatty acids within skeletal muscle, we hypothesized that observed changes in skeletal muscle fat metabolism following exercise may be due, in part, to changes in skeletal muscle ACSL protein abundance. In support of this hypothesis, ACSL1 and ACSL6 expression can be upregulated by peroxisome proliferator-activated receptor alpha (PPARα) and sterol regulatory binding element-1c (SREBP-1c) (Durgan et al., 2006; Martin et al., 1997; Schoonjans et al., 1995; Teodoro et al., 2017), respectively, and both transcription factors are upregulated by exercise (Cresci et al., 1996; Horowitz et al., 2000; Ikeda et al., 2002). In contrast to our hypothesis, protein abundance of ACSL isoforms was largely unchanged in the 2 hours following acute moderate intensity-exercise, with the exception of a transient increase in ACSL5. The functional significance of such transient changes in ACSL5 remain to be determined; however, overexpression of ACSL5 in cultured human muscle cells provide evidence that this isoform may be important for fat oxidation (Kwak et al., 2019). Our previous findings in mice demonstrated isoform-specific differences in ACSL protein abundance within skeletal muscle following aerobic exercise training, whereby ACSL4 was lower and ACSL6 was greater compared with remaining sedentary (Stierwalt et al., 2020). It remains possible that acute exercise-induced changes in ACSL protein abundance may require longer to manifest (i.e., more than 2 hours), repeated exercise stimuli or perhaps exercise of a greater duration and/or intensity. Nevertheless, we interpret our current findings to indicate ACSL isoform

protein abundance is largely unchanged in the first few hours following an acute bout of moderate-intensity exercise.

Genetically altered model systems have helped elucidate the potential role of ACSL isoforms 1, 5 and, 6 in skeletal muscle fat metabolism (Jung & Bu, 2020; Kwak et al., 2019; Li et al., 2015; Teodoro et al., 2017). Previous evidence demonstrates ACSL1 is critical for normal skeletal muscle fat oxidation (Li et al., 2015). We therefore anticipated that ACSL1 protein abundance may be related to measures of whole-body fat oxidation. Consistent with our previous findings in mice, however (Stierwalt et al., 2020), ACSL1 protein abundance was not related to resting measures of whole-body fat oxidation in humans. These findings highlight the need for direct measurement of skeletal muscle fat oxidation at rest, given our current evidence demonstrates ACSL1 is positively related with whole-body fat oxidation during exercise, when skeletal muscle is a major determinant of whole-body substrate oxidation. Neither skeletal muscle ACSL4 nor ACSL5 were related to any measures of fat metabolism in the current study. Several reports, including our previous study in mice (Jung & Bu, 2020; Stierwalt et al., 2020; Teodoro et al., 2017), have demonstrated ACSL6 may be a critical determinant for skeletal muscle fat storage. Our current findings further support this conclusion and demonstrate ACSL6 positively relates to resting measures of skeletal muscle TAG concentration (i.e., fat storage) in humans. Interestingly, the strongest correlation among our unbiased analysis was between ACSL1 and ACSL6 protein abundance. Recognizing these two isoforms belong to the same subfamily of ACSLs (Grevengoed et al., 2014; Mashek et al., 2007), we took several steps to evaluate non-specific antibody binding as a potential explanation for this finding. In brief, ACSL1 and ACSL6

were measured on separate membranes, prior evidence using the same ACSL1 antibody detected no protein abundance in skeletal muscle ACSL1 knockout mice (Li et al., 2015), and we previously used these same antibodies in mice demonstrating notable differences between the two isoforms (Stierwalt et al., 2020). Although there is a possibility of some non-specific binding, previous findings generated by us and others using these antibodies in addition to rigorous validation testing by the manufacturer make us confident our findings demonstrate accurate representations of the stated ACSL isoforms within skeletal muscle (Li et al., 2015; Stierwalt et al., 2020). Collectively, we interpret our findings to further support the importance of ACSL1 and ACSL6 in skeletal muscle fat metabolism.

Although our current findings provide more insight into potential isoform-specific roles of skeletal muscle ACSLs in humans, our measures of protein abundance may not reflect ACSL activity. Critical next steps will include identifying functional consequence of identified post-translational modifications to ACSL proteins (Frahm et al., 2011), determining the effects of exercise training on isoform-specific ACSL activity (Cortright et al., 2006), and investigating ACSL isoforms in more diverse study populations which may provide further insight into roles for ACSLs in health and disease. For example, skeletal muscle TAG synthesis across various populations is positively associated with insulin sensitivity and it is unknown if ACSL6 is a contributing mechanism to observed changes in TAG synthesis within skeletal muscle of different populations (Bergman et al., 2018). Additionally, altered skeletal muscle fat oxidation is associated with various disease states which could be, in part, due to changes in ACSL1 function (Koves et al., 2008; Turcotte, Swenberger, Zavitz Tucker, & Yee, 2001). Overall, there is much that

still remains unknown in regard to the role and regulation of ACSL isoforms within skeletal muscle and this is an area ripe for future research in human performance and the prevention of metabolic disease.

In conclusion, protein abundance for 4 of 5 known ACSL isoforms was readily detected in human vastus lateralis muscle. Skeletal muscle ACSL protein abundance was largely unchanged following a single session of acute exercise; however, ACSL5 abundance transiently increased 15-minutes post-exercise. ACSL1 protein abundance tended to be positively related to measures whole-body fat oxidation during moderate-intensity exercise, indicating potential contribution to fat oxidation with increased energetic demand within skeletal muscle. ACSL6 protein abundance was positively related to measures of skeletal muscle TAG content, suggesting contribution to regulation of lipid storage. Collectively, we interpret our evidence to further support the role of ACSLs in skeletal muscle fat oxidation and fat storage in humans.

Acknowledgements

We thank Kathleen Harrison, Ph.D., Karin Zemski Berry, Ph.D., and Bryan Bergman, Ph.D. at the University of Colorado Anschutz Medical Campus for their skilled assistance with lipidomic measures. We also thank Anthony Bouranis for his skilled assistance with R studio in generating the correlation matrix figure. Lastly, we would like to thank the participants and the helpful staff at Samaritan Athletic Medicine Center, including Drs. Nicholas Phillips, Craig Graham and Joshua Lenhof. The results of the present investigation do not constitute endorsement by the American College of Sports

Medicine. We declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Disclosures

The authors have no conflict of interest to declare.

Grants

This project was partly supported by the ACSM Foundation Doctoral Student Research Grant from the American College of Sports Medicine Foundation awarded to H.D.S. This project was also supported by KL2TR002370 awarded to S.A.N. as part of the Oregon Clinical & Translational Research Institute's Clinical and Translational Science Award from the National Institutes of Health, the Collins Medical Trust awarded to S.A.N. and M.M.R., and the John C. Erkkila, M.D. Endowment for Health and Human Performance awarded to S.A.N. and M.M.R. M.M.R. was supported by DK103829 from the National Institutes of Health. H.D.S. and S.E.E. are supported by fellowships from Oregon State University.

Author Contributions

H.D.S., M.M.R. and S.A.N. designed study and performed experiments. S.E.E. performed experiments. H.D.S. performed the analysis and drafted the manuscript.

S.E.E., M.M.R. and S.A.N. edited the manuscript. S.A.N. is the guarantor of this work and, as such, takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Chapter 5 – AMPK-specific TBC1D1 phosphorylation, not Rac1 signaling contribute to the insulin sensitizing effects of acute exercise in humans

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AMPK-specific TBC1D1 phosphorylation, not Rac1 signaling contribute to the insulin sensitizing effects of acute exercise in humans

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Title characters (including spaces): 130
Abstract (words): 282
Word count: 4662

Abstract

A single session of moderate-intensity exercise improves insulin sensitivity in most humans; however, the mechanisms are incompletely understood. Insulin or muscle contraction promotes GTP binding and activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) that reorganizes actin filaments and promotes glucose uptake. It remains unknown in humans if enhanced insulin-stimulated activation of Rac1 contributes to the insulin sensitizing effects of exercise. Sedentary lean adults (n=14 [4M/10F], BMI 22.2±2.1 kg/m^2, VO_2max 32.2±4.5 ml/kg/min) completed two metabolic study visits in a randomized crossover design of 1-hour of moderate-intensity cycling exercise (65% VO_2max) or rest. Insulin sensitivity was determined as glucose infusion rate during the final 30 min of a 3-hour hyperinsulinemic-euglycemic clamp (insulin infused at 2.3 mU/kg FFM/min) started at 120 min post-exercise or rest. Vastus lateralis muscle biopsies were obtained 15 min post-exercise or rest, 120 min post-exercise/rest, and 1 h into a hyperinsulinemic-euglycemic clamp (180 min post-exercise/rest) to determine Rac1-GTP binding by ELISA and insulin signaling by western blotting. Rac1-GTP binding and activation of downstream p21-activated kinase (PAK) were increased 15 min post-exercise (P<0.05 vs. rest); whereas Akt phosphorylation (Thr308 or Ser473) was not altered (P>0.20 vs. rest for both). Exercise increased glucose infusion rates (+12±16.5%, P=0.03 vs. rest) following exercise compared with rest. Despite improved insulin sensitivity, exercise did not enhance insulin-stimulated phosphorylation of Akt (P=0.40), Rac1-GTP binding (P=0.36) or phosphorylation of PAK (P=0.71) compared with rest. However, AMPK-induced activation of TBC1D1 remained increased 180-min post-exercise (P=0.04 vs. 915 Rest) which was greater compared to rest trial

(*P*=0.01 for EX vs. Rest). We interpret these findings to indicate mechanisms independent of Rac1 signaling, such as AMPK-induced activation of TBC1D1, contribute to the insulin sensitizing effects of exercise in healthy adults.

Introduction

Skeletal muscle accounts for up to 80% of insulin-stimulated glucose disposal in the postprandial state and is thus a significant determinant of systemic glucose metabolism (DeFronzo & Tripathy, 2009). Impairments in skeletal muscle insulin sensitivity disrupt glucose homeostasis and act as a major contributor to insulin resistance and type 2 diabetes (DeFronzo & Tripathy, 2009). A single session of exercise can improve insulin sensitivity; however, the mechanisms are incompletely resolved. Identifying these mechanisms may provide necessary information to develop therapeutic interventions to promote skeletal muscle insulin sensitivity of at-risk populations.

A single session of exercise can increase insulin-stimulated glucose disposal into skeletal muscle across a range of intensities and populations (Devlin & Horton, 1985; D. S. King et al., 1988; Newsom et al., 2013). The canonical insulin signaling involves activation of insulin receptor substrate-1 (IRS-1) then phosphatidylinositol 3-kinase (PI3K) activation of protein kinase B (Akt). Yet, the increase in glucose uptake occurs despite varied proximal insulin signal events, suggesting multiple signaling cascades. For example, improvements in skeletal muscle insulin action post-exercise appear to be independent of changes to proximal insulin signaling including Akt phosphorylation despite greater insulin-stimulated glucose uptake (Bonen, Tan, & Watson-Wright, 1984; Fisher, Gao, Han, Holloszy, & Nolte, 2002; Hamada, Arias, & Cartee, 2006; Wojtaszewski et al., 2000; Wojtaszewski, Hansen, Kiens, & Richter, 1997). Increased skeletal muscle insulin action post-exercise may therefore be due to exercise-induced improvements in insulin signaling that are distal to, or independent of, Akt.

Ras-related C3 botulinum toxin substrate 1 (Rac1), a small Rho-family GTPase is one such mechanism for insulin-stimulated glucose uptake independent of Akt (Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014b; Ueda et al., 2010). Rac1 is active in the GTP-bound state and induces reorganization of actin-filaments near the sarcolemmal membrane which facilitate the exocytosis of glucose transporter 4 (GLUT4) vesicles to the cell surface during insulin stimulation (Chiu et al., 2011). Importantly, Rac1 is also activated by mechanical stretch (i.e. muscle contraction) (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b), making Rac1 a target of interest for investigating improvements in insulin-stimulated glucose uptake post-exercise.

Activation of Rac1 by insulin and exercise may occur via independent mechanisms, and therefore may be additive. Insulin-dependent activation of Rac1 occurs via PI3K dependent action. Inhibition of PI3K blocks insulin-stimulation of Rac1-GTP binding (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Conversely to what is known regarding Rac1 activation by insulin, exercise-induced activation of Rac1 remains largely unknown. Ex vivo studies indicate exercise-induced Rac1 activation is occurs via mechanical stress following muscle stretch or contraction (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b; Sylow, Møller, Kleinert, Richter, & Jensen, 2015). Prior exercise may therefore enhance insulin-stimulated Rac1 activation in skeletal muscle and contribute to improvements in insulin sensitivity observed post-exercise. Recent findings demonstrate inducible knock out of Rac1 (specific to skeletal muscle) did not impair the insulin-sensitizing effects of exercise, indicating Rac1 is dispensable for this effect (Sylow, Møller, et al., 2016a). Nevertheless, such findings do

not rule out the possibility that Rac1 contributes to the insulin-sensitizing effects of exercise in humans with functional Rac1.

The primary aim of this study was to identify the combined effects of acute exercise and insulin stimulation on Rac1 activation and contribution to insulin sensitivity in humans . We hypothesized that prior moderate-intensity exercise would increase insulin-stimulated Rac1 activation post-exercise compared with remaining sedentary. In contrast to our hypothesis, the improvement in insulin sensitivity after an acute bout of exercise was not associated with enhanced insulin-stimulated activation of Rac1 or its downstream effector p21-activated kinase (PAK). In contrast, we identified AMPK-dependent activation of TBC1D1 remained increased in the hours after exercise and may contribute to the improvements in insulin sensitivity after exercise (Kjøbsted et al., 2017; 2019). We interpret our evidence to indicate mechanisms independent of Rac1 signaling contribute to the acute insulin sensitizing effect of exercise among healthy humans.

Methods

Participants

Participants were generally healthy and free of any major medical conditions. All participants were nonsmokers, weight stable (± 2 kg for ≥ 6 months), and sedentary (less than 60 minutes of purposeful exercise per week for ≥ 6 months). Exclusionary criteria consisted of structured physical activity (>60 minutes per week), changes in body weight (>2 kg in previous 6 months), hyperglycemia (fasting glucose >126 mg/dl),

hypertension, cancer, heart disease, pregnancy, un-treated hypo- or hyperthyroid, and allergy to lidocaine. Exclusionary medications included insulin, metformin, thiazolidinediones, statins, chronic non-steroidal anti-inflammatory medications, hypertensive treatments and hyperlipidemic treatments. Regular use of oral contraceptive medications was permitted. Females were studied during early follicular phase of menstrual cycle for each metabolic study day.

Study Design

The study protocol was approved by the Institutional Review Board at Oregon State University (IRB #7605) and registered at clinicaltrials.gov (#NCT02987491). All study procedures were performed at the Samaritan Athletic Medical Center on the campus of Oregon State University. The four study visits were for medical screening, maximal aerobic capacity (VO2max) and two metabolic study days. During the medical screening visit, a member of the study team explained all procedures to the participant prior to obtaining written consent. A fasting blood draw was collected for clinical blood panel (analyzed at Samaritan Regional Medical Center). Anthropometrics were collected including height, weight, waist circumference, and body composition was assessed by dual energy x-ray absorptiometry (Hologic Horizon). Females were not pregnant as verified using point-of-care urine testing. Eligible participants returned for a graded exercise test on a cycle ergometer with 12-lead electrocardiogram. Participants then completed study visits three and four, which consisted of two separate metabolic study visits (i.e., one sedentary “rest” trial and one exercise trial), in a randomized crossover design separated by ≥ 1 week.

VO_2max Test

The VO_2max test was performed on an electronically braked stationary cycle ergometer (Lode Corival) with indirect calorimeter (Parvo Medics, Sandy, UT). The protocol consisted of a 2-minute warm up at 50 W, intensity then increased 25 W each minute for males and 15 W for females until volitional fatigue (~8-12 minutes). Participants were verbally encouraged throughout the test. Ratings of perceived exertion (6-20 on Borg scale) and blood pressure were collected every 3 minutes. VO_2max was defined as peak oxygen consumption during 30-second averaging achieved with 1) failure to maintain pedaling cadence of at least 60 revolutions per minute, 2) reaching approximately 10% of age-predicted maximal heart rate, and 3) respiratory exchange ration greater than 1.1.

Experimental Protocol

The experimental protocol for each metabolic study visit is presented in Figure 5.1.

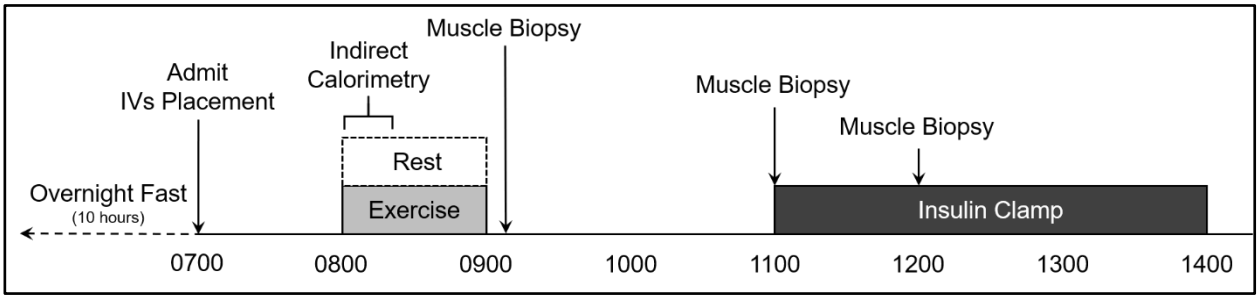

Figure 5.1: Study Design.

Metabolic study visits were identical other than completing 1 h of moderate intensity cycling exercise (60 minutes, 65% VO2max) or remaining sedentary. Insulin clamp, hyperinsulinemic euglycemic clamp.

Each participant completed two separate metabolic study visits separated by ≥ 1 week in a randomized crossover design. Participants recorded a 24-hour diet log the day prior to their first metabolic study visit which they were asked to repeat prior to their second metabolic study visit. Participants were admitted to the Samaritan Athletic Medicine Center at 0700 h following a 10-hour overnight fast. Upon arrival participants had two intravenous catheters placed: one in the left antecubital vein for hormone and substrate infusion and one in the right antecubital vein with an electric heating pad wrapping the lower portion of the arm to obtain arterialized blood samples (M. D. Jensen & Heiling, 1991). Between 0800 and 0900 h, participants either continued to rest in bed or completed a single session of exercise on a stationary cycle ergometer (Lode Corival) at 65% of their pre-determined VO_2max. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured at 0800 h by indirect calorimetry (Parvo Medics TrueOne 2400) during both trials for calculation of energy expenditure, whole-body substrate oxidation, and to verify exercise intensity. Exercise intensity was adjusted via modification of cycle ergometer resistance to achieve the desired 65% of VO_2max. During the Rest trial, indirect calorimetry was measured while participants lay in a quiet low-lit room for 30 minutes using a ventilated-hood. During the Exercise trial, indirect calorimetry was measured during the first ~20 minutes of exercise using a two-way valve mouthpiece. Vastus lateralis skeletal muscle biopsies were obtained at 0915 h (15-minutes post rest or exercise), 1100 h (120-minutes post rest or exercise), and 1200 h (180-minutes post rest or exercise). A local anesthetic (2% lidocaine) was used on the biopsy site before a 6-8 mm incision was made through the skin and fascia. The muscle biopsy sample was obtained using a 5 mm Bergström needle with light suction, with the

0915, 1100, and 1200 h biopsies obtained from contralateral legs, with counterbalancing of biopsies from each leg among trials. Importantly, the 1200 h biopsy was sampled a minimum of 2 inches proximal to the 0915 h biopsy to minimize impact of inflammation, etc. (Cotter et al., 2013). For this study, the 1100 h muscle biopsy sample from the rest trial was not processed to preserve human muscle sample by eliminating duplication of resting measures. Muscle biopsy samples were dissected free of adipose and connective tissue, blotted dry, frozen in liquid nitrogen, and stored at –80˚C until further analysis. Immediately after the 1100 h muscle biopsy, a 3-h hyperinsulinemic euglycemic clamp was performed between 1100 h and 1400 h to assess insulin sensitivity during both trials. The clamp was performed using a constant insulin infusion rate of 2.3 mU/kg FFM/min for 3 hours. Blood glucose concentrations were measured in duplicate every 10 minutes during the clamp using a handheld glucometer (Accu-Chek Performa), while a 20% dextrose solution was infused at a variable rate to maintain a target glycemia of 90 ± 5 mg/dL. Steady state plasma insulin and glucose infusion rates were determined by averaging the values obtained in the final 30 minutes of the hyperinsulinemic euglycemic clamp.

Blood sampling and insulin concentrations

In addition to blood sampling to determine blood glucose concentration every 10 minutes, larger blood samples were collected at 1100, 1200, 1300, 1330, 1340, 1350, and 1400 h to measure plasma insulin concentration. Collected blood samples were transferred to either potassium-ethylenediaminetetraacetic acid (K2EDTA) or sodium-heparin spray-coated collection tubes (BD Vacutainer). Collection tubes were

centrifuged at 1,000 × *g* for 10 minutes to separate plasma which was stored at –80˚C until further analysis. Plasma insulin concentrations were measured by immunosorbent assay according to the manufacturer recommendations (ab200011, Abcam).

Energy expenditure and substrate oxidation

Energy expenditure during rest and exercise was calculated from VO_2 and VCO_2 measurements using the Weir equation (Mansell & Macdonald, 1990). Resting metabolism was determined via ventilated hood (Parvo) for 20 minutes while participants rested quietly with lights dimmed. Whole-body substrate oxidation was calculated from VO_2 and VCO_2 measurements using equations of Frayn (Frayn, 1983). At minimum, the first 5 minutes of indirect calorimetry data acquisition were not used for calculations to allow for equilibration. The average values for VO_2 and VCO_2 measures were obtained over the course of ~10-15 minutes once steady state was achieved.

Rac1 activation

Skeletal muscle Rac1-GTP binding was measured by immunosorbent assay as previously described and according to the manufacturer recommendations (BK128, Cytoskeleton Inc.) (Stierwalt et al., 2018). Vastus lateralis muscle biopsy samples (~5 mg) were homogenized by a handheld micro-tube homogenizer in lysis buffer plus protease inhibitors. Tissue lysates were centrifuged for 1 min at 10,000 x *g* and immediately flash frozen in liquid nitrogen. Sample protein concentrations were equalized and 50 ug of protein was loaded into wells coated with Rac1-GTP binding

domain. GTP-bound Rac1 was determined following a colorimetric reaction using antibodies toward Rac1 linked with horseradish peroxidase activity.

Western Blotting

Vastus lateralis muscle biopsy samples (~30 mg) were homogenized using glass-glass homogenizers as described previously by our group (Newsom et al., 2017; Stierwalt et al., 2018; 2020). In brief, samples were homogenized 1:10 wt/vol in lysis buffer with protease inhibitors (20 mM Tris HCL, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1x Sigma Protease Inhibitor Cocktail no. P8340). Homogenates rotated at 4ºC for 20 minutes and then centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatant was stored at -80°C until further analysis. Approximately 30 µg protein was separated on bis-tris gels and transferred to nitrocellulose membranes. Each gel was loaded with the same internal control sample in 2 lanes, the average density of both lanes was used to normalize band density between gels. Ponceau staining of membranes was used to verify equal loading and transfer of protein. Membranes were blocked in 5% bovine serum albumin in tris-buffered saline with tween (TBST) and incubated in primary antibodies at 4°C. Following primary incubation, membranes were washed in TBST and incubated in secondary antibody diluted in blocking buffer at room temperature. Images were generated using infrared detection (LI-COR Odyssey). Primary antibodies used included Rac1 (product no. ARC03) from Cytoskeleton Inc., PAK1 (product no. 2602), pPAK 1/2$^{Thr423/402}$ (product no. 2601), Akt (product no. 2920), pAkt^{Thr308} (product no. 9275), pAkt^{Ser473} (product no. 9271), AMPK (product no. 2793), pAMPK^{T172} (product no. 2535) from Cell

Signaling Technology, TBC1D1 (product no. 229504) from Abcam, and pTBC1D1^{Ser231} (product no. 07-2268) from Millipore Sigma. The secondary antibodies used were anti-rabbit-700 (product no. 926-68071) and anti-mouse-800 (product no. 926-32212) from LICOR diluted 1:10,000.

Statistical analysis

The effect of exercise on steady state plasma insulin, blood glucose, and insulin-stimulated glucose infusion rate was determined by comparing the sedentary rest and exercise trials using paired two-tailed student's t-tests. The effects of exercise on insulin signaling were determined by repeated-measures one-way analysis of variance (ANOVA) tests with specific post-hoc comparisons determined *a priori;* these comparisons investigated the effects of insulin and exercise by comparing 915 R vs. 1200 R, 915 EX, 1100 EX, and 1200 EX biopsy samples and the insulin sensitizing effects of exercise by comparing the 1200 R and 1200 EX biopsy samples. Statistical significance was set as $P \leq 0.05$. Statistical analysis and generation of figures were performed using Prism version 8 (GraphPad Software). Data are presented as mean and standard deviation, in accordance with recommendations for the field (Curran-Everett, 2008).

Results

Participant characteristics and exercise characterization

Characteristics of the participants that completed this study were recently reported (Dissertation Chapter 4). Study participants (n=14; 10 females and 4 males) were younger adults (28 ± 7 years of age), relatively lean (BMI 22 ± 2 kg/m^2), yet sedentary (32.2 ± 4.5 ml/kg/min). All participants successfully completed the 1-hour moderate-intensity exercise session as planned. Exercise energy expenditure and substrate oxidation are reported in Table 1. Exercise intensity was well controlled (mean intensity 63 ± 3% of VO_2max) and participants expended an average of 382 ± 75 kcals.

Table 5.1. Energy expenditure at rest and during exercise	
Resting energy expenditure (kcal/day)	1501 ± 262
Exercise energy expenditure (kcal)	382 ± 75
Exercise respiratory exchange ratio (RER)	0.94 ± 0.04
Exercise fat oxidation (µmol fatty acid/kg)	503 ± 203
Exercise carbohydrate oxidation (µmol glucose/kg)	7322 ± 1517
Data are presented as mean ± standard deviation.	

Plasma insulin concentration and glucose infusion rates

Hyperinsulinemic-euglycemic clamps were successfully completed as designed. A continuous insulin infusion rate of 2.3 mU/kg FFM/min resulted in hyperinsulinemia (Figure 2A) with no difference in steady state plasma insulin concentrations between rest and exercise trials (*P*=0.35 vs. Rest, Figure 5.2A). Euglycemia was maintained at ~90 mg/dl, with no difference in steady state glucose concentrations between trials (Figure 5.2B). Steady state glucose infusion rates were significantly greater following moderate-intensity exercise (+12 ± 16% vs. Rest, *P*=0.03, Figure 5.2C), indicating improved insulin sensitivity following a single session of moderate-intensity exercise compared with remaining sedentary.

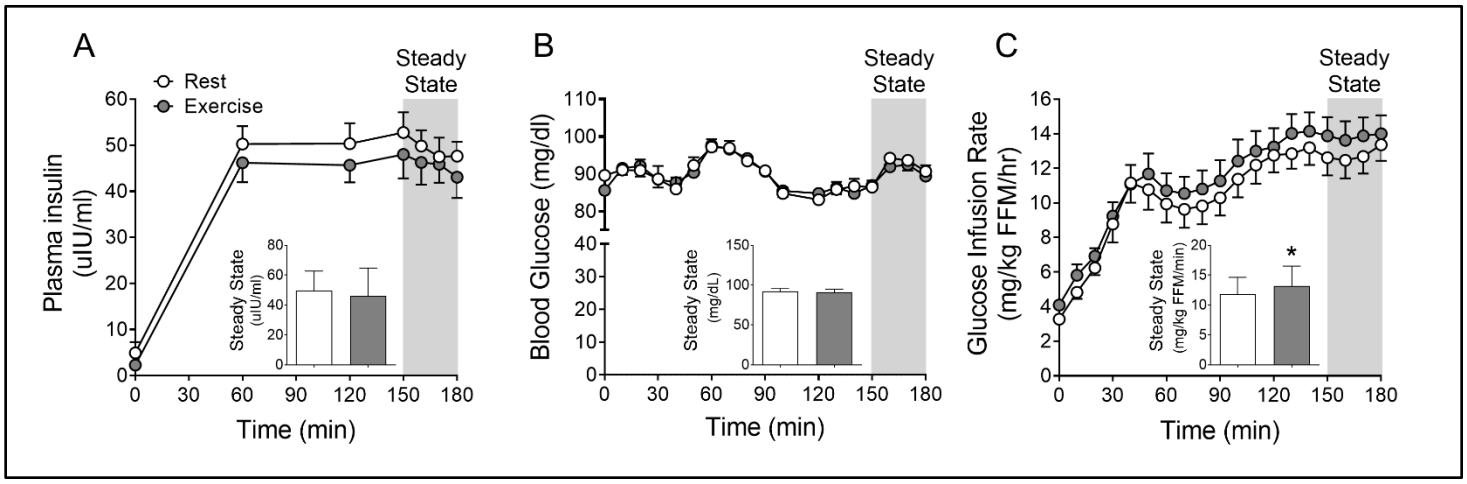

Figure 5.2: Hyperinsulinemic euglycemic clamp.

Participants underwent a 3-hour hyperinsulinemic euglycemic clamp to determine whole-body insulin sensitivity at rest and after a single session of moderate intensity exercise. **A)** Plasma insulin concentration over the course of the hyperinsulinemic euglycemic clamp. **B)** Blood glucose was measured in duplicate was maintained at ~90 mg/dl via **C)** variable glucose infusion rates during the clamp. Panels A-C are presented as mean and standard error over the course of the hyperinsulinemic euglycemic clamp with steady state measures for each variable displayed as an inset. *$P \leq 0.05$ vs. 915 R for paired t-test. n=14 (female/male: 10/4).

Rac1 and PAK activation during rest and post-exercise

We next measured Rac1-GTP binding and activation of its downstream effector PAK during rest, after exercise, and during insulin stimulation. Rac1-GTP binding was significantly increased 15-minutes post-exercise ($P<0.01$ for 915 EX vs. 915 R, Figure 5.3A) which returned to basal levels by 120-minutes after exercise ($P=0.30$ for 1100 EX vs. 915 R). Rac1-GTP binding was also significantly increased 1 hour into the hyperinsulinemic-euglycemic clamp compared with basal resting measures ($P=0.02$ for 1200 R vs. 915 R). Contrary to our hypothesis, there was no difference in insulin-stimulated Rac1-GTP binding between the rest and exercise trials ($P=0.52$ for 1200 R vs. 1200 EX). These findings were corroborated by similar effects of exercise and insulin on activation of PAK, a downstream effector of Rac1 (Bokoch, 2003; Sylow,

Jensen, Kleinert, Højlund, et al., 2013a), measured by phosphorylation at Threonine 423 and 402 (pPAK1/2$^{Thr423/402}$). pPAK1/2$^{Thr423/402}$ was increased 15-minutes post-exercise (P<0.01 for 915 EX vs. 915 R, Figure 5.3C) and 1 hour into the hyperinsulinemic-euglycemic clamp (P<0.01 for 1200 R vs. 915 R), with no difference in insulin-stimulated activation between the rest and exercise trials (P=0.28 for 1200 R vs. 1200 EX). Total protein abundance for Rac1 and PAK1 was not altered by insulin or exercise (Figure 5.3). Collectively, our findings suggest that insulin-stimulated Rac1 signaling is not further enhanced by prior exercise.

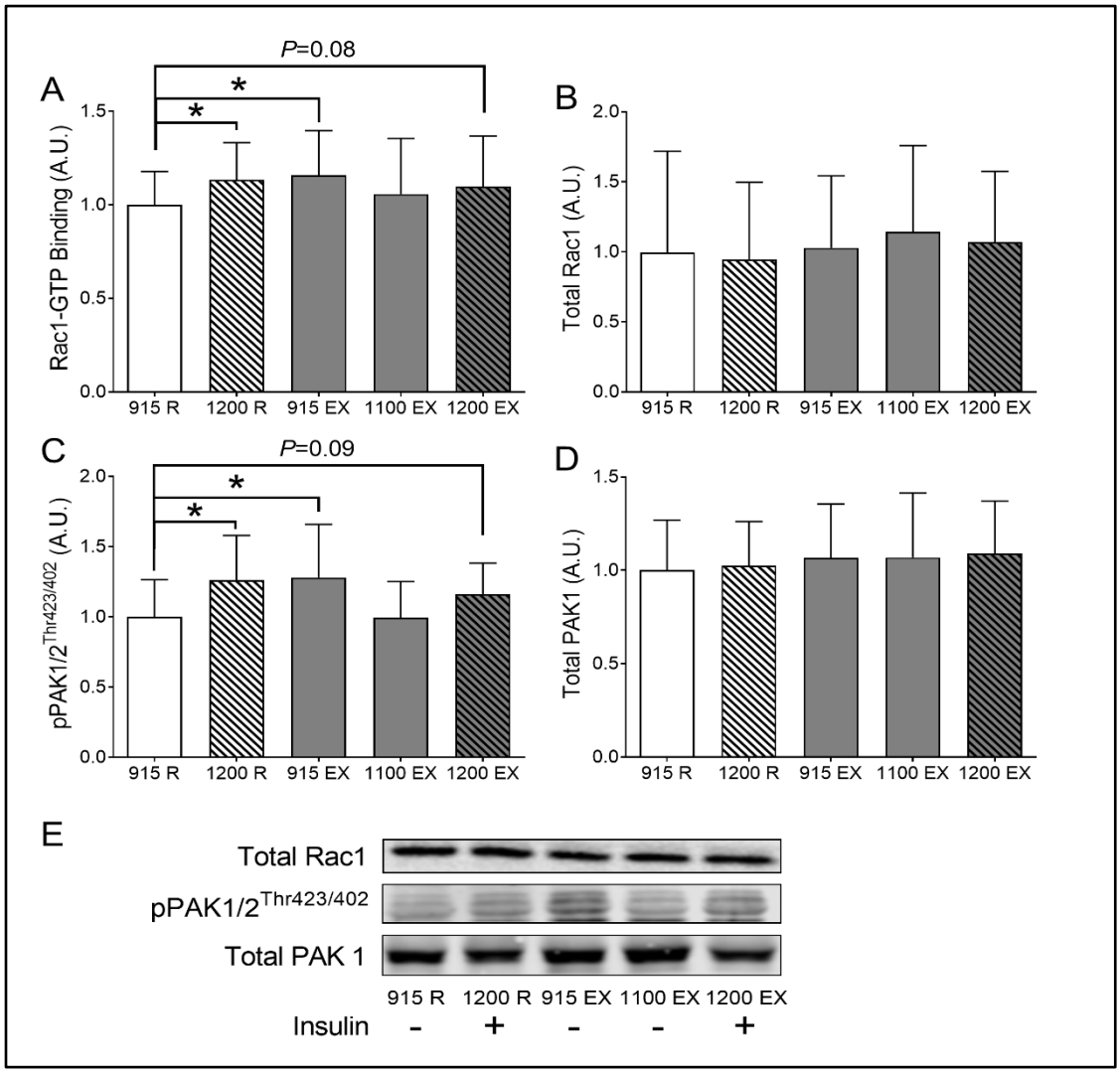

Figure 5.3: Rac1 signaling during rest and after exercise.

Rac1 activation and activation of its downstream effector PAK in vastus lateralis muscle samples during rest (915 R), rest with insulin (1200 R), 15 minutes after acute exercise (915 EX), 120 minutes after acute exercise (1100 EX), and 180 minutes after acute exercise with insulin (1200 EX). **A)** Rac1 activation via GTP binding measured using enzyme-linked immunoassay (see methods), **B)** total protein abundance for Rac1, **C)** PAK activation via phosphorylation at Threonine 423/402, **D)** total protein abundance for PAK1, with **E)** representative western blot images. Hatched bars indicate insulin stimulation. Full blot and ponceau images for each representative image were made available during the review process. The effects of insulin and exercise were analyzed by repeated measures one-way analysis of variance models, with post-hoc comparisons analysis determined *a priori*. Data are presented as mean and standard deviation. *$P \leq 0.05$ vs. 915 R. n=14 (female/male: 10/4).

Akt activation during rest and post-exercise

Insulin-stimulated activation of Akt is required for insulin-stimulated glucose uptake and occurs independent from and parallel to Rac1 signaling (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). We therefore determined if insulin-stimulated activation of Akt was enhanced post-exercise. As expected, Akt activation was not increased 15- or 120-minutes post-exercise compared with rest (Figure 5.4), as evidenced by no change in either Threonine 308 ($pAkt^{Thr308}$) or Serine 473 ($pAkt^{Ser473}$) phosphorylation (all comparisons $P>0.25$). Insulin increased Phospho-Akt compared to rest ($P<0.01$ for 1200 R vs. 915 R for both $pAkt^{Thr308}$ and $pAkt^{Ser473}$), but previous exercise did not further increase in insulin-stimulated Akt activation ($P>0.15$ for 1200 R vs. 1200 EX for both $pAkt^{Thr308}$ and $pAkt^{Ser473}$). Total protein abundance for Akt was not altered by insulin or exercise (Figure 5.4). Our findings in humans demonstrate insulin-stimulated activation of Akt activation is not enhanced following an acute bout of exercise.

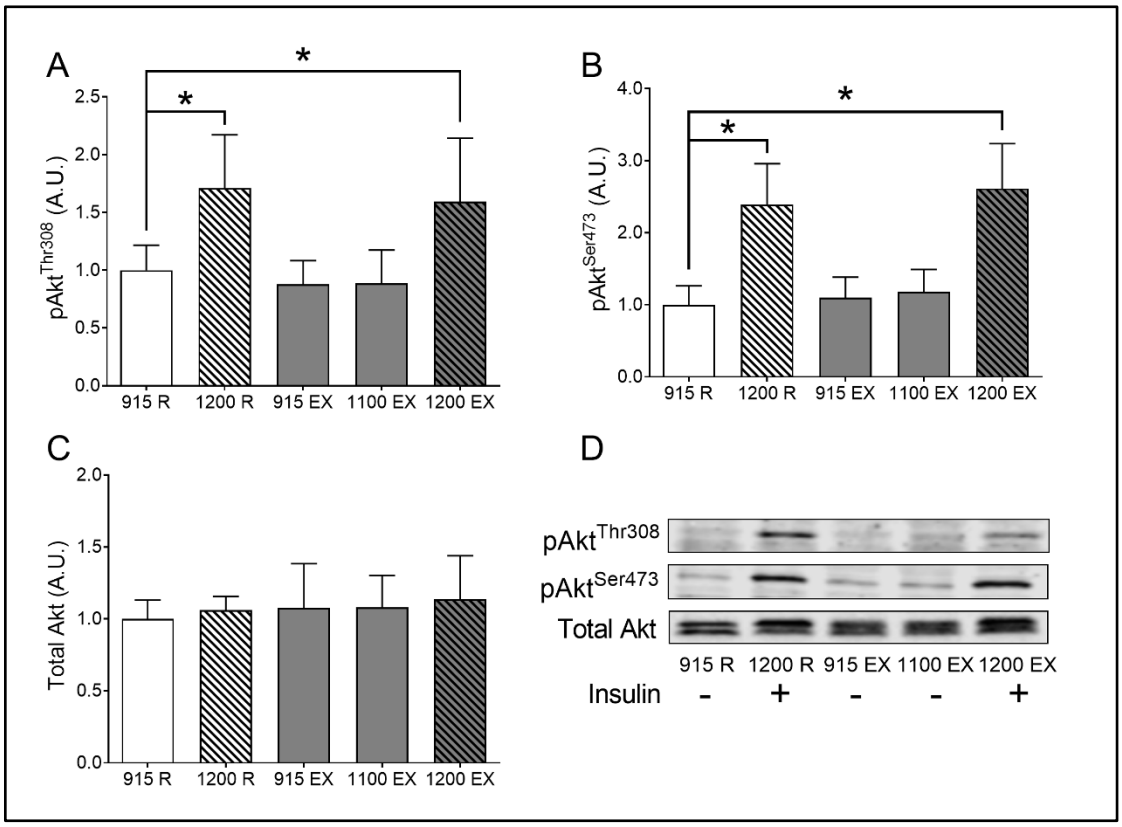

Figure 5.4: Akt activation during rest and after exercise.

Akt activation in vastus lateralis muscle samples during rest (915 R), rest with insulin (1200 R), 15 minutes after acute exercise (915 EX), 120 minutes after acute exercise (1100 EX), and 180 minutes after acute exercise with insulin (1200 EX). **A)** Akt activation via Threonine 308 phosphorylation, **B)** Akt activation via Serine 473 phosphorylation **C)** total protein abundance for Akt, with **D)** representative western blot images. Hatched bars indicate insulin stimulation. Full blot and ponceau images for each representative image were made available during the review process. The effects of insulin and exercise were analyzed by repeated measures one-way analysis of variance models, with post-hoc comparisons analysis determined *a priori*. Data are presented as mean and standard deviation. *$P \leq 0.05$ vs. 915 R. n=14 (female/male: 10/4).

AMPK and TBC1D1 activation during rest and post-exercise

Exercise-induced AMPK activation contributes to greater skeletal muscle glucose uptake post-exercise (Kjøbsted et al., 2019). We therefore measured activation of AMPK and its downstream effector TBC1D1 during rest and after exercise. Exercise

stimulated AMPK, as evidenced by greater Threonine 172 phosphorylation (pAMPK^{Thr172}) measured 15 minutes post-exercise compared with rest (*P*=0.05 915 EX vs. 915 R, Figure 5.5A). Exercise increased pAMPK^{Thr172} at both 120 and 180 minutes compared to rest (*P*=0.03 for 1100 EX vs. 915 R; *P*=0.06 for 1200 EX vs. 915 R). Insulin did not alter pAMPK^{Thr172} (*P*=0.38 for 1200 R vs. 915 R), and total AMPK protein abundance was not altered by rest or exercise (Figure 5.5B).

AMPK-induced phosphorylation of TBC1D1 at serine 231 (pTBC1D1^{Ser231}) contributes to increased glucose uptake post-exercise, with such effects appearing to be independent of insulin action (Kjøbsted et al., 2019). In agreement with AMPK activation, pTBC1D1^{Ser231} increased 15 minutes post-exercise (*P*<0.01 for 915 EX vs. 915 R, Figure 5.5C) and remained elevated 120 minutes post-exercise (*P*=0.02 for 1100 EX vs. 915 R). As expected, pTBC1D1^{Ser231} was not increased by insulin (*P*=0.66 1200 R vs. 915 R); however, it remained phosphorylated at 180 minutes post-exercise compared with basal measures (*P*=0.04 for 1200 EX vs. 915 R), and insulin-stimulation during the resting trial (*P*=0.01 for 1200 EX vs. 1200 R). Neither insulin nor exercise altered total TBC1D1 protein abundance (Figure 5.5D). Taken together, our findings demonstrate exercise-induced activation of skeletal muscle TBC1D1 may contribute to improved insulin sensitivity measured after exercise compared with remaining sedentary.

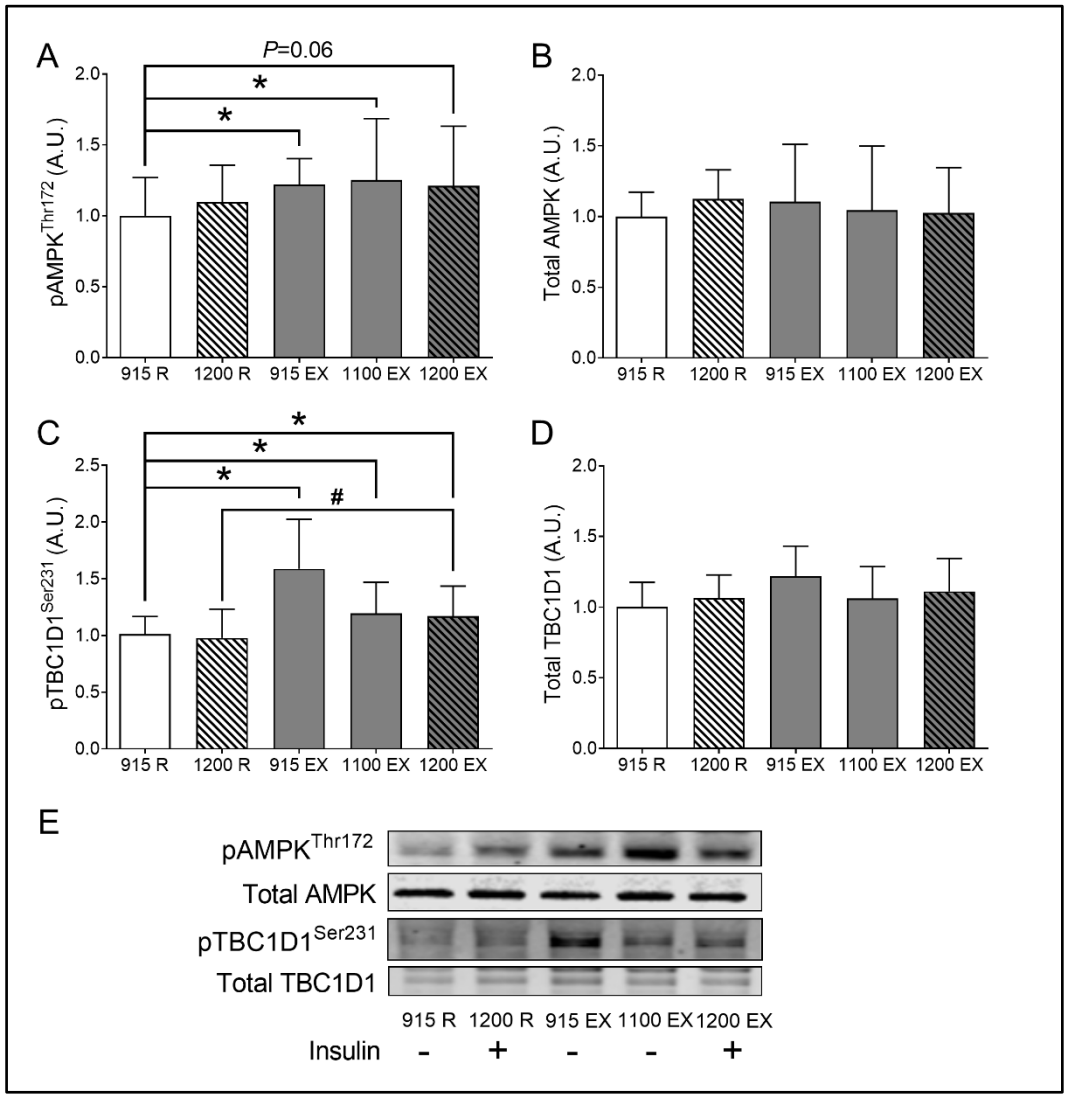

Figure 5.5: AMPK signaling during rest and after exercise.

AMPK activation and activation of its downstream effector TBC1D1 in vastus lateralis muscle samples during rest (915 R), rest with insulin (1200 R), 15 minutes after acute exercise (915 EX), 120 minutes after acute exercise (1100 EX), and 180 minutes after acute exercise with insulin (1200 EX). **A)** AMPK activation via Threonine 172 phosphorylation, **B)** total protein abundance for AMPK, **C)** TBC1D1 activation via phosphorylation Serine 231, **D)** total protein abundance for TBC1D1, with **E)** representative western blot images. Hatched bars indicate insulin stimulation. Full blot and ponceau images for each representative image were made available during the review process. The effects of insulin and exercise were analyzed by repeated measures one-way analysis of variance models, with post-hoc comparisons analysis determined *a priori*. Data are presented as mean and standard deviation. *$P \leq 0.05$ vs. 915 R. #$P \leq 0.05$ vs. 1200 R. n=14 (female/male: 10/4).

Discussion

The primary purpose of this study was to investigate the combined effects of acute moderate-intensity exercise and insulin on skeletal muscle Rac1 activation and its contribution to improvements in insulin sensitivity post-exercise. Moderate-intensity exercise improved steady state glucose infusion rates in relatively lean healthy adults, indicating improved insulin sensitivity after exercise compared with remaining sedentary. However, insulin-stimulated Rac1 signaling in skeletal muscle was not enhanced by prior exercise, suggesting it may not contribute to the insulin sensitizing effects of exercise. Insulin-stimulated Akt activation, which occurs parallel to Rac1 signaling in skeletal muscle, also was not further enhanced by exercise. In contrast the lack of effects observed for skeletal muscle insulin signaling, we report that AMPK-specific activation of TBC1D1, which contributes to glucose uptake post-exercise independent of insulin action *per se*, was significantly increased 15 minutes after exercise and remained increased for at least 180 minutes. We interpret our findings to indicate mechanisms independent of Rac1, such as AMPK-induced activation of TBC1D1, contribute to improvements in glucose metabolism in the hours after exercise in relatively lean healthy adults.

We report a single session of moderate-intensity exercise improves insulin sensitivity in the hours after exercise, which is consistent with previous reports that acute bouts of exercise of various intensities can improve whole-body insulin action among most individuals (Devlin & Horton, 1985; Newsom et al., 2013). Exercise is considered an effective treatment for prediabetes and type 2 diabetes due to its beneficial effects on insulin action and glucose regulation (Colberg et al., 2010).

Importantly, acute exercise is not always associated with an improvement in insulin sensitivity (Steenberg et al., 2020). A recent elegant study by Steenberg et al. suggests this discrepancy in the effects of exercise on whole-body insulin sensitivity may be due, at least in part, to decreased rates of glucose uptake into skeletal muscles that were inactive during exercise, thereby offsetting increased insulin-stimulated glucose uptake into skeletal muscles that were recruited during exercise (Steenberg et al., 2020). Nevertheless, we interpret our current findings to provide further support that exercise – even a single session – can improve whole-body insulin sensitivity.

Rac1 is activated by both exercise and insulin (Sylow et al., 2015; Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Sylow, Jensen, Kleinert, Mouatt, et al., 2013b), but the length of activation in response to such stimuli remains to be completely resolved. Rac1 activation evidenced by GTP-binding was increased immediately post-exercise in skeletal muscle of mice and humans (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b). We further add to the literature by demonstrating Rac1 activation was sustained 15 minutes post-exercise in humans, yet returned to basal levels by 120 minutes. We reported Rac1 activation was transient following insulin treatment in muscle cell cultures, with insulin-stimulated Rac1-GTP binding returning to basal levels within 15 minutes in spite of continuous incubation with insulin (Stierwalt et al., 2018). In our current study however, increases in insulin-stimulated Rac1 activation were sustained 1 hour into a hyperinsulinemic-euglycemic clamp. These findings are consistent with previous reports indicating Rac1 activation by insulin remains elevated immediately after a 2-hour hyperinsulinemic-euglycemic clamp in humans (Sylow, Jensen, Kleinert, Højlund, et al., 2013a). Although not the focus of the current study, an area of future

interest is identifying mechanisms for transient vs. sustained activation of Rac1 by insulin and exercise.

We sought to determine the interaction of insulin and exercise on Rac1 signaling to better understand mechanisms of exercise improvements to insulin sensitivity in humans. Rac1 is required for normal insulin-stimulated glucose uptake into skeletal muscle and this mechanism of activation is relatively established, likely occurring via PI3K dependent mechanisms. For example, the inhibition of PI3K in skeletal muscle completely ablated insulin-stimulated Rac1 activation in mice (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Conversely, though the activation of Rac1 by exercise is implicated as a critical regulatory element for controlling glucose uptake during exercise (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b), the mechanisms responsible for such activation remain to be completely resolved. Much evidence suggests the exercise-induced activation occurs independent of AMPK signaling (Sylow et al., 2017), whereby AICAR-induced glucose uptake requires AMPK but not Rac1 (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b). Additionally, exercise-induced Rac1 activation is normal in muscles from AMPK α2KD mice and the same is true for AMPK activation by exercise in muscle specific Rac1 knockout mice (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b; Sylow, Nielsen, et al., 2016b). However, few studies suggest the exercise-induced activation of Rac1 is AMPK-dependent by demonstrating siRNA-mediated knockdown of AMPK blocked contraction-induced GTP loading of Rac1 (Yue et al., 2020). Our evidence adds to the literature indicating there were no additive effects of prior exercise on insulin-stimulated Rac1 activation in human skeletal muscle. Similarly, muscle specific Rac1 knockout in

mice does not impair the insulin sensitizing effects of exercise (Sylow, Nielsen, et al., 2016b). We interpret our results to suggest that Rac1 activation does not contribute to the insulin sensitizing effects of exercise.

Insulin-stimulated glucose uptake requires activation of Akt in parallel, yet independent, to Rac1 (Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). Our current results indicate that previous exercise did not enhance insulin-stimulated activation of Akt. Our findings are consistent with previous reports that improved insulin sensitivity post-exercise occurs independent of changes to skeletal muscle Akt activation (Brozinick & Birnbaum, 1998; Lund et al., 1998; Soos et al., 2001). However, strong evidence indicates previous exercise enhances distal elements of insulin-stimulated Akt signaling via enhanced activation of TBC1D4 (i.e., AS160). We probed our samples for TBC1D4 activation via threonine 642 phosphorylation with unsuccessful results (data not shown). Using non-commercially available antibodies, others have demonstrated that prior exercise results in AMPK-specific phosphorylation on TBC1D4 contributing to its enhanced activation by insulin (Treebak et al., 2009). Additionally, TBC1D4 activation in rat skeletal muscle was sustained in the hours after exercise and tracked with elevated insulin-stimulated glucose transport (Arias, Kim, Funai, & Cartee, 2007; Funai, Schweitzer, Sharma, Kanzaki, & Cartee, 2009). Therefore, the insulin sensitizing effects of exercise may be due, in part, to enhanced insulin-stimulated TBC1D4 activation.

Exercise increases glucose uptake into skeletal muscle, even in the absence of insulin, with most of this effect typically returning to basal by 3 to 4 hours post-exercise

(Cartee et al., 1989). The transient effect of exercise on skeletal muscle glucose uptake is in part regulated by AMPK and its downstream effector TBC1D1 (Kjøbsted et al., 2017; 2019). Importantly, AMPK-induced activation of TBC1D1 delays the endocytosis of GLUT4 vesicles (Kjøbsted et al., 2019), thereby enhancing glucose uptake by increasing the time GLUT4 remains at the sarcolemma. We report that the exercise-induced activation of TBC1D1 was increased 15 minutes post-exercise, and remained increased for at least 180 minutes. The combined effects of delayed endo- and increased exocytosis on GLUT4 via exercise-induced TBC1D1 activation and insulin-stimulated activation of Rac1 and Akt, respectively, may result in greater GLUT4 at the sarcolemma and thereby contribute to the insulin sensitizing effects of exercise. Measures of sarcolemmal GLUT4 abundance could not be completed in the current study due to remaining biopsy tissue being insufficient for fractionation. However, recent findings by Knudsen et al. support this notion by indicating exercise augments skeletal muscle insulin-stimulated GLUT4 translocation to the sarcolemma (Knudsen et al., 2020). We report that lasting effects of exercise on AMPK-specific TBC1D1 activation may act as a contributing mechanism to insulin sensitivity in the hours after exercise.

Exercise improves skeletal muscle insulin action in obese and insulin resistant individuals (Devlin & Horton, 1985; Newsom et al., 2013). Obesity-induced insulin resistance is strongly linked to the accumulation of bioactive signaling lipids within skeletal muscle, such as diacylglycerols and ceramides (Erion & Shulman, 2010; Perreault et al., 2018; Stratford et al., 2004). Diacylglycerols are hypothesized to inhibit insulin action through the binding of various C1 domains (Szendroedi et al., 2014) and there is also evidence for the ability to ceramides to bind C1 domains (Yin et al., 2009).

Several proteins of the Rac1 signaling cascade have C1 binding domains suggesting a potential mechanism by which diacylglycerols and ceramides could impair Rac1 activation. In agreement, previous reports demonstrate impaired insulin-stimulated Rac1-GTP binding in cultured muscle cells following ceramide C2 analog incubation (JeBailey et al., 2007). Additionally, Rac1 signaling is impaired in obese insulin resistance mice and humans but the underlying mechanisms were not investigated (Sylow, Jensen, Kleinert, Højlund, et al., 2013a). We reported overnight fatty acid induced insulin resistance did not impair insulin-stimulated Rac1-GTP binding, suggesting the accumulation of diacylglycerol and ceramides do not impair Rac1 activation by insulin (Stierwalt et al., 2018). Nevertheless, mechanisms that contribute impaired Rac1 signaling by insulin with obesity and whether this may be rescued by previous exercise remains an area of future interest.

In conclusion, a single session of moderate intensity exercise improved whole-body insulin sensitivity that was independent of changes to Rac1 signaling or Akt activation in relatively lean healthy adults. AMPK-specific activation of TBC1D1 increased immediately after exercise and remained elevated for at least 3 hours. Collectively we interpret our evidence to indicate skeletal muscle Rac1 does not contribute to improvements in insulin sensitivity post-exercise. The insulin sensitizing effects of exercise may be due to the combined effects of insulin-dependent (e.g., Rac1 and Akt activation) and insulin-independent (e.g., AMPK and TBC1D1 activation) mechanisms in the hours after exercise.

Acknowledgements

We would like to thank the participants and the helpful staff at Samaritan Athletic Medicine Center, including Drs. Nicholas Phillips, Craig Graham and Joshua Lenhof. We appreciate the contributions of Emily Burney and Bergen Sather for assistance during metabolic study days.

Disclosures

The authors have no conflict of interest to declare.

Grants

Study funding was provided by KL2TR002370 awarded to S.A.N. as part of the Oregon Clinical & Translational Research Institute's Clinical and Translational Science Award from the National Institutes of Health, the Collins Medical Trust awarded to S.A.N. and M.M.R., and the John C. Erkkila, M.D. Endowment for Health and Human Performance awarded to S.A.N. and M.M.R.. H.D.S. and S.E.E. were supported by fellowships from Oregon State University. MMR was supported by K01DK103829.

Author Contributions

H.D.S., M.M.R. and S.A.N. designed study and performed experiments. S.E.E. performed experiments. H.D.S. performed the analysis and drafted the manuscript. S.E.E., M.M.R. and S.A.N. edited the manuscript. S.A.N. is the guarantor of this work and, as such, takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Chapter 6 – General conclusions

Overall problem

Changes in skeletal muscle fat metabolism are linked to insulin sensitivity. For example, altered skeletal muscle fat metabolism induced by obesity is associated with impaired insulin action (Bergman et al., 2012; Newsom et al., 2017; Perreault et al., 2018). Conversely, a single session of exercise and exercise training also result in adaptations to fat metabolism within skeletal muscle, yet are associated with improvements in insulin sensitivity (Dubé et al., 2008; Kawanishi et al., 2018; Schenk & Horowitz, 2007). The mechanisms that contribute these observed alterations in skeletal muscle fat metabolism and/or insulin sensitivity with obesity and exercise are incompletely understood. In order to better understand these relationships, we must first investigate mechanisms that contribute to altered skeletal muscle fat metabolism and insulin sensitivity. Thereby the primary aim of this collection of studies was to identify underlying mechanisms that promote changes in skeletal muscle fat metabolism and/or insulin sensitivity observed with obesity, exercise training, and a single session of exercise.

Contributions to the field

Obesity is linked to the accumulation of bioactive signaling lipids such as DAG and ceramides which act as negative regulators to skeletal muscle insulin signaling (Bergman et al., 2012; Schubert et al., 2000; Szendroedi et al., 2014). Negative regulation of insulin signaling results in decreased insulin-stimulated GLUT4 translocation and subsequent glucose uptake (Stierwalt et al., 2018). Rac1 is a novel

regulator of insulin-stimulated glucose uptake by facilitating the translocation of GLUT4 (Sylow, Jensen, Kleinert, Højlund, et al., 2013a; Ueda et al., 2010). Evidence suggests Rac1 signaling is impaired with obesity (Sylow, Jensen, Kleinert, Højlund, et al., 2013a). However, in this report Rac1 activation was measured indirectly and the underlying mechanisms were not investigated (Sylow, Jensen, Kleinert, Højlund, et al., 2013a). The primary aim of chapter 2 addressed this gap in knowledge by investigating the potential negative regulation of insulin-stimulated Rac1-GTP binding by DAG and ceramide in cultured muscle cells. Our findings indicate overnight fatty acid (i.e., palmitate) treatment in cultured muscle cells resulted in DAG and ceramide accumulation which blunted insulin-stimulated translocation of GLUT4. Lipid accumulation was not associated with impairments to insulin-stimulated Rac1-GTP binding, whereas in agreement with previous findings, insulin-stimulated PAK and Akt activation were impaired (Schubert et al., 2000; Sylow, Jensen, Kleinert, Højlund, et al., 2013a). Our findings contribute to the field by demonstrating the blunting of GLUT4 translocation by lipid accumulation in cultured muscle cells occurs independent of impairments to insulin-stimulated Rac1-GTP binding.

Obesity and aerobic exercise training induce adaptations to skeletal muscle fat metabolism evidenced by changes in fat oxidation and fat storage (Kiens, 2006). However, mechanisms that contribute to such changes are incompletely understood. Fatty acids entering skeletal muscle are activated by ACSLs before undergoing further metabolism (Mashek et al., 2007). Genetically modified model systems indicate skeletal muscle ACSL isoforms 1 and 6 contribute to fat oxidation and fat storage, respectively (Jung & Bu, 2020; Li et al., 2015; Teodoro et al., 2017), and mRNA tissue

characterization in rodents suggest ACSL isoforms 1, 3, and 6 are expressed in skeletal muscle (Mashek et al., 2006). The primary aim of chapter 3 was to investigate the effects of diet-induced obesity and aerobic exercise training on skeletal muscle ACSL isoform protein abundance and determine relationships between ACSLs and measures of fat metabolism. We detected protein abundance for 4 of 5 known ACSL isoforms in mouse gastrocnemius muscle with ACSL3 being below limits of detection. High fat diet-induced obesity resulted in greater ACSL1 and ACSL6 protein abundance. Aerobic exercise training decreased ACSL4 protein abundance but resulted in greater abundance for ACSL6. Skeletal muscle ACSL1 was not related to fasting measures of whole-body fat oxidation, whereas ACSL6 was positively associated with intramyocellular lipid content. Our findings contribute to the field by indicating a disconnect between ACSL mRNA and protein expression within skeletal muscle, that ACSLs undergo isoform specific regulation by diet and exercise, and further support the role for ACSL6 in skeletal muscle fat storage.

A single session of exercise is sufficient to alter skeletal muscle fat oxidation and fat storage, but mechanisms contributing to such observations remain to be completely elucidated (Schenk & Horowitz, 2007; Votruba et al., 2003). Skeletal muscle ACSLs are emerging as critical regulators of fat oxidation and storage, but this remains underexplored in humans (Adeva-Andany et al., 2018; Watt & Hoy, 2012). The primary aim of chapter 4 was to investigate the acute effects of exercise on skeletal muscle ACSL isoform protein abundance and identify relationships between ACSLs and measures of fat metabolism in humans. Consistent with our findings in mice, we detected 4 of 5 ACSL isoforms in human skeletal muscle. Skeletal muscle ACSL

isoforms were largely unaltered by acute exercise aside from transient increases in ACSL5. ACSL1 protein abundance tended to be related to be positively associated with whole-body fat oxidation during exercise but not at rest. Skeletal muscle ACSL6 was positively associated with triacylglycerol concentrations (i.e., fat storage). We contribute to the field by indicating skeletal muscle ACSL protein abundance is largely unaltered by a single session of exercise and further support the role for ACSLs in fat metabolism in humans.

A single session of exercise is sufficient to improve insulin sensitivity in most adults at various exercise intensities and different populations (Devlin & Horton, 1985; Newsom et al., 2013). Improved insulin sensitivity post-exercise occurs independent to changes in proximal insulin signaling including insulin-stimulated Akt activation (Wojtaszewski et al., 2000). Insulin-stimulated Rac1 activation occurs independent from Akt signaling and is also activated by exercise (Sylow, Jensen, Kleinert, Mouatt, et al., 2013b; Sylow, Kleinert, Pehmoller, Prats, Chiu, Klip, Richter, & Jensen, 2014a). However, it is unknown whether prior exercise further enhances insulin-stimulated Rac1 activation in humans. The primary aim of chapter 5 was to investigate the potential interaction of insulin and exercise on Rac1 activation in humans. Exercise improved insulin sensitivity independent of enhanced insulin-stimulated Rac1 activation, phosphorylation of its downstream effector PAK, or insulin-stimulated Akt phosphorylation. Exercise increased phosphorylation of AMPK and its downstream effector TBC1D1 which increase glucose uptake independent of insulin. AMPK-induced phosphorylation of TBC1D1 remained increased 180 minutes post-exercise during insulin stimulation suggesting it may contribute to improved insulin sensitivity. Our

findings contribute to the field by demonstrating insulin-stimulated Rac1 activation does not contribute to the insulin sensitizing effects of exercise in relatively lean healthy adults.

Future direction

We provide further insight into the regulation of insulin-stimulated Rac1 activation in the context of lipid induced insulin resistance. However, there is much that remains unknown regarding regulation of Rac1 and downstream signaling by insulin. For example, overnight palmitate treatment in cultured muscle cells decreased insulin-stimulated GLUT4 translocation in the absence of impaired Rac1 activation by insulin. However, insulin-stimulated phosphorylation of a downstream effector PAK was blunted by palmitate suggesting impaired Rac1 signaling. Similar reports in obese insulin resistant mice and humans also exhibit lower insulin-stimulated PAK phosphorylation (Sylow, Jensen, Kleinert, Højlund, et al., 2013a). Importantly, PAK activation requires both the binding of Rac1 and then phosphorylation by phosphoinositide dependent kinase 1 (PDK1) for complete activation (C. C. King et al., 2000). Overnight palmitate treatment provides indirect evidence of impaired PDK1 activity via decreased PDK1-specific Akt phosphorylation at Threonine 308. However, this has yet to be tested directly. Identifying the underlying mechanisms that impair insulin-stimulated PAK phosphorylation independent of Rac1-GTP binding may yield a potential therapeutic target by which restoring PAK phosphorylation could, in part, restore insulin-stimulated glucose uptake under states of lipid-induced insulin resistance. Additionally, others report impaired insulin-stimulated Rac1 activation following incubation of a ceramide C2 analog suggesting that lipids may negatively regulate Rac1-GTP binding (JeBailey et

al., 2007). Therefore, the effects of lipid-induced insulin resistance on Rac1-GTP binding and subsequent signaling remains an area of future interest.

We report novel insight into the potential regulation of skeletal muscle fat metabolism by ACSL isoforms. However, measures of total protein abundance may not indicate activity. ACSLs exhibit numerous post-translational modifications but their functional role remains unknown (Frahm et al., 2011). A single session of exercise increases triacylglycerol (TAG) synthesis within skeletal muscle and protects against lipid-induced insulin resistance in humans but contributing mechanisms are incompletely understood (Schenk & Horowitz, 2007). Additionally, TAG synthesis is positively associated with insulin sensitivity across numerous populations (Bergman et al., 2018). We and others continue to provide evidence indicating a role for ACSL6 in skeletal muscle TAG synthesis (Jung & Bu, 2020; Stierwalt et al., 2020; Teodoro et al., 2017). Identifying post-translational modifications that regulate ACSL6 activity may provide a promising therapeutic target. Inducing increased ACSL6 activity could upregulate the trafficking of fatty acids toward a neutral lipid storage pool, and limit the accumulation of bioactive signaling lipids identified to impair insulin signaling (Schenk & Horowitz, 2007). Regardless, our understanding of skeletal muscle ACSLs in fat metabolism is still in its infancy, and remains an area of high interest in the context of human performance and disease prevention.

Our findings indicate that insulin-stimulated Rac1 activation is not enhanced by prior exercise. However, our findings are limited to relatively lean healthy adults and the underlying mechanisms for exercise-induced Rac1 activation remain largely unknown.

Insulin-stimulated Rac1 signaling is impaired in obese insulin resistant mice and humans (Sylow, Jensen, Kleinert, Højlund, et al., 2013a). Exercise improves insulin sensitivity and although we demonstrate it occurs independent of enhanced Rac1 activation by insulin in a healthy population, this remains to be investigated in populations exhibiting insulin resistance. Our previous findings indicate lipid-induced insulin resistance results in dysfunctional PAK activation by insulin, a downstream effector of Rac1 (Stierwalt et al. 2018). Exercise increases PAK activation and may perhaps restore the dysfunctional signaling observed with insulin resistance and contribute to improved insulin sensitivity post exercise, but this remains to be tested. Additionally, Rac1 is required for glucose uptake during exercise (Sylow, Nielsen, et al., 2016b). Identifying mechanisms that contribute to Rac1-dependent glucose uptake during exercise could yield a potential therapeutic intervention for restoring glucose homeostasis in insulin resistant adults. Therefore, the activation of Rac1 by exercise and the potential interaction between insulin and exercise on Rac1 activation in insulin resistant populations remains an area for future study.

Overall conclusion

This collection of studies indicates the accumulation of bioactive signaling lipids that negatively regulate insulin-stimulated GLUT4 translocation, occurs independent of impaired insulin-stimulated Rac1 activation. Altered skeletal muscle fat metabolism observed with diet-induced obesity and/or aerobic exercise training may be due, in part, to ACSL-dependent trafficking of fatty acids. The acute effects of exercise on skeletal muscle fat oxidation and/or fat storage are not likely dependent on changes to ACSL protein abundance. Lastly, the insulin sensitizing effects of exercise occur independent

of changes to insulin-stimulated Rac1 signaling. These independent observations further our understanding of mechanisms that contribute to altered skeletal muscle fat metabolism and insulin action in various states of insulin resistance. The interaction between fat metabolism and insulin sensitivity within skeletal muscle remains an area ripe for future study.

Chapter 7 – Bibliography

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