EFFECTS OF EXERCISE AND ELEVATED FATTY ACID AVAILABILITY ON MUSCLE LIPID METABOLISM AND INSULIN SENSITIVITY

by

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Table of Contents

List of Figures

List of Tables

List of Appendices

ABSTRACT

Effects of exercise and elevated fatty acid availability on muscle lipid metabolism and insulin sensitivity

by

Sean Alec Newsom

Chair: Jeffrey F. Horowitz

Elevated systemic fatty acid availability is a key factor underlying obesity-related insulin resistance and type 2 diabetes. A single session of exercise can protect against fatty acid-induced insulin resistance, possibly via altered muscle lipid metabolism. The overall purpose of this dissertation was to examine the effects of exercise and/or elevated fatty acid availability on the regulation of muscle lipid metabolism and changes in insulin sensitivity. The major findings of my dissertation studies were as follows: In STUDY 1, compared with a saline infusion after exercise, an overnight (16h) lipid infusion/heparin infusion after a single session of exercise increased muscle triacylglycerol (TAG) concentration by ~30%, without any change in muscle diacylglycerol (DAG) or ceramide. Despite minimal changes in activity of TAG-synthesis enzymes, greater membrane-associated abundance of the fatty acid transporter FAT/CD36 may have facilitated the enhanced muscle TAG storage. In STUDY 2, a modest single session of low intensity exercise (70 min at 50% VO_2peak) was sufficient to improve insulin sensitivity into the next day in obese adults. Although we did not find any change in

muscle lipids after exercise, reduced systemic fatty acid uptake after exercise may have been important for the improvement in insulin sensitivity. Unlike STUDY 1 and STUDY 2, which were performed in human subjects, STUDY 3 was conducted in cultured C2C12 muscle cells, *in vitro*. In STUDY 3 we found that compared with the deleterious effects of palmitate incubation, muscle cells acutely (12h) incubated with physiologic mixtures of several fatty acids showed little impairment in insulin signaling, likely due to a robust capacity to store the available fatty acid as TAG and accumulate virtually no DAG. Importantly, providing a relatively high proportion of saturated fatty acids in this fatty acid mixture did not abnormally affect fatty acid "partitioning" or insulin signaling. Together these major findings of my dissertation projects suggest that preferential storage of excess fatty acid as muscle TAG may limit accumulation of harmful lipid intermediates and protect against fatty acid-induced insulin resistance; however, the mechanisms underlying preferential TAG storage remain elusive.

CHAPTER 1

Statement of the Problem

Approximately one-third of the US population is obese (11). The burden of this epidemic is evidenced in part by the very high prevalence of obesity-related complications, including Type 2 Diabetes Mellitus (T2DM) (17). T2DM is a chronic disease characterized by insulin resistance resulting in poor blood glucose regulation. Approximately 16.5 million people in the United States have been diagnosed with T2DM, and the estimated total economic cost of T2DM was nearly 160 billion dollars in 2007 (1, 9). Upwards of 6 million more United States adults are projected to have T2DM but remain undiagnosed at this time, contributing an additional estimated 18 billion dollars of economic burden (9, 25). What is more alarming is that an additional 57 million Americans, including 2 million adolescents, are considered to be pre-diabetic (24), many of whom will likely soon develop T2DM, adding greatly to the incidence of diabetes and the costs for treatment. Even in the absence of frank T2DM, the insulin resistance associated with pre-diabetes is a strong predictor of many acquired chronic diseases such as hypertension, coronary heart disease, stroke, and certain cancers (10). Not surprisingly, the vast majority of pre-diabetics are obese, as obesity is considered a primary factor contributing to the development of insulin resistance (16-17). Considering the immense economic burden and extraordinarily poor health outcomes associated with obesity-related insulin resistance, understanding the mechanism(s) underlying insulin resistance in obesity as well as the treatments for insulin resistance in obesity is of great importance.

It is well known that excessive systemic fatty acid availability, a hallmark of obesity, can induce insulin resistance (2, 4-6, 14, 21-22). Within skeletal muscle, accumulation of fatty acid intermediates (e.g., long-chain acyl-CoA [LCACoA], diacylglycerol [DAG],

and ceramide) and subsequent activation of proinflammatory pathways (e.g., c-jun N-terminal kinases [JNK] and nuclear factor-κB [NF-κB]) are thought to underlie obesity-related insulin resistance (for reviews, see (13, 19)). In short, the accumulation of fatty acid intermediates in skeletal muscle can occur when the excessive uptake of fat by myocytes is not matched by the metabolism of fatty acids within the cell (i.e., oxidation in the mitochondria or storage within the muscle as intramuscular triacylglycerol [IMTG]). We have recently reported that performing a single session of exercise can prevent fatty acid-induced insulin resistance, at least in part by altering the metabolic fate of fatty acids within skeletal muscle (20, 22). Specifically, increased storage of excess fatty acid as IMTG after exercise protected against the accumulation of fatty acid intermediates and proinflammatory stress (20, 22). Despite this evidence that a single session of exercise may alter skeletal muscle lipid metabolism, the regulation of skeletal muscle lipid metabolism after exercise was still poorly understood. Gaining a better understanding of the regulation of muscle lipid metabolism when fatty acid availability is high after exercise, as in obesity, has provide insight into potential mechanisms by which exercise offers protection against fatty acid-induced insulin resistance.

Although it is very clear that a single session of exercise can profoundly improve insulin sensitivity, the minimal "dose" of exercise required to enhance insulin sensitivity the next day in obese adults was not known. The few studies that had examined the influence of exercise intensity on blood glucose regulation in obesity not only present conflicting data, but also failed to directly measure insulin sensitivity (15), isolate the effects of a single session of exercise (3), or account for the caloric expenditure of the exercise bout (23). It was clear that there is a need for a well controlled examination of the effect of exercise intensity on the ability of a single session of exercise to enhance insulin sensitivity in obese adult humans. Furthermore, it was also important to examine putative mechanisms for the insulin sensitizing effects of exercise, including exercise-induced alterations in muscle lipid metabolism. Whether exercise-induced alterations in muscle lipid metabolism are influenced by the intensity of the exercise bout in obese humans was unknown.

Finally, evidence concerning the importance of specific fatty acid species and the role of saturated *vs.* unsaturated fatty acids in fatty acid-induced insulin resistance was conflicting. Several independent studies had reported that saturated fatty acids readily induced insulin resistance and that unsaturated fatty acid can actually protect against palmitate-induced insulin resistance *in vitro* (7-8, 18), whereas lipid-infusion studies in humans report rapid induction of insulin resistance even when the lipid emulsion was almost entirely (~90%) composed of unsaturated fatty acids (2, 5-6, 12, 22). Perhaps more importantly, it should be noted that not one of the aforementioned models of elevated fatty acid availability provided an accurate reflection of the elevated fatty acid availability common to obesity (i.e., an unbiased increase of the mixture of many different fatty acids comprising adipose tissue TAGs). Determination of the effects of increasing fatty acid availability on muscle cell insulin signaling and lipid metabolism in a way that more closely resembled the elevated fatty acid availability commonly found in obesity (i.e., a mixture of the most abundant plasma fatty acids), yielded new insight into the regulation of muscle lipid metabolism and changes in muscle cell insulin signaling.

My dissertation studies were designed to address the following issues:

1) Whether increasing fatty acid availability after a single exercise session altered the regulation of skeletal muscle lipid metabolism the day after exercise in adult humans
2) The effect of mild (50% VO_2peak) and moderate (65% VO_2peak) exercise intensity during a single session of exercise on systemic fatty acid availability, the accumulation of muscle lipids, and insulin sensitivity measured the next day in obese adults
3) The effect of increased availability of physiologic mixtures of fatty acids on muscle cell insulin signaling and lipid accumulation, and whether increased fatty acid availability altered lipogenic, lipolytic, and/or fatty acid transport proteins in cultured muscle cells

The overarching goal of my dissertation was to determine the effects of exercise and fatty acid availability on muscle lipid metabolism and insulin sensitivity. The specific gaps in

knowledge outlined above were addressed through the use of both human studies and cell culture methodology. Together these studies greatly aid our understanding of the mechanism(s) underlying insulin resistance in obesity as well as the treatments for insulin resistance in obesity.

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CHAPTER 2

Review of Literature

The following review of literature outlines current knowledge regarding muscle lipid metabolism as it pertains to obesity-related insulin resistance and the role that exercise plays in improving insulin resistance in obesity. This review will describe the effects of elevated fatty acid availability as a key mediator of obesity-related insulin resistance, summarize support for the role of inflammation in lipid-induced insulin resistance, and highlight recent studies demonstrating that acute exercise protects against lipid-induced insulin resistance. Finally, specific changes in muscle lipid metabolism after exercise that may underlie some of the exercise-induced improvement in insulin resistance in obesity will be addressed.

Obesity-related insulin resistance is linked with altered lipid metabolism

Obesity is a condition characterized by excessive adiposity resulting from a chronic surplus of energy intake relative to energy expenditure. Obesity is also characterized by elevated systemic fatty acid availability (52, 56). Abdominally obese humans release fatty acid into the circulation at rates nearly two-fold greater than lean counterparts after an overnight fast (56). Importantly, the excessive fatty acid availability in obesity occurs independently of high dietary fat intake and positive energy balance (i.e., energy intake exceeds energy expenditure) (52). Chronically elevated systemic fatty acid availability has been linked to many obesity-related health complications (82-83). The role of elevated systemic fatty acid availability in obesity-related insulin resistance will be discussed below.

It has long been appreciated that high fatty acid availability can alter glucose metabolism (13, 99), however, it was not until much more recently that dysregulated fatty acid

metabolism began to take center stage as the likely mediator of insulin resistance associated with obesity (77-78, 100). For the purposes of this review insulin resistance will be discussed in regard to dysregulated glucose metabolism, whereby there is an impairment in the plasma glucose lowering effects of insulin. In this context, healthy insulin sensitive lean individuals become insulin resistant within as little as 3-4 hours of being exposed to high rates of fatty acid availability (via triacylglycerol emulsion plus heparin infusion) similar to that of abdominally obese individuals, thereby implicating elevated fatty acid availability as an important mediator of obesity-related insulin resistance (8, 14-15, 41, 110). However, if elevated fatty acid availability is indeed an important mediator of obesity-related insulin resistance, experimental models that reduce fatty acid availability in obese individuals should enhance insulin sensitivity despite the persistence of obesity. Nicotinic acid and the niacin derivative acipimox are potent inhibitors of lipolysis that can be used clinically to treat hyperlipidemia and experimentally to substantially lower systemic fatty acid availability. Indeed, acute acipimox administration has repeatedly been shown to potently enhance insulin sensitivity in insulin resistant obese individuals and those with T2DM (106, 121). Thus, independent of any change in obesity, *per se*, a reduction in systemic fatty acid availability is sufficient to "restore" insulin sensitivity. Weight loss is similarly effective at attenuating systemic fatty acid availability and enhancing insulin sensitivity (7, 60, 120). In fact, our laboratory has recently reported that much of the insulin sensitizing effect of weight loss can be attributed to lowered systemic fatty acid availability, as restoration of pre-weight loss fatty acid availability (via acute lipid plus heparin infusion) completely reverses the effect of weight loss on insulin sensitivity (109). From this, we conclude that elevated fatty acid availability is a key mediator of obesity-related insulin resistance.

Elevated systemic fatty acid availability in obesity is paralleled by increased skeletal muscle fatty acid uptake (17). Fatty acid uptake occurs largely via facilitated transport (16, 29), and increased skeletal muscle fatty acid transporters at the sarcolemmal membrane is an important contributor to the elevated rate of skeletal muscle fatty acid uptake found in obesity (2, 16-17). For example, the rate of fatty acid uptake in skeletal

muscle is proportional to the quantity of fatty acid transporters at the cell surface membrane (2, 16-17). Fatty acid translocase (FAT/CD36) is an important fatty acid transporter in skeletal muscle (2, 16-17, 29), and obesity is associated with increased membrane-associated FAT/CD36 (2, 17). In general, there are three main metabolic fates for fatty acids entering the myocyte: mitochondrial β-oxidation, storage as triacylglycerol, or they may be partially metabolized, forming fatty acid intermediates (Figure 2-1). The rate of β-oxidation of fatty acids in any myocyte is almost certainly a function of the energy status within the cell (i.e., ADP/ATP content), the availability of substrate, and the hormonal milieu. Whether mitochondrial function or oxidative capacity plays a role in the rate of skeletal fatty acid oxidation will be discussed in detail below (see the second paragraph under the heading, *A single session of exercise protects against lipid-induced insulin resistance*). Storage of fatty acid within skeletal muscle as intramyocellular triacylglycerol (IMTG) occurs through the succession of four reactions. The first committed step of triacylglycerol synthesis is regulated by the enzyme glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the production of lysophosphatidic acid (LPA) from fatty acyl-CoA and glycerol-3-phosphate. The final key step in the triacylglycerol synthesis pathway is regulated by the enzyme diacylglycerol acyltransferase (DGAT), which catalyzes the esterification of a third fatty acyl-CoA to diacylglycerol (DAG) to create a triacylglycerol. Many other lipid molecule biosynthetic pathways are also operating within skeletal muscle, including synthesis pathways for phospholipids, retinol and cholesterol esters, sphingolipids, etc. Intermediary lipid molecules in the triacylglycerol pathway and several of these other synthesis pathways, namely long-chain acyl-CoA (LCACoA), ceramide, and diacylglycerol (DAG), herein globally referred to as "fatty acid intermediates," serve as another broadly classified metabolic fate of skeletal muscle fatty acids. Because skeletal muscle fatty acid uptake in obesity is generally believed to exceed the energetic need for fatty acid oxidation (51), lipid tends to accumulate such that skeletal muscle lipid content is much greater in sedentary obese compared with sedentary lean adults. People with abdominal obesity have been reported to have high IMTG content (39, 67), such that IMTG content in obese individuals often two-fold greater than that of sedentary lean individuals (38, 75). Not surprisingly, IMTG content has also been found to inversely correlate with insulin

sensitivity in sedentary populations (38-39, 67, 93-94). Importantly, evidence from our lab (36, 108, 110) and others (8, 38, 70, 97) indicates that elevated IMTG content is not causally related to insulin resistance (see the first paragraph under the heading, *A single session of exercise protects against lipid-induced insulin resistance*). Skeletal muscle content of lipid intermediates such as LCACoA, ceramide, and DAG is also elevated in sedentary obese humans (1, 9, 53, 88, 114), again reflecting the general imbalance between fatty acid availability and metabolism. It is now widely believed that the accumulation of lipid intermediates in skeletal muscle rather than IMTG is an important mediator of obesity-related insulin resistance. How lipid accumulation in skeletal muscle impairs insulin sensitivity is not completely understood, however, considerable evidence suggests that these intermediates interfere with the insulin signaling cascade.

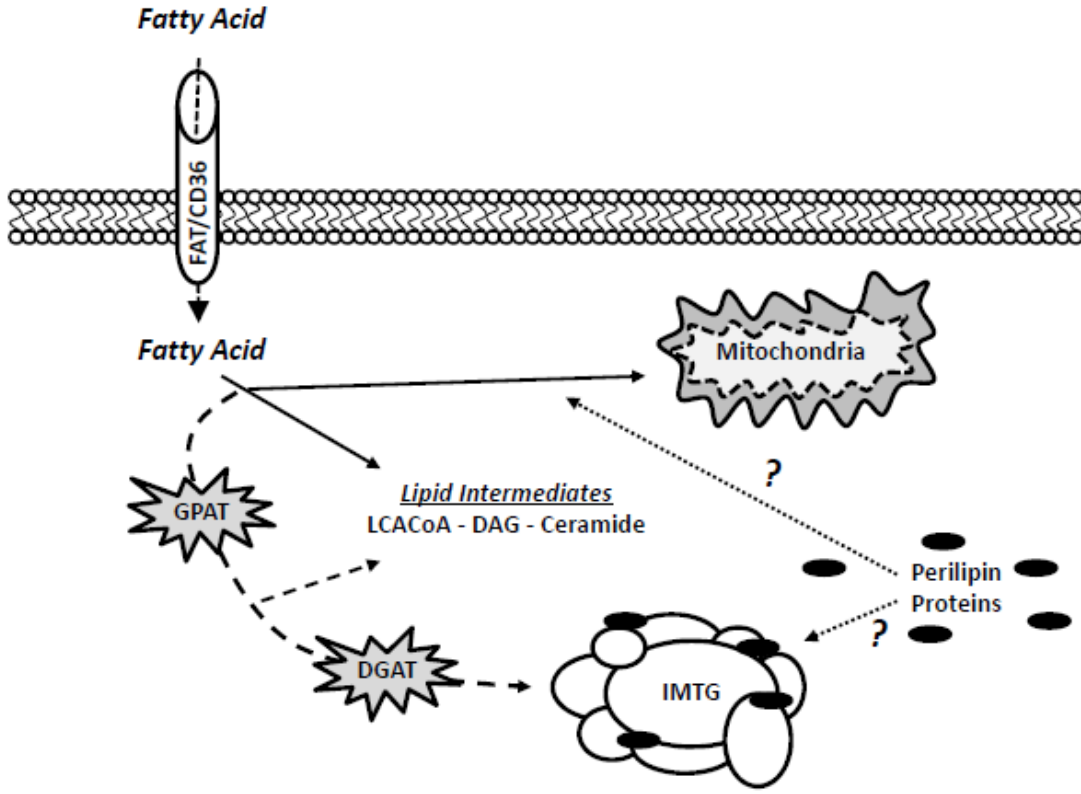

Figure 2-1. Primary metabolic fates of fatty acids in skeletal muscle: oxidation, storage as intramyocellular triacylglycerol (IMTG), and formation/accumulation of fatty acid intermediates (e.g., long-chain acyl-CoA (LCACoA), diacylglycerol (DAG), and ceramide). Perilipin proteins may act to facilitate lipid trafficking in skeletal muscle, and thereby may help regulate intramyocellular lipid oxidation and/or storage. FAT/CD36, fatty acid translocase; GPAT, glycerol-3-phosphate acyltransferase; DGAT, diacylglycerol acyltransferase.

Lipid-induced insulin resistance is marked by inflammation and poor insulin signaling

In skeletal muscle, insulin signaling transduction events culminate with translocation of glucose transporters (GLUT4) to the sarcolemmal membrane, resulting in enhanced glucose transport. The major signaling events of this cascade are highlighted here, and also summarized in Figure 2-2. The insulin receptor is a receptor tyrosine kinase that, upon insulin binding at the cell surface, autophosphorylates several cytosolic domain tyrosine residues and subsequently recruits and tyrosine phosphorylates adaptor insulin receptor substrate (IRS) proteins (89, 104, 115). Tyrosine phosphorylated IRS proteins bind and activate phosphatidylinositol 3-kinase (PI3K) (22). Activated PI3K migrates to the sarcolemmal phospholipid bilayer and phosphorylates PI-(4,5)-biphosphate (PIP2) to generate PI-(3,4,5)-triphosphate (PIP3) (122-123). The formation of PIP3 lipids attracts proteins containing pleckstrin homology domains, including phophoinositide-dependent kinase-1 (PDK1) and Akt/protein kinase B (herein referred to as Akt) (5, 69). PDK1 phosphorylates Akt at Thr^{308}, thereby activating the serine/threonine kinase (3-4), while phosphorylation of Akt at Ser^{473} by mammalian target of rapamycin complex 2 (mTORC2) reportedly stabilizes activity of the kinase (107). Collectively these initial insulin signaling events (i.e., insulin binding its receptor through induction of Akt) will be referred to as "proximal" insulin signaling events. There are many known Akt targets, but phosphorylation of Akt Substrate of 160 kDa (AS160, or TBC1D4) appears to play an important role in insulin stimulated glucose transport in skeletal muscle (21, 68, 105). In short, it is believed that unphosphorylated AS160 acts as a brake, inhibiting GLUT4 transporter vesicles from translocating to the cell surface (24). In the presence of insulin, activated Akt phosphorylates AS160, inhibiting the Rab-GTPase-activating protein (Rab-GAP) domain activity of the protein, thereby "releasing the brake" on Rab-dependent GLUT4 translocation (68, 105). Upon GLUT4 docking and insertion into the sarcolemmal membrane, glucose molecules can diffuse across the cell membrane in a concentration dependent manner. Importantly, far less is known about these "distal" insulin signaling events (i.e., downstream of Akt activation) compared with proximal insulin signaling. In summary, this complex sequence of signaling events is believed to be important for normal insulin-stimulated glucose transport in skeletal muscle.

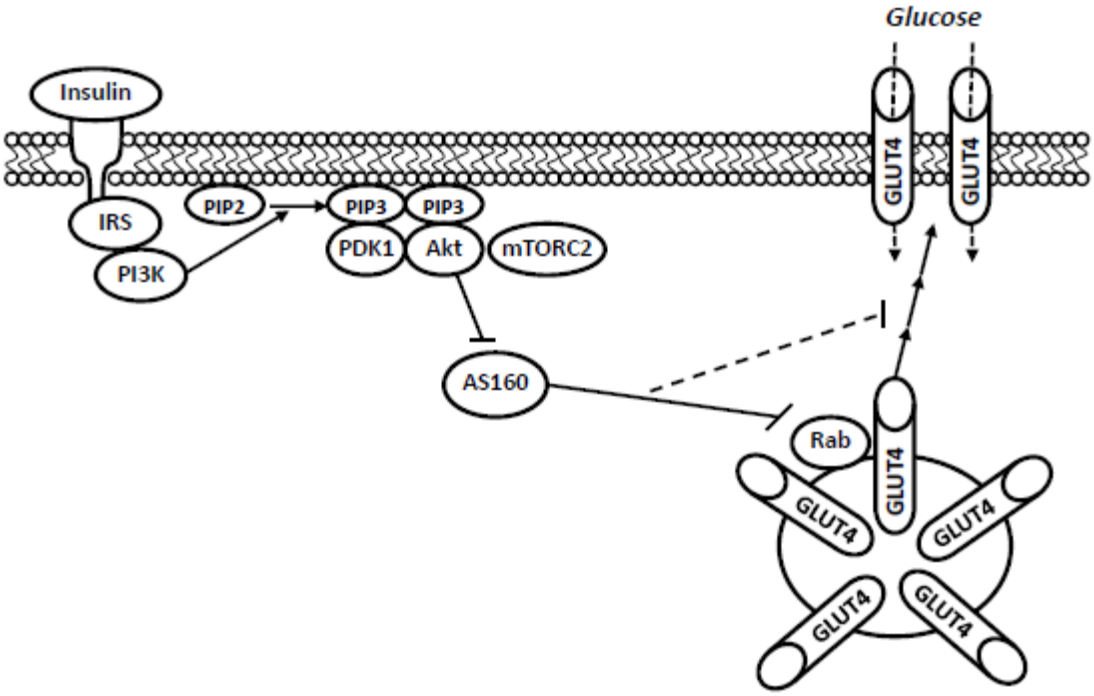

Figure 2-2. Major signaling events of insulin stimulated glucose uptake. Insulin binding to its receptor initiates a signaling cascade involving insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase-1 (PDK1), mammalian target of rapamycin complex-2 (mTORC2), and Akt. Akt phosphorylates AS160, inhibiting its RabGAP activity that allows subsequent Rab-dependent GLUT4 translocation to the cell surface membrane (represented by the dashed line). PIP2, phosphatidylinositol 4,5-phosphate; PIP3, phosphatidylinositol 3,4,5-phosphate.

Obesity is associated with impaired insulin signaling. These effects do not appear to be due to altered ability of insulin to bind its receptor, or insulin receptor autophosphorylation activity (43, 45, 117). However, insulin stimulated tyrosine phosphorylation of IRS-1 and IRS-1-associated PI3K activity are significantly attenuated in obese compared with lean individuals (11, 40, 98, 117). Additionally, artificial elevation of fatty acid availability in lean individuals similarly attenuates proximal insulin signaling (35, 42). Despite clear evidence of impaired insulin signaling upstream of Akt in obesity and in models of elevated fatty acid availability, the role of impaired Akt activation in insulin resistance is less apparent (64-65). Nonetheless, insulin stimulated phosphorylation of AS160 has been found to be reduced in skeletal muscle of individuals with T2DM (57). Furthermore, these observations in insulin resistant obese adults and experimental models of elevated systemic fatty acid availability are both

ultimately characterized by attenuated GLUT4 abundance at the muscle cell membrane (45, 117). Therefore, high fatty acid availability may be a key factor underlying impaired insulin signaling and GLUT4 translocation in obesity. Importantly, these deleterious effects are not likely the result of excess availability of fatty acids, *per se*, but rather the intracellular accumulation of lipid intermediates.

DAG and ceramide are the most well studied lipid intermediates believed to negatively regulate insulin signaling. DAGs are substrate for several enzymes in skeletal muscle, including DGAT of the triacylglycerol synthesis pathway and DAG-kinase for the synthesis of phosphatidic acid, a major membrane lipid. Genetic manipulation of these enzymes has been used to study the impact of altered skeletal muscle DAG content on insulin sensitivity. Mice with skeletal muscle specific overexpression of DGAT1 demonstrate increased accumulation of IMTG, low muscle DAG content, and are protected against high fat diet-induced insulin resistance (72). Conversely, mice with DAG-kinase-δ haploinsufficiency present with increased muscle DAG content and muscle-specific insulin resistance (28). These studies suggest that muscle DAG accumulation is likely important for the development of fatty acid-induced insulin resistance. It should be noted that some DGAT1-deficient mouse models (e.g., Agouti yellow, A^{Y}/a) are protected against high fat diet-induced obesity and insulin resistance (27). However, in this model it is likely that protection against weight gain (driven by altered energy balance) is at least partially responsible for protection against high fat diet-induced insulin resistance. Indeed, DGAT1-deficiency on a leptin-deficient background does not protect against weight gain, and these mice are not protected against high fat diet-induced insulin resistance (27). This indicates that protection against obesity, rather than DGAT1 deficiency, *per se*, may be mediating protection against insulin resistance in Agouti yellow DGAT1 deficient mice. Animal models have also been used to examine the role of ceramide accumulation in skeletal muscle insulin resistance. In a seminal paper from the laboratory of Scott Summers (49), induction of insulin resistance in rats via glucocorticoid exposure (dexamethasone injection), saturated lipid infusion, or a genetic obesity (Zucker diabetic fatty rats) were all associated with increased skeletal muscle ceramide content. To demonstrate a causal relationship between ceramide

accumulation and the onset of insulin resistance these authors showed that inhibition of ceramide synthesis via myriocin administration prevented both the accumulation of ceramide and induction of insulin resistance in skeletal muscle in each of these experimental models (49). Similar findings were recently reported in a rodent model of high fat diet-induced insulin resistance, whereby myriocin treatment was able to prevent accumulation of skeletal muscle ceramide content and preserve normal insulin sensitivity (119).

The effects of these lipid intermediates are thought to be the result of activation of several proinflammatory stress pathways, including protein kinase C (PKC; particularly PKCθ) (42, 55), inhibitor of IκB kinase (IKK)/nuclear factor-κB (NFκB) (6, 62, 113, 130), and/or c-Jun N-terminal kinase (JNK) (71, 92, 98). In brief, phosphorylation of serine residues on IRS-1 (e.g., $Ser^{307/312}$) appears to be a primary mechanism by which these various serine/threonine kinases inhibit insulin signaling (19, 85-86). Serine phosphorylation of IRS-1 alters the ability of the protein to serve as a substrate for the insulin receptor, leading to reduced insulin stimulated tyrosine phosphorylation of IRS-1 and subsequent propagation of the insulin signaling cascade (73, 81). Indeed, high fatty acid availability is associated with elevated activation of these proinflammatory pathways, increased serine phosphorylation of IRS-1, and attenuated insulin signaling (40, 55, 92, 98, 117). Furthermore, muscle-specific serine-to-alanine mutation of key IRS-1 residues that prevent serine phosphorylation of the protein provide protection against lipid-induced insulin resistance (85). Genetic manipulation and pharmacological studies also provide further support for the importance of these proinflammatory pathways in lipid-induced insulin resistance. Pharmacological inhibition of PKC (111) and knockout of PKCθ in mice (61) enhance insulin sensitivity and prevent lipid-induced insulin resistance. Similarly, knockout (6) and pharmacological inhibition (37, 54, 62, 130) of the IKK/NFκB pathway also protects against lipid-induced insulin resistance. Finally, both knockdown of JNK (92) and knockout of JNK-1 (47) prevent high fat diet-induced insulin resistance. Although discretely defined pathways of various proinflammatory activation via accumulation of skeletal muscle fatty acids have yet to be mapped, it is clear that finding therapies aimed at attenuating skeletal muscle lipid

accumulation and subsequent proinflammatory activation will be very important for the treatment of obesity-related insulin resistance.

A single session of exercise protects against lipid-induced insulin resistance

Exercise is a cornerstone prescription for the treatment of insulin resistance. Although regular exercise training potently enhances insulin sensitivity, much of the insulin sensitizing effect of "training" can be attributed to the most recent session(s) of exercise (33). In fact, a single session of exercise is sufficient to greatly enhance insulin sensitivity for hours and even days in healthy lean (79, 101, 110) and obese insulin resistant (34) adult humans. For the remainder of this review, the effects of only a single session of exercise (rather than exercise training) will be discussed unless specifically stated otherwise. In addition to providing further support for the insulin sensitizing effects of a single session of exercise, we have recently reported that a single session of exercise protects against lipid-induced insulin resistance (108, 110). In a recent study from our laboratory lean sedentary women performed two identical trials that differed only by the contents of an overnight infusion (108). In both trials subjects performed a single session of aerobic exercise during the morning of day one and were subsequently fed an identical diet designed to replenish muscle glycogen stores and maintain energy balance. During one trial, subjects received an overnight triacylglycerol plus heparin infusion designed to raise plasma fatty acid availability to a high physiologic level, while during the other trial subjects were infused with saline. Although several previous studies had found insulin sensitivity to be greatly reduced in response to a similar lipid infusion (14-15, 35), insulin sensitivity was identical the next morning in these two trials, suggesting that a single session of exercise protected against fatty acid-induced insulin resistance. Importantly, IMTG content was increased ~30% in response to the lipid infusion compared with saline. It has been previously demonstrated that individuals with the greatest capacity to partition fatty acids toward IMTG synthesis have the smallest impairment in insulin sensitivity when fat availability is increased through either diet or lipid plus heparin infusion (8). Therefore, in contrast to the previously discussed inverse relationship between IMTG content and insulin sensitivity in *sedentary* adult humans (38-39, 67, 93-94), we surmised that augmented storage of excess lipid as triacylglycerol

following a single session of exercise may limit the accumulation of harmful lipid intermediates known to inhibit insulin signaling, thereby providing protection against lipid-induced insulin resistance. To address this hypothesis, in another recent study from our laboratory sedentary women were again recruited to participate in two overnight experimental trials. These trials were identical other than that during one trial subjects remained sedentary, while during the other subjects performed a single session of exercise as in our previous study (110). To determine the effects of the lipid infusion on the accumulation of muscle lipid intermediates and insulin sensitivity after a single session of exercise compared with remaining sedentary, insulin sensitivity was measured both during the morning of day one (before exercise) and again after overnight lipid plus heparin infusion the next morning. As designed, the overnight lipid plus heparin infusion readily induced a ~30% reduction in insulin sensitivity when subjects remained sedentary (110). Impressively, the single session of exercise not only prevented a lipid-induced lowering of insulin sensitivity, but significantly enhanced insulin sensitivity the next morning despite overnight exposure to elevated fatty acid availability. Compared with remaining sedentary, muscle accumulation of DAG and ceramide was attenuated while IMTG content was increased nearly 50% the morning after a single session of exercise. Muscle GPAT1 and DGAT1 protein abundance was elevated the morning after exercise compared with remaining sedentary, and may have contributed to the augmented partitioning of fatty acids toward triacylglycerol synthesis. Measures of skeletal muscle proinflammatory stress paralleled these differences in muscle lipid accumulation such that inflammatory activation was greatly reduced the morning after exercise compared with remaining sedentary (e.g., ↓pJNK and ↑IκB). The results of these studies support our working hypothesis that a single session of exercise favorably alters the partitioning of excess fatty acids toward storage as triacylglycerol, thereby limiting excessive accumulation of harmful lipid intermediates that are known to inhibit insulin signaling via proinflammatory stress pathway activation.

It has been proposed that an impaired ability to oxidize fatty acids may underlie an accumulation of lipid intermediates and the resultant suppression of insulin action in obesity (58-59, 63, 102). In brief, the concept of “mitochondrial dysfunction,” either

inherent or as a consequence of obesity, describes the impaired ability of skeletal muscle to increase fatty acid oxidation to meet the delivery of fatty acid substrate resulting in muscle lipid accumulation and insulin resistance. Accordingly, several studies have suggested that increasing oxidative disposal of fatty acids provides important protection against lipid-induced insulin resistance (59, 74, 87). While the simplistic nature of this theory is attractive and seemingly logical, there are fundamental flaws and contradictory evidence worth acknowledging. Much of the following argument was recently well outlined by Dr. John Holloszy (50) and others (51), and is only briefly reviewed here. Most notably, the roughly 30% decrement in mitochondrial capacity observed in some insulin resistant populations (12, 86, 95) should have little to no effect on the ability of the skeletal muscle mitochondria to accommodate resting rates of fatty acid oxidation. Conservative estimates of maximal oxygen consumption within skeletal muscle of insulin resistant individuals with mitochondrial dysfunction are still 30-40 fold greater than resting values (50). In this context there is little reason to suggest that even substantial differences in maximal mitochondrial capacity could reasonably limit resting fat oxidation in obesity and/or insulin resistance. Additional evidence contrary to the notion that mitochondrial dysfunction underlies insulin resistance can be taken from studies of insulin resistant Asian Indians that exhibit higher mitochondrial capacity compared with insulin sensitive adults of northern European decent (90). Furthermore, high fat feeding in rodents has been shown to concurrently induce mitochondrial biogenesis and insulin resistance (44, 118). Finally, there is emerging evidence that decrements in mitochondrial capacity may in some instances present secondary to insulin resistance (18, 48). Therefore it is our belief that mitochondrial dysfunction, or more specifically low maximal mitochondrial oxidative capacity, does not underlie obesity-related insulin resistance. This is not to say that fat oxidation plays no role in protecting against lipid-induced insulin resistance. Acute exercise is often (110, 112, 124-125), but not always (91), associated with a subsequent increase in resting whole-body fat oxidation. It is likely that augmented oxidative disposal of fatty acids after exercise helps prevent accumulation of lipid intermediates within the myocyte; however, it is important to recognize that this change in fatty acid oxidation is likely to be quantitatively small relative to the availability and uptake of fatty acid. In summary, decrements in maximal

mitochondrial capacity are not likely responsible for the induction of insulin resistance in obesity, and increased fat oxidation is not sufficient to protect against lipid-induced insulin resistance.

It is clear that a single session of exercise is sufficient to greatly enhance insulin sensitivity for several hours and even into the next day in obese insulin resistant adult humans (34, 131), and this exercise-induced enhancement of insulin sensitivity is the result of increased insulin stimulated GLUT4 translocation to the cell surface membrane (46). Interestingly, there is very little evidence to support a role for enhanced insulin signaling after a session of exercise, as most human and animal data report little to no change in proximal insulin signaling (i.e., upstream of Akt) after exercise (for review, see (23)). Studies of obese humans are limited, but similarly suggest that acute exercise may not improve proximal insulin signaling despite enhanced insulin stimulated glucose uptake. For example, 24 h after a single session of moderate cycle ergometer exercise obese insulin resistant adults demonstrated improved insulin stimulated glucose uptake compared with remaining sedentary, yet exhibited no change in insulin stimulated IRS1-associated PI3K activity (30). In contrast, some groups have reported robust improvements in activation of insulin signaling proteins following a single session of exercise. A group of Brazilian investigators (103) has previously demonstrated that prolonged swim exercise (6 h) was able to completely restore normal insulin signaling (e.g., tyrosine phosphorylation of the insulin receptor and IRS1, serine phosphorylation of Akt) in skeletal muscle from high fat diet-induced obese rats incubated in a supraphysiological insulin concentration. This group recently extended these findings in a less prolonged, yet more intense bout of weighted swim exercise, showing similarly effective restoration of normal insulin signaling in high fat diet-induced obese rats following either exercise task (31). It is important to recognize that although these data are promising, these exercise protocols do not reflect practical exercise prescriptions for an insulin resistant human population and may not reflect *in vivo* human physiology. To this end, the role of enhanced insulin signaling in an exercise-induced improvement in insulin sensitivity remains unclear.

Regulation of muscle lipid metabolism and its relation to insulin sensitivity are poorly understood

The projects of my dissertation attempt to clarify the regulation of muscle lipid metabolism after exercise and how this may impact insulin sensitivity.

Dissertation Project I

Our working hypothesis is that a single session of exercise protects against lipid-induced insulin resistance in part by favorably altering the partitioning of excess fatty acids toward storage as triacylglycerol. Although it is clear that high plasma fatty acid availability after exercise (as in obesity) provides more substrate for IMTG synthesis, how elevated fatty acid availability alters the regulation of intramyocellular fatty acid metabolism after exercise is not completely understood. We have previously reported that a single session of exercise increased skeletal muscle protein abundance of DGAT1 and GPAT1 (110). However, whether elevated fatty acid availability after exercise is associated with increased GPAT and/or DGAT enzyme activity had not been determined. Regulation of fatty acid flux into the myocyte to provide the necessary substrate for IMTG synthesis may also be important for altered fatty acid partitioning, but the effect of elevated fatty acid availability after exercise on myocyte fatty acid transport capacity was not known. Emerging evidence indicates that IMTG accumulation as hydrophobic lipid droplets within the cytosol may be under the regulation of a family of proteins that are known to be associated with intracellular lipid droplets, now collectively referred to as perilipins (66). Perilipin proteins have been suggested to be involved in the metabolic regulation of the triacylglycerols within the lipid droplet (e.g., storage (127, 129), lipolysis (20, 116), oxidation (32, 128)), as well as involved in trafficking the lipid droplet toward specific sites and/or signaling pathways within the cell (for review, see (126)). Still, the specific roles of each of the perilipin proteins in muscle had yet to be completely elucidated. For example, the improvement in insulin sensitivity during weight loss has been associated with increased muscle perilipin 2 relative to IMTG content (96); while in contrast, it has recently been reported that the improvement in insulin sensitivity after thiazoladinedione treatment was accompanied by a reduction in the abundance of perilipin proteins relative to the IMTG content (80). Examination of

the putative regulators of muscle lipid triacylglycerol accumulation during conditions of high fatty acid availability after exercise helped to determine which of these adaptations may be key contributors to the improvement in insulin sensitivity found after a single session of exercise in obesity. Project 1 of my dissertation determined whether elevated fatty acid availability alters the regulation of muscle lipid metabolism after exercise.

Dissertation Project II

The minimal "dose" of exercise required to enhance insulin sensitivity the next day in obese adults had not been identified. Furthermore, whether the intensity of the exercise performed plays a role in exercise-induced changes in insulin sensitivity had not been well studied. In what may have been the most comprehensive study regarding the effect of the intensity of a single session of exercise on insulin sensitivity the next day, Zhang, et al. (131) found that 1 h of exercise at 60% and 70% of VO_2peak significantly reduced the Homeostatic Model Assessment (HOMA-IR), while exercise at 40% did not. However, HOMA-IR provided only a very crude index of insulin resistance, and because they did not control for exercise energy expenditure or energy balance in their study (i.e., the higher the exercise intensity, the greater the negative energy balance) these data did not distinguish the effects of exercise, *per se*, from the insulin sensitizing effects of an energy deficit. In contrast to these findings, 1 h of lower intensity exercise (35% of cycle ergometry W_{max}) had been found to significantly lower 24 h glycemia compared with remaining sedentary in subjects with T2DM, while the modest reduction in 24 h glycemia following 30 min of isoenergetic exercise at a higher intensity (70% of cycle ergometry W_{max}) did not reach statistical significance (76). It is important to note that glycemia was determined by a number of factors, and cannot be interpreted as a measure of insulin sensitivity. In a separate study, lower intensity exercise (40-50% VO_2peak) during 8 mo of an exercise training program enhanced insulin sensitivity in overweight and obese adults to a greater degree than higher intensity exercise (65-80% VO_2peak) designed to illicit the same total energy expenditure from exercise (10). However, the lack of a measure of insulin sensitivity in the former study (76) and inability to distinguish the effects of "training" from the most recent session(s) of exercise in the latter study (10) limited the interpretation of these findings. Given the state of disagreement among

studies in this area, it was clear that there was a need for a well controlled examination of the effect of exercise intensity on the ability of a single session of exercise to enhance insulin sensitivity in obese adult humans. Furthermore, it was important to examine putative mechanisms for the insulin sensitizing effects of exercise, including exercise-induced alterations in muscle lipid metabolism. There had been no studies in obese adult humans that had examined the role of exercise intensity in altered muscle lipid metabolism after a single bout of exercise, and determined whether these changes relate to improved insulin sensitivity. Project 2 of my dissertation determined the effect of mild (50% VO_2peak) and moderate (65% VO_2peak) exercise intensity during a single session of exercise on systemic fatty acid availability, the accumulation of muscle lipids, and insulin sensitivity measured the next day in obese adults.

Dissertation Project III

Excessive fatty acid availability, as found in obesity, impairs insulin sensitivity largely due to the accumulation of intramyocellular lipid intermediates. Surprisingly little was known about the role of fatty acid availability on the regulation of muscle lipid metabolism, including the effect(s) of graded doses of fatty acid on lipid accumulation, the regulation of lipid metabolism, and the importance of these changes on insulin signaling and insulin action in muscle. Furthermore, evidence concerning the importance of specific fatty acid species and the role of saturated *vs.* unsaturated fatty acids in fatty acid-induced insulin resistance was conflicting. Several independent studies had reported that saturated fatty acids (i.e., palmitate) readily induced insulin resistance and that unsaturated fatty acid (i.e., oleate) could actually protect against palmitate-induced insulin resistance *in vitro* (25-26, 84), whereas lipid-infusion studies in humans have reported rapid induction of insulin resistance even when the lipid emulsion was almost entirely (~90%) composed of unsaturated fatty acids (8, 14-15, 41, 110). There had been no explanation for these paradoxical findings *in vitro* compared with *in vivo*. Perhaps more importantly, it should be noted that not one of the aforementioned models of elevated fatty acid availability provided an accurate reflection of the elevated fatty acid availability common to obesity (i.e., an unbiased increase of the mixture of many different fatty acids comprising adipose tissue TAGs). Determination of the effects of

increasing fatty acid availability on muscle cell insulin signaling and lipid metabolism in a way that might more closely resemble the elevated fatty acid availability commonly found in obesity (i.e., a mixture of the most abundant plasma fatty acids), yielded new insight into the regulation of muscle lipid metabolism and changes in muscle cell insulin signaling. Project 3 of my dissertation was designed to determine the effect of increasing availability of physiologic mixtures of fatty acids on muscle cell insulin signaling and lipid accumulation, and to examine whether increasing fatty acid availability alters lipogenic, lipolytic, and/or fatty acid transport proteins in cultured muscle cells.

Summary of Review of Literature

The overarching goal of my dissertation was to determine the effects of exercise and fatty acid availability on muscle lipid metabolism and insulin sensitivity. My working hypothesis was that exercise-induced alterations in skeletal muscle lipid metabolism provide protection against fatty acid induced (i.e., obesity-related) insulin resistance. The regulation of muscle lipid metabolism during elevated fatty acid availability and in obesity was poorly understood. It was not known whether the availability of fatty acid alters the regulation of muscle lipid metabolism after exercise, or if the intensity of an exercise session in an obese population differentially affected insulin sensitivity and/or muscle lipid metabolism. In this context, it was unclear if altered skeletal muscle lipid metabolism is important for the exercise induced improvement in insulin sensitivity in obesity, and how these changes may mechanistically drive the improvement in insulin sensitivity. It also remained to be seen how an increase in availability of a physiologic mixture of fatty acids influences muscle lipid metabolism and fatty acid partitioning, and how these responses may relate to muscle cell insulin signaling. These gaps in knowledge were addressed in three separate projects. Completion of these projects has enhanced our understanding of the mechanism(s) underlying insulin resistance in obesity, and identified a refined exercise prescription for the treatment of insulin resistance in obesity.

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CHAPTER 3

High fatty acid availability after exercise alters the regulation of muscle lipid metabolism

Abstract

We previously reported that a single exercise session protects against fatty acid (FA)-induced insulin resistance, perhaps in part through augmented intramyocellular triacylglycerol (IMTG) synthesis. The aim of this study was to examine the effect of elevated FA availability after exercise on factors regulating IMTG metabolism. After exercise (90min, 65% VO_2peak), 7 healthy women (body mass index: 23±1 kg/m^2) were infused overnight (16h) with either a lipid and heparin solution (LIPID; 0.11 g fat/kg/h) or saline (SALINE). We measured resting FA oxidation (indirect calorimetry) and obtained a skeletal muscle biopsy sample the next morning. The 4-fold increase in overnight plasma FA concentration during LIPID increased IMTG by ~30% during LIPID vs. SALINE (49±3 vs. 38±3 μmol/g dw; P=0.04). This was accompanied by ~25% greater membrane-associated abundance of the FA transporter FAT/CD36 (P<0.01), and ~8% increase in the activity of the IMTG synthesis enzyme glycerol-3-phosphate acyltransferase (GPAT; P<0.01). In contrast, resting FA oxidation was not affected. We also found no difference in the protein abundance of GPAT1 and diacylglycerol acyltransferase-1 (DGAT1), DGAT activity, or the abundance of the lipid droplet coat proteins (perilipins 2, 3, 4, 5) between treatments. Our findings suggest augmented capacity for FA flux into muscle (i.e., via membrane-associated FAT/CD36), perhaps together with a slight, yet significant increase in activity of a key IMTG synthesis enzyme (GPAT) may enhance IMTG storage when FA availability is high after exercise. The importance of the absence of a change in perilipin protein abundance despite increased muscle lipid storage remains to be determined.

Introduction

Excessive fatty acid availability is a primary contributor to the insulin resistance found in obesity (2, 28), and we have demonstrated a single session of exercise can protect against fatty acid-induced insulin resistance (27, 29). We attributed at least part of this protective effect of exercise to an increase in intramyocellular triacylglycerol (IMTG) synthesis for several hours after the exercise session (27, 29). While it is clear that high plasma fatty acid availability after exercise (as in obesity) provides more substrate necessary for IMTG synthesis, how elevated fatty acid availability alters the regulation of intramyocellular fatty acid metabolism after exercise is not completely understood.

The synthesis of IMTG occurs through the succession of four reactions. The first committed step of this process is regulated by the enzyme glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the production of lysophosphatidic acid (LPA) from fatty acyl-CoA and glycerol-3-phosphate. The final key step in the triacylglycerol synthesis pathway is regulated by the enzyme diacylglycerol acyltransferase (DGAT), which catalyzes the esterification of a third fatty acyl-CoA to DAG to create a triacylglycerol. We found that a single session of exercise was sufficient to increase the protein abundance of both GPAT and DGAT (29). However, whether elevated fatty acid availability after exercise is associated with increased GPAT and/or DGAT enzyme activity remains to be determined.

In addition to changes in activity of the triacylglycerol synthesis pathway enzymes, increased fatty acid availability may also influence other factors that can regulate IMTG metabolism after exercise. Perhaps most important is the regulation of fatty acid flux into the myocyte, to provide the necessary substrate for IMTG synthesis. Fatty acid translocase (FAT/CD36) is a principal skeletal muscle fatty acid transporter (5, 9), and the rate of fatty acid uptake is proportional to the abundance of FAT/CD36 on the plasma membrane (1, 6). Furthermore, because IMTG accumulate largely in hydrophobic lipid droplets within the cytosol, regulation of IMTG metabolism may also be affected by a family of proteins that are known to be associated with intracellular lipid droplets (now collectively referred to as “perilipins” (20)). Although the role of this family of five

perilipin proteins on lipid metabolism has predominantly been studied in adipocytes (for reviews, see (4, 33)), most of the perilipin proteins have now also been identified in skeletal muscle, and have been the subject of several recent studies . However, whether high availability of fatty acid after exercise augments muscle fatty acid transport capacity (i.e., increased fatty acid transporter abundance at the muscle membrane) or the abundance of the perilipin proteins changes in parallel with IMTG accumulation is not clear. The primary objective of this study was to determine if the accumulation of IMTG that we observed in response to a high availability of fatty acids after exercise (27), was accompanied by: 1) increased activity of key enzymes of the muscle triacylglycerol esterification pathway (i.e., GPAT and DGAT), 2) increased fatty acid transport capacity in muscle (i.e., fatty acid transporter abundance at the muscle membrane), and 3) changes in the abundance of perilipin proteins within skeletal muscle.

Methods

Subjects

Seven sedentary but otherwise healthy women (age 27 ± 4 years, body mass 62.6 ± 3.7 kg, body mass index 22.9 ± 1.0 kg/m^2) volunteered to participate in this study. Subjects were not taking any medications (except oral contraceptives), and all subjects underwent a comprehensive medical examination, including a history and physical examination, a 12-lead electrocardiogram, and standard blood and urine tests. All subjects were non-smokers, weight stable (i.e., ± 2 kg), had been sedentary (regular exercise < 2 h/wk) for at least 6 months before the study. Any history of metabolic or cardiovascular disease resulted in exclusion from participation. All of these subjects also participated in a previous study in our laboratory (27). Written, informed consent was obtained from all subjects before initiating participation. All procedures of this study were approved by the University of Michigan Institutional Review Board.

Preliminary testing

Prior to initiating the experimental protocol, subjects underwent an incremental peak oxygen uptake test (VO_{2peak}; 40.9 ± 2.3 ml/kg/min) on a stationary bicycle ergometer to assess aerobic fitness, and hydrostatic weighing was used to assess body composition

(body fat 28.7 ± 1.5 %). This preliminary exercise test was performed at least one week before the subjects' first experimental trial.

Experimental protocol

All subjects performed two experimental trials, separated by ≥ 7 days, and all trials were completed during the follicular phase of the subjects' menstrual cycle (i.e., within the first 2 weeks after the onset of menses). The order of the trials was randomized, and the two trials differed only by the contents of the overnight infusion (see below). The day before each trial, subjects received a standardized evening meal (2.25 g carbohydrate/kg, 0.5 g fat/kg, 0.375 g protein/kg) prepared by the Michigan Clinical Research Unit (MCRU) that was eaten at home and completed at 2130 h. The next morning (Day 1) subjects were admitted to the MCRU at 0830 h, after an overnight fast. Beginning at 1000 h subjects began 90 min of exercise at ~65% VO_2peak. Exercise consisted of 45 min of treadmill exercise, immediately followed by 45 min of exercise on a cycle ergometer. Low fat meals were provided after the exercise session at 1200, 1400, and 2030 h (total content of the three meals: 8 g carbohydrate/kg, 0.3 g fat/kg, 1.1 g protein/kg). Meal energy intake was calculated to match the estimated energy expenditure during Day 1 of each trial. At ~1415 h two intravenous catheters were placed, one in an antecubital vein for use during the overnight infusion and the other in a hand vein in the contra-lateral arm for blood sampling. The overnight infusion began at 1500 h and continued until 0700 h the next morning. The content of this infusion was the only difference between the two experimental trials. On one occasion (LIPID), subjects were infused overnight with a 20% lipid emulsion (Abbott Laboratories, North Chicago, IL; [0.55 mL/kg/h]) and heparin (Elkins-Sinn, Inc., Cherry Hill, NJ; [5 U/kg/h]) with the goal of increasing overnight plasma fatty acid concentration to a high physiologic level (~1.0 mmol/L). Because our subjects were eating a weight maintaining diet, this lipid infusion resulted in a total positive energy balance. However, even when consuming a weight-maintain diet, systemic fatty acid availability is very high in obesity. Therefore, while an obese individual may be in a state of neutral energy balance (i.e., energy intake = energy expenditure) their systemic energy availability is very high compared with a lean person,

at least in part because of their high lipolytic rates. It was the objective of our study design to mimic this condition.

During the other trial (SALINE), subjects were infused overnight with normal saline (0.55 mL/kg/h). Indeed, plasma fatty acid concentration was elevated to an overnight average of 0.84 ± 0.14 mmol/L during the LIPID compared with 0.22 ± 0.04 mmol/L during SALINE (27). The next morning, resting oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured (DeltaTrac, SensorMedics Inc., Yorba Linda, CA) at 0630 h to assess rates of substrate oxidation. At 0900 h, a muscle biopsy was obtained from the vastus lateralis muscle of the thigh using the percutaneous biopsy technique. Muscle biopsy samples were dissected free of adipose and connective tissue, rinsed in saline, dried and then frozen in liquid nitrogen. Muscle samples were stored at -80ºC until biochemical analysis.

Analytical Procedures

Muscle DAG and ceramide concentration

Muscle DAG and ceramide content were assessed using the DAG-kinase assay as previously described (25). In brief, lipid was extracted from ~5 mg (dry weight) of lyophilized muscle for each sample using a chloroform-methanol-water (1:2:0.8) homogenization buffer. The reaction was carried out for 2 h at room temperature by adding DAG-kinase and ^{32}P-ATP to the lipid extracts. The reaction was stopped with chloroform-methanol (2:1). The organic phase was dried, re-dissolved in 65 µl chloroform-methanol (2:1), and spotted for thin layer chromatography (TLC; Whatman Inc.). Lipids were separated in chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10) and ^{32}P-labelled phosphatidic acid and ceramide-1-phosphate spots were visualized via radiography, scraped, and counted in scintillation fluid (Tri-Carb 2800TR, Perkin Elmer, Waltham, MA).

Muscle GPAT and DGAT enzyme activity

The enzyme activity of GPAT and DGAT were assessed in partially purified membrane fractions similar to as previously described (32, 37). Briefly, ~20 mg of each muscle

sample was homogenized in buffer solution (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM DTT, 250 mM sucrose for GPAT and 20 mM HEPES [pH 7.4], 1 mM $CaCl_2$, 1 mM DTT, 250 mM sucrose for DGAT). A mixture of protease inhibitors was also added to each of the homogenization buffers. Following a 30 min incubation, homogenates were centrifuged at 1500 g for 10 min at 4^{o}C. Pellets were discarded and the supernatants were centrifuged for 2 h at 38,000 rpm (> 150,000 g) at 4^{o}C. Supernatant was saved from the DGAT preparations for immunoblot analysis of cytosolic proteins (see below). Pellets were manually homogenized and re-dissolved in the homogenization buffer. Protein content of the resultant solution was measured (Pierce BCA Protein Assay, Thermo Scientific). For total GPAT activity, the reaction was carried out using 10 µg protein in a 200 µl reaction mixture containing 75 mM Tris pH 7.5, 1 mg/ml BSA (fatty acid-free), 4 mM $MgCl_2$, 1 mM DTT, 8 mM NaF, 80 µM palmitol-CoA and 414 mM ^{14}C glycerol 3-phosphate (SA >20,000 dpm/nmol) for 20min at 37^{o}C H_2O bath with agitation. The organic phase containing ^{14}C-labelled LPA was dried, reconstituted in scintillation fluid, and measured for radioactivity. For total DGAT activity, the reaction was carried out using 10 µg protein in a 200µl reaction mixture containing 100 mM Tris pH 7.5, 250 mM sucrose, 1 mg/ml BSA (fatty acid-free), 150 mM $MgCl_2$, 0.8 mM EDTA, 0.25 mM DAG, and 25 µM palmitoyl-CoA with 0.1 µCi ^{14}C palmitoyl-CoA (SA >30,000 dpm/nmol) at 37^{o}C for 20 min in a water bath with agitation. The reaction was stopped with 0.75 ml chloroform-methanol (2:1). After a 2 h room temperature lipid extraction, 0.375ml of 1 mM H_2SO_4/17 mM NaCl was added to facilitate lipid-aqueous phase separation. The organic phase was dried, re-dissolved in 30 µl chloroform, and spotted for TLC. Lipids were separated in chloroform-acetic acid (96:4) and triacylglycerol spots were visualized with iodine vapor, scraped, and counted in scintillation fluid.

Western blotting

Cytosolic and crude membrane fractions of muscle from DGAT activity preparations (see above) were used for immunoblot analysis of protein contents in muscle. Proteins from the centrifugation supernatant of the DGAT activity preparation were concentrated using Amicon Ultra Centrifugal Filters (MWCO 3KD, Millipore) and used for electrophoresis

analysis of cytosolic proteins. 35 µg of cytosolic proteins or 25 µg of membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Crude membrane protein fractions were used to assess GPAT1 and DGAT1 protein abundance. For all other immunoblot analyses, the use of cytosolic and/or membrane protein fractions is indicated throughout the manuscript. Blots were probed with the following antibodies: α-NADH-ubiquinol oxidoreductase (COX-I; Molecular Probes, A21344), α-FAT/CD36 (Santa Cruz Biotechnology, sc-9154), α- perilipin 1, 2, 3, and 4 (all gifts from P.E. Bickel and N.E. Wolins), α-perilipin 5 (American Research Products, 03-GP31), α-GPAT1 (a gift from R.A. Coleman), and α-DGAT1 (Novus Biologicals, NB110-41487). Membranes were incubated with appropriate secondary antibodies and developed using enhanced chemiluminescence (Amersham Biosciences). Bands were imaged and then quantified via densitometry (AlphaEaseFC, Alpha Innotech Corp.). All within-subject comparisons were made using the same blot.

Calculations

Respiratory exchange ratio and fat oxidation

Respiratory exchange ratio (RER) was calculated as the ratio of VCO_2 to VO_2. Whole body fat/triacylglycerol oxidation (g/min) was calculated from VO_2 and VCO_2 measurements using the equations of Frayn (15). Whole body fatty acid oxidation was calculated by dividing triacylglycerol oxidation by an estimated molecular weight of triacylglycerol (860 g/mol) and multiplying by 3.

Muscle GPAT enzyme activity

Muscle GPAT enzyme activity was calculated as:

$$\frac{counts\ of\ samples\ (dpm)}{conversion\ factor\ (\frac{dpm}{pmol})} X \frac{1}{proteins\ (mg)} X \frac{1}{20min} X \frac{1}{fraction\ of\ 14C\ G3P}$$

The conversion factor of ^{14}C glycerol 3-phosphate (G3P) was calculated by dividing the counts of 1µl ^{14}C-G3P (dpm), by ^{14}C-G3P concentration (pmol/µl). The fraction of ^{14}C-G3P refers to the ratio of ^{14}C-G3P to total G3P (mol) in the reaction mixture. GPAT activity during LIPID was calculated relative to GPAT activity during SALINE, in a within-subject manner, with the mean of these calculations expressed relative to one.

Muscle DGAT enzyme activity

Muscle DGAT enzyme activity was calculated as:

$$\frac{counts\ of\ samples\ (dpm)}{conversion\ factor\ (\frac{dpm}{pmol})} X \frac{1}{proteins\ (mg)} X \frac{1}{20min} X \frac{1}{fraction\ of\ 14C\ palmitoyl-CoA}$$

The conversion factor was calculated by dividing counts of 1µl ^{14}C palmitoyl-CoA (dpm), by ^{14}C palmitoyl-CoA concentration (pmol/µl). The fraction of ^{14}C palmitoyl-CoA is the percentage of ^{14}C palmitoyl-CoA in total palmitoyl-CoA (mol) in the reaction mixture. DGAT activity during LIPID was calculated relative to DGAT activity during SALINE, in a within-subject manner, with the mean of these calculations be presented relative to one.

Statistical analysis

A paired, two-tailed Student's *t*-test was used to test for significant differences in all outcome variables between trials. Pearson Product Moment Correlation analysis was used to examine the relationship between outcome variables. Due to limited muscle sample acquisition during some trials, analysis of DAG and ceramide (n = 4) and GPAT1 protein content (n = 5) could not be performed using tissue from all subjects (n = 7). Statistical significance was defined as $P < 0.05$. All results are presented as means ± standard error (SE).

Results

Muscle lipids

We have previously reported (27) that IMTG concentration was significantly increased in response to elevated fatty acid availability during LIPID compared with SALINE. In parallel with the marked increase in IMTG concentration, presently we found GPAT enzyme activity in skeletal muscle to be slightly, yet significantly greater during LIPID compared with SALINE (P = 0.01; Figure 3-1a). However, the small increase in muscle GPAT1 protein abundance between trials did not reach statistical significance (Figure 3-1b). We found no differences in muscle DGAT activity or DGAT1 protein abundance between trials (Figure 3-2). In contrast to the elevated IMTG concentration with the lipid

infusion, muscle concentrations of the lipid intermediates, DAG (1839 ± 251 vs. 1494 ± 229 pmol/mg protein for LIPID and SALINE, respectively; $P = 0.35$) and ceramide (665 ± 37 vs. 636 ± 72 pmol/mg protein for LIPD and SALINE, respectively; $P = 0.52$) were not different between trials.

FAT/CD36 protein abundance

The lipid infusion significantly increased membrane-associated FAT/CD36 ($P = 0.005$; Figure 3-3), while cytosolic FAT/CD36 protein content was not affected (3.7 ± 0.6 vs. 3.8 ± 0.5 arbitrary units (AU) for LIPID and SALINE, respectively; $P = 0.59$). Interestingly, we found membrane-associated FAT/CD36 protein content to be positively correlated with IMTG concentration (Figure 3-4).

Fatty acid oxidation

Despite the increase in muscle membrane-associated FAT/CD36 abundance and a near three-fold increase in overnight plasma fatty acid concentration during LIPID compared with SALINE, neither RER (0.83 ± 0.02 vs. 0.81 ± 0.02, respectively, $P = 0.38$) nor whole body fatty acid oxidation (3.0 ± 0.6 vs. 3.5 ± 0.4 μmol/kg/min, respectively, $P = 0.35$) were different between trials the next morning. COX-I protein content in the muscle membrane fraction, an indicator of oxidative capacity, was also identical between trials (1.2 ± 0.2 vs. 1.2 ± 0.2 AU for LIPID and SALINE, respectively; $P = 0.49$).

Perilipin proteins

Perilipin 1 was not detected in muscle homogenates, indicating that our muscle samples were free of adipose tissue contamination (Figure 3-5a). We did detect perilipins 2, 3, 4, and 5 in the skeletal muscle samples from both trials. Interestingly, the augmented IMTG concentration during LIPID vs. SALINE was not accompanied by elevated concentrations of any of these perilipin proteins within either the cytosolic or membrane fractions (Figure 3-5b and 3-5c). We did not detect any perilipin 5 protein in the membrane fractions of any of our muscle samples (Figure 3-5c).

Discussion

We have previously demonstrated that alterations in muscle lipid metabolism in the several hours after exercise can help offset insulin resistance stemming from the excessive fatty acid availability commonly found in obesity (27, 29). However, the influence of elevated fatty acid availability on specific changes in intramyocellular lipid metabolism after exercise is not completely understood. Here we found that the accumulation of IMTG resulting from high systemic fatty acid availability after exercise was accompanied by a small but significant increase in the activity of GPAT, which is a key regulating step in the triacylglycerol esterification pathway. Perhaps more importantly, we found the lipid infusion increased the abundance of FAT/CD36 in the membrane fraction from skeletal muscle, suggesting that the capacity to transport fatty acids into the cell was enhanced. In fact, the abundance of membrane-associated FAT/CD36 was significantly correlated with IMTG concentration. Additionally, we found that the abundance of lipid droplet coating proteins (i.e., perilipins) was not increased despite the marked elevation in lipid storage within muscle.

High systemic fatty acid availability after exercise is known to augment triacylglycerol resynthesis in muscle and elevate IMTG concentration (14, 27, 29). Because fatty acids largely enter skeletal muscle via protein-mediated transport (30), the abundance of fatty acid transporters at the myocyte membrane largely dictates the capacity for fatty acid transport into the cell (1, 6). Our finding that FAT/CD36 abundance in muscle membrane fractions was greater after the lipid infusion compared with saline despite no difference in the cytosolic fraction between trials suggests that augmented fatty acid likely increased the total abundance of FAT/CD36 protein, but that the additional FAT/CD36 was exclusively localized at the muscle membrane. This expands on previous studies that have reported augmented muscle membrane FAT/CD36 in obesity (1, 6), by suggesting the chronic elevation in fatty acid availability found in obesity may be responsible for the increased abundance and altered basal localization of muscle FAT/CD36. How fatty acid may be inducing this effect has yet to be determined, as pharmacological activation of the fatty acid ligand inducible transcription factors peroxisome proliferator-activated receptors (PPARs) α and γ does not augment

FAT/CD36 transcription in rat skeletal muscle (3), despite the known presence of a peroxisome proliferator response element (PPRE) in the promoter region of the FAT/CD36 gene (26). Because our measurement of FAT/CD36 in crude membrane preparations did not allow us to determine the specific localization of FAT/CD36, we do not have definitive evidence to support that this increased abundance of FAT/CD36 in our study occurred within the plasma membrane. However, based on the previous finding of increased sarcolemmal FAT/CD36 protein content in obese compared with lean individuals (1, 6), it is likely that the lipid infusion in our study increased FAT/CD36 within the plasma membrane. We surmise that elevated fatty acid availability can increase membrane-associated FAT/CD36, thereby augmenting long-chain fatty acid uptake capacity into the myocyte. The significant correlation we observed between membrane-associated FAT/CD36 and IMTG concentration suggests that the increased abundance of FAT/CD36 in the plasma membrane may be a key step in augmenting IMTG accumulation when circulating fatty acid availability is elevated after exercise, and is in agreement with other recent studies highlighting the role of FAT/CD36 in muscle lipid accumulation (1, 6).

Accompanying the greater capacity for fatty acid flux into muscle in response to the lipid infusion, we also found a slight, yet significant increase in the activity of the enzyme that catalyzes the first committed step of the triacylglycerol synthesis pathway in muscle (i.e., GPAT). Conversely, we found no effect of the lipid infusion on DGAT activity, which catalyzes the final step of the esterification pathway. The relatively small increase in total GPAT activity that we found occurred in absence of a significant increase in GPAT1 protein content, which could suggest that either the intrinsic activity of the enzyme was increased, or isoforms of GPAT other than GPAT1 were increased. Alternatively, it is possible that a small increase in GPAT1 protein abundance was simply not detected with our immunoblotting technique, as GPAT activity was identical between trials when expressed relative to GPAT1 protein abundance (8.3 ± 0.4 vs. 8.3 ± 0.5 AU, respectively). We previously reported that a prior session of exercise increased the protein abundance of both GPAT1 and DGAT1 in skeletal muscle in a similar time frame as in this study (29). Because our subjects performed a session of exercise the day before

the muscle biopsy in both the LIPID and SALINE trials of this study, it is important to acknowledge that this session of exercise may have increased GPAT and DGAT protein abundance (and activity) during both trials compared with if no exercise had been performed.

It has been proposed that an impaired ability to oxidize fatty acids may underlie an accumulation of lipid intermediates, and the resultant suppression in insulin action in obesity (18-19). Accordingly, several studies have suggested that increasing oxidative disposal of fatty acids provides important protection against lipid-induced insulin resistance (19, 21). However, whether or not fat oxidation plays a key role in the accumulation of intramyocellular lipids and insulin resistance in obesity is controversial (7, 13, 16-17). We recently reported that in these same subjects a single session of exercise protected against the lipid-induced insulin resistance (27), and here we confirm that this protection occurred in absence of an increase in fat oxidation. Moreover, our present finding that muscle DAG and ceramide concentrations were no greater after LIPID compared with SALINE, indicates that an increase in fatty acid oxidation is not required to prevent the accumulation of these lipid intermediates. Together, these data suggest that exercise-induced protection against lipid-induced insulin resistance is not dependent on an increase in fat oxidation.

Intramyocellular lipids are mainly stored in lipid droplets that are coated by specialized proteins. The largest family of these lipid droplet-associated proteins are now collectively referred to as perilipins (20). Perilipins help establish and maintain the partition between insoluble triacylglycerols and the aqueous cytosol, while retaining a physical connection between the phases. Perilipin proteins have been suggested to be involved in the metabolic regulation of the triacylglycerols within the lipid droplet (e.g., storage (34, 36), lipolysis (8, 31), oxidation (10, 35)), as well as involved in trafficking the lipid droplet toward specific sites and/or signaling pathways within the cell (for review, see (33)). Still, the specific roles of each of the perilipin proteins in muscle have yet to be completely elucidated. Our findings indicate that the ~30% increase in IMTG concentration during LIPID was not paralleled by an increased protein abundance of

perilipin 2, 3, 4 or 5. This suggests that the perilipin coat surrounding the intramyocellular lipid droplets was less dense during LIPID compared with SALINE. This finding was somewhat unexpected given that the expression of perilipins 2, 4 and 5 is known to be upregulated by PPARs α and γ (11-12). One potential functional outcome associated with a lower perilipin density is that with less of this protein coat, the lipid droplet may be more susceptible to lipase activity. However, we did not observe an increase in DAG concentration as one might expect if IMTG lipolytic rate was accelerated. The effect of changes in perilipin density on insulin sensitivity is also equivocal. The improvement in insulin sensitivity during weight loss has been associated with increased muscle perilipin 2 relative to IMTG content (24). In contrast, it has recently been reported that the improvement in insulin sensitivity after thiazoladinedione treatment was accompanied by a reduction in the abundance of perilipin proteins relative to the IMTG content (22). Therefore, whether a change in perilipin density relative to IMTG content is important for the metabolic regulation of lipid storage and downstream effects of insulin sensitivity remains to be determined.

In summary, our findings suggest that an increased fatty acid transport capacity (as indicated by greater membrane-associated FAT/CD36 transporter protein abundance), together with a slight, yet significant increase in muscle GPAT activity underlie the increased accumulation of IMTG when fatty acid availability is high after exercise. Because partitioning of fatty acids toward neutral lipid (i.e., IMTG) has been proposed to protect against insulin resistance (2, 27, 29), these adaptations may be key contributors to the improvement in insulin sensitivity found after a single session of exercise in obesity. However, the relatively small increase in muscle GPAT activity with no rise in DGAT activity during the lipid infusion, suggests that direct adaptations within the triacylglycerol esterification pathway may be secondary to the increase fatty acid transport capacity. The significant correlation we observed between membrane-associated FAT/CD36 and IMTG concentration does not prove causality, but helps support the potential impact of augmented transport capacity on IMTG synthesis. Additionally, our novel finding that the protein abundance of perilipin 2, 3, 4 and 5 did not increase in parallel with IMTG accumulation suggests that elevating fatty acid

availability after exercise reduced the IMTG perilipin coating density. While changes in perilipin content of a lipid droplet can impact the metabolic fate of cellular lipids, the functional significance of the lower perilipin density we observed here has yet to be determined.

Acknowledgements

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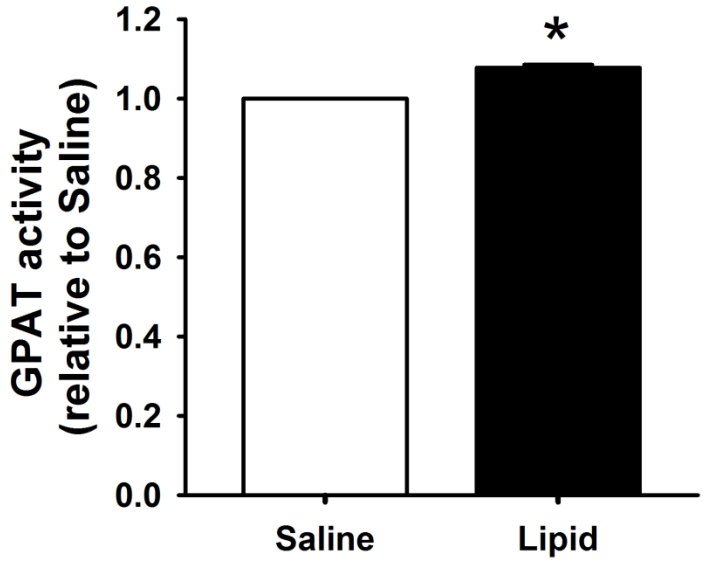
A.



B.

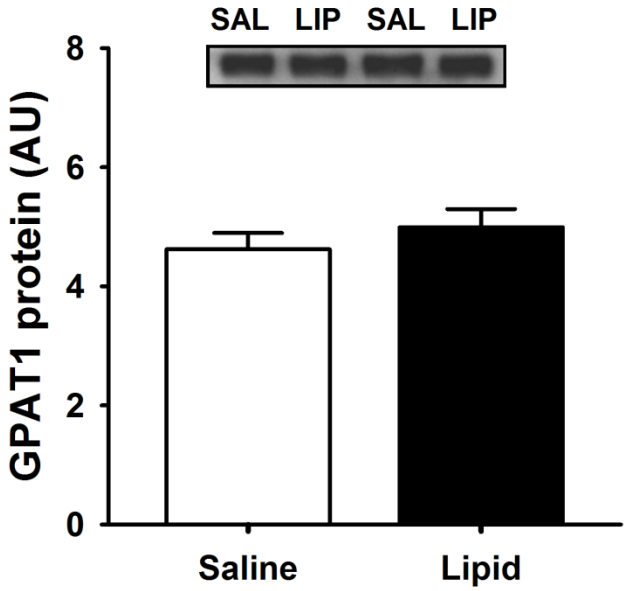
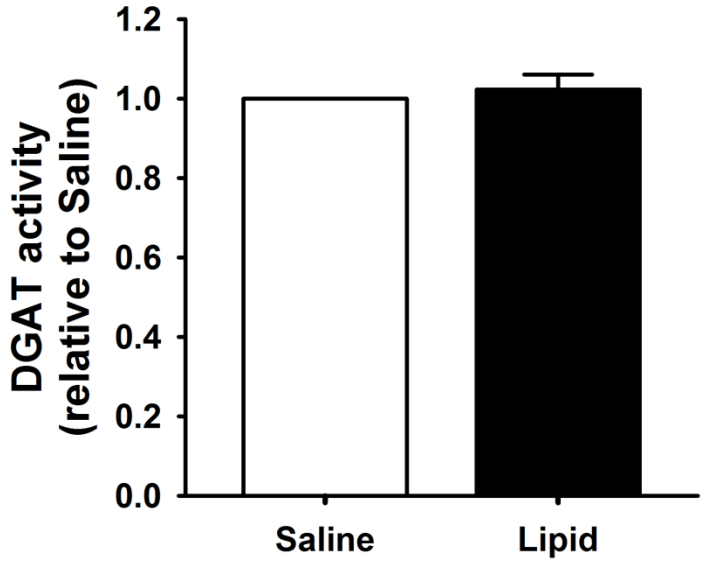


Figure 3-1. (A) Total muscle GPAT activity and (B) muscle GPAT-1 protein abundance the morning after the overnight infusion. The inset figure is a representative western blot for two subjects. *$P \leq 0.01$ for LIPID compared with SALINE. GPAT, glycerol-3-phosphate acyltransferase; SAL, SALINE; LIP, LIPID; AU, arbitrary units.

A.



B.

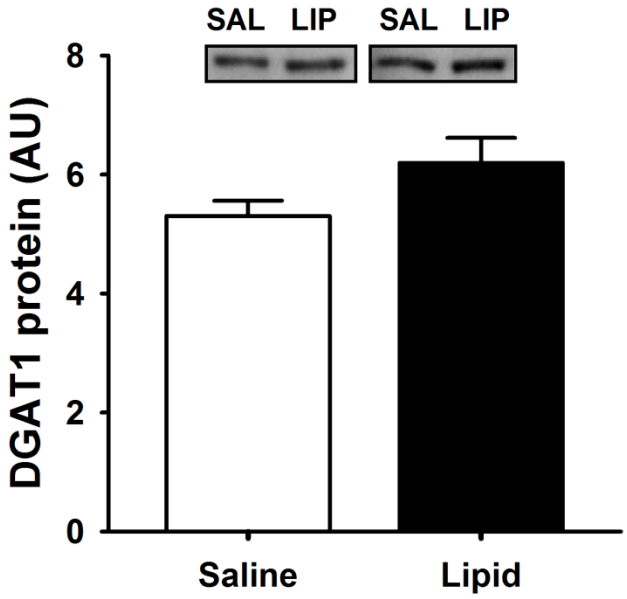


Figure 3-2. (A) Muscle DGAT activity and (B) muscle DGAT-1 protein abundance the morning after the overnight infusion. The inset figures are representative western blots for two subjects. DGAT, diacylglycerol acyltransferase; SAL, SALINE; LIP, LIPID; AU, arbitrary units.

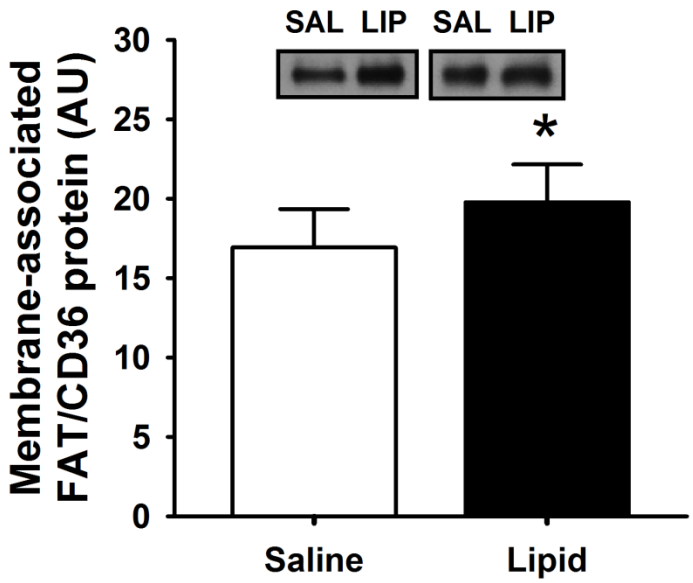


Figure 3-3. Muscle membrane-associated FAT/CD36 protein abundance the morning after the overnight infusion. Inset figures are representative western blots for two subjects. *$P \leq 0.01$ for LIPID compared with SALINE. SAL, SALINE; LIP, LIPID; AU, arbitrary units.

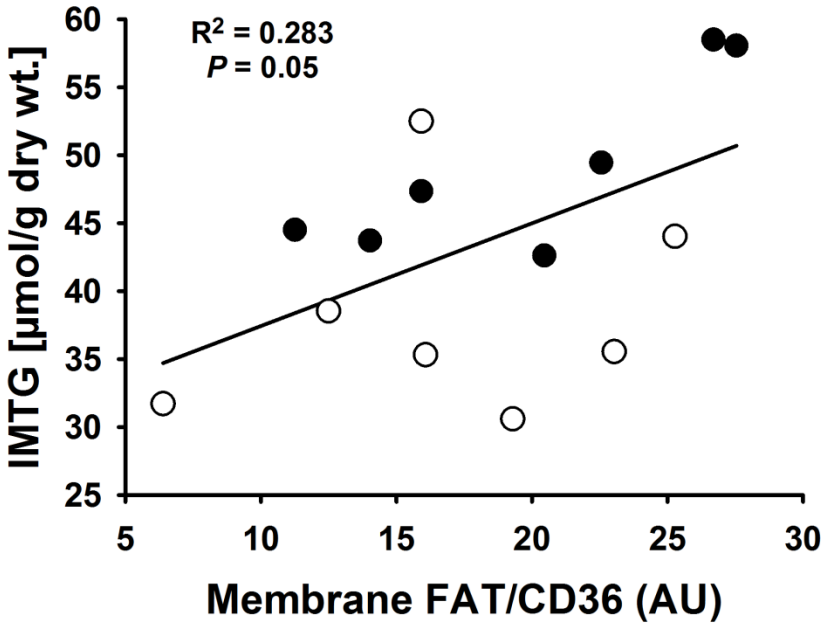


Figure 3-4. The relationship between IMTG and membrane-associated FAT/CD36 protein abundance the morning after the overnight infusion. Open circles represent SALINE, whereas closed circles represent LIPID. IMTG, intramyocellular triacylglycerol; AU, arbitrary units.

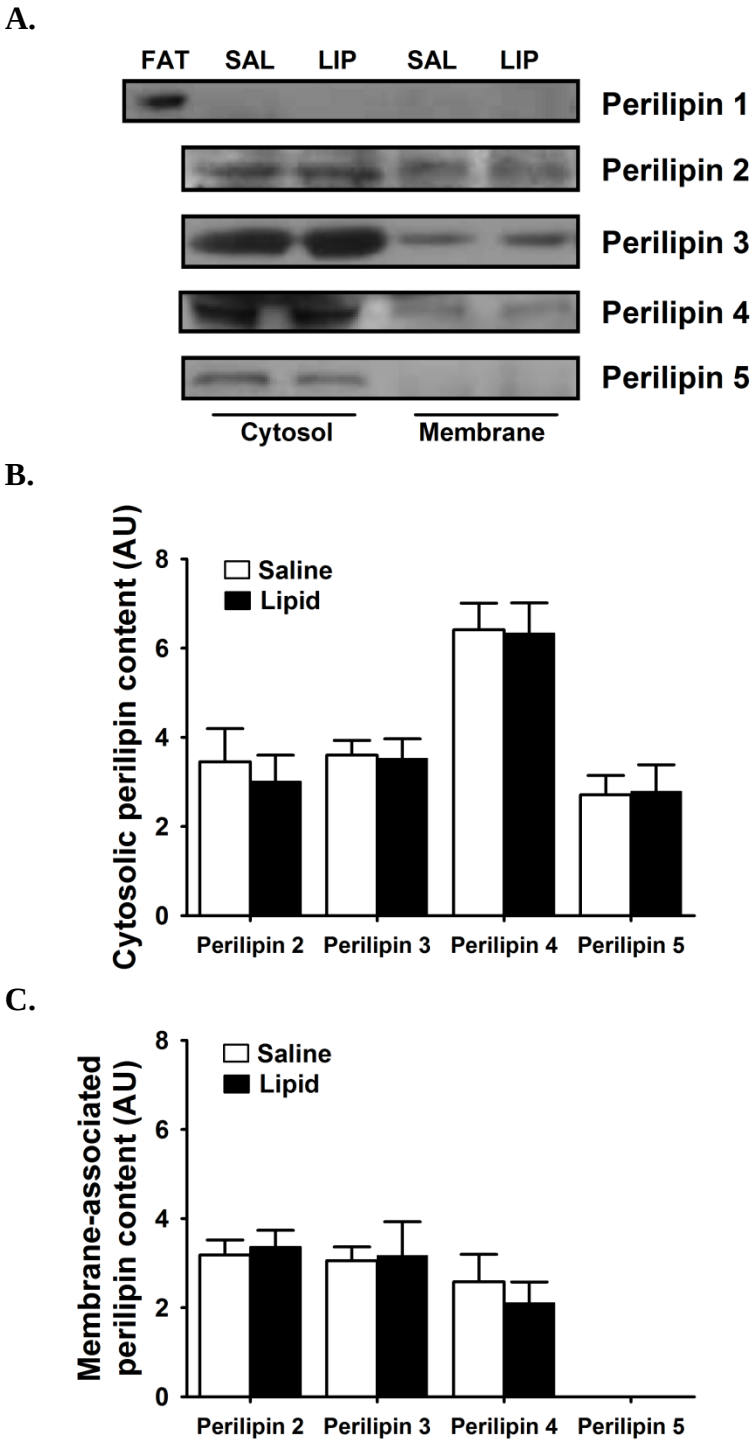


Figure 3-5. (A) Representative western blots from two subjects for perilipins 1, 2, 3, 4, and 5. (B) Muscle protein abundance of cytosolic and (C) membrane-associated perilipins 2, 3, 4 and 5. Perilipin 5 was not detected in muscle membrane preparations. FAT, a control adipose tissue homogenate; SAL, SALINE; LIP, LIPID; AU, arbitrary units.

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CHAPTER 4

Improved insulin sensitivity is accompanied by reduced fatty acid uptake the day after a modest session of exercise in obese adults

Abstract

A single session of vigorous endurance exercise improves insulin sensitivity into the next day, and alterations in lipid metabolism may be important for this response. It is not clear whether a relatively modest session of exercise has similar effects. The purpose of this study was to determine the effect of a relatively modest session of exercise on insulin sensitivity and fatty acid uptake the next day in obese adults. Eleven sedentary obese adults (M/F: 3/8; BMI: 37±1 kg/m^2; VO_2peak: 20±1 ml/kg/min) completed three experimental trials. On two occasions, subjects exercised to expend 350 kcals in the afternoon. The next morning we measured insulin sensitivity (hyperinsulinemic-euglycemic clamp) and whole-body fatty acid uptake (palmitate rate of disappearance from plasma (Rd)). These two exercise trials were identical except for the exercise intensity (50% VO_2peak [EX50] and 65% VO_2peak [EX65]) and the duration of exercise necessary to expend 350 kcals (EX50= ~70min; EX65= ~55min). Subjects also completed a control trial [CON], without exercise. A relatively modest exercise session did indeed increase insulin sensitivity the next day, but while the 35% improvement after EX50 compared with CON was statistically significant (P=0.01), the 20% improvement after EX65 was not (P=0.17). Interestingly, systemic fatty acid uptake tended to be lower (~15%, P=0.07) after EX50 compared with CON, but this effect was essentially absent after EX65. Accordingly, the change in fatty acid uptake after exercise compared with CON was negatively correlated to the change in insulin sensitivity for all trials (r=-0.60, P=0.003). In summary, a relatively modest session of exercise in obese adults improved insulin sensitivity the next day, and a reduction in systemic fatty acid uptake in the

several hours after exercise may be important for this exercise-induced improvement in insulin sensitivity in obese adults.

Introduction

Exercise is a cornerstone treatment for obesity-related metabolic complications, including insulin resistance (16), which is a primary symptom of type 2 diabetes and many other chronic diseases. Importantly, much of the insulin-sensitizing effect of exercise can be attributed to the most recent session(s) of exercise, rather than to an accumulated effect of training and/or "fitness" (22, 29). Even a single session of exercise can greatly enhance insulin sensitivity in insulin resistant, obese individuals (23), however, this beneficial effect is typically short-lived (i.e., 24-48h) (22, 29, 38). For these reasons, we contend that exercise prescriptions aimed at improving insulin sensitivity in obese populations should be tailored to maximize the beneficial effects that occur in the several hours after *each* session of exercise.

Surprisingly, the minimal "dose" of exercise required to significantly enhance insulin sensitivity is not known. Devlin and Horton (23) were the first to demonstrate that a single session of vigorous exercise (e.g., high intensity interval exercise until fatigue) could significantly improve insulin sensitivity measured the next day in insulin resistant obese adults. Clearly this level of strenuous exercise does not translate into a viable exercise prescription for most obese people, yet little is understood about the effects of a lower exercise stimulus (e.g., lower intensity, duration) on insulin sensitivity in obesity. The very few studies that have attempted to examine the metabolic benefit of less intense and/or shorter exercise sessions in obese subjects have yielded inconsistent results (6, 42, 67). The use of indirect assessments of insulin sensitivity (e.g., 24h glycemia, Homeostatic Model Assessment of Insulin Resistance [HOMA-IR]), and variations in the control of the energy expended during the exercise sessions likely contributed to these equivocal findings. The primary aim of our study was to examine the insulin sensitizing effects of an exercise session performed at either a rather mild intensity (50% peak oxygen uptake [VO_2peak]) or a slightly more intense exercise session (65% VO_2peak) in obese adults who are at risk for developing type 2 diabetes. Importantly, the energy expended during exercise was identical between our two exercise treatments (350kcal), and these exercise sessions were far less rigorous than those previously used to demonstrate improved insulin sensitivity in obesity (13, 23, 36, 53).

An additional objective of this study was to examine factors that may underlie the improvement in insulin sensitivity in the hours after a modest session of exercise. It has been clearly demonstrated that the improvement in insulin sensitivity after exercise is related to the exercise-induced reduction in skeletal muscle glycogen content, especially in lean humans (46), and animals (14). Our laboratory has recently demonstrated that altered skeletal muscle lipid metabolism may also be important for the improvement in insulin sensitivity after exercise, particularly when systemic fatty acid availability is elevated (56, 58), as is commonly found in obesity (31, 34). Specifically, our previous findings suggested that a single session of exercise increased fatty acid incorporation into triacylglycerol storage (in lean individuals exposed to an overnight lipid infusion), thereby limiting accumulation of fatty acid intermediates (e.g., ceramide and diacylglycerol [DAG]) that are known to negatively regulate insulin signaling proteins (15, 30, 41, 64-65). Because obese adults are often found to have high fatty acid availability and uptake (31, 34), increased skeletal muscle ceramide and DAG content (3, 33, 45, 62), and impaired skeletal muscle insulin signaling (7, 28, 51, 63), these effects of exercise on "fatty acid partitioning" within muscle may be particularly relevant in obesity. However, the role of altered skeletal muscle lipid metabolism in the insulin sensitizing effect of exercise in obesity is not well understood. Therefore, the secondary aim of this investigation was to evaluate the relationship between exercise-mediated changes in skeletal muscle lipid metabolism and alterations in insulin sensitivity after exercise in obese adults.

Methods

Subjects

A total of 11 obese women and men (female/male: 8/3; BMI: 30-45 kg/m^2; age: 18-45 years; fasting blood glucose concentration: <125 mg/dl) were recruited to participate in study (Table 4-1). Subjects were not taking any medications (consistent use of oral contraceptives was permitted), and all subjects underwent a comprehensive medical examination, including a history and physical examination, a 12-lead electrocardiogram, and standard blood and urine tests. All subjects were non-smokers, weight stable (i.e., ±

2 kg for ≥ 6 months), and sedentary (exercise < 2 h/wk for ≥ 6 months). Any history of metabolic or cardiovascular disease resulted in exclusion from participation. Written, informed consent was obtained from all subjects before initiating participation. All procedures of this study were approved by the University of Michigan Institutional Review Board.

Preliminary testing

At least one week before the experimental protocol, subjects performed an incremental peak oxygen uptake test (VO_2peak) on a stationary bicycle ergometer (Examiner; Lode B.V., Groningen, Netherlands) to assess aerobic fitness using a metabolic cart (MaxII; Physio-Dyne Instrument Corp., Quogue, NY). In addition, dual energy X-ray assessment (DEXA; Lunar Prodigy Advance; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was used to assess body composition.

Experimental protocol

All subjects participated in three separate experimental trials (i.e., two exercise trials and one no-exercise "control" trial; Figure 4-1). The evening before each trial, subjects ingested a standardized meal at 1900h (55% carbohydrate, 30% fat, and 15% protein; one-third of total daily caloric requirements estimated from fat-free mass as previously described (18)). The next morning (Day 1), after an overnight fast, subjects were admitted to the Michigan Clinical Research Unit at 0830h. Subjects were provided a standardized breakfast at 0930h and lunch at 1230h (see "Study Diets" section, below). Because the duration of exercise varied between the two exercise trials, subjects began exercise at different times so that the exercise session in both trials was completed at 1600h. Subjects exercised at either 50% or 65% of their pre-determined VO_2peak for the duration calculated to be required to expend 350kcal. Energy expended during exercise was divided equally between treadmill and cycle ergometry exercise, with no rest provided between these modes of exercise. To ensure subjects were exercising at the appropriate intensity and to quantify energy expenditure during exercise, we measured VO_2 and VCO_2 using a metabolic cart (Physio-Dyne Instrument Corp.) at the beginning of exercise and approximately every 20 min thereafter. During the "no-exercise" trials,

subjects remained seated quietly. After the exercise period (1600h), a retrograde intravenous catheter was placed in a hand vein for blood sampling. Exactly 1h after exercise (1700h) a "meal tolerance test" was conducted by providing a standardized meal (20% of total daily caloric requirements; 55% carbohydrate, 30% fat, and 15% protein), and collecting blood samples every 15 min for 2h to measure plasma glucose and insulin concentrations. Another meal was provided at 2000h and an evening snack was eaten at 2200h (see "Study Diets" section, below). After consumption of the evening snack a second intravenous catheter was placed in a forearm vein for infusions that began the next morning. Subjects remained sedentary in the hospital until completion of the trial the next day.

Beginning at 0450h the next morning, 3 blood samples were taken in 5 min intervals (i.e., 0450h, 0455h, and 0500h) from the heated hand vein to obtain "arterialized" blood samples (35), for determination of background enrichment of [6,6 d_2]glucose (Sigma Aldrich Inc., St. Louis, MO) and [1-^{13}C]-palmitate (Cambridge Isotopes, Andover, MA). At 0500h we began a primed, constant rate infusion of [6,6 d_2]glucose (35 µmol/kg priming dose; 0.41 µmol/kg/min continuous infusion). We next measured resting energy expenditure (and fat oxidation) using indirect calorimetry for 30 min starting at 0700h (indirect calorimetry, ventilated hood; Vmax Encore; CareFusion, San Diego, CA). At 0730h we obtained a skeletal muscle sample (~100 mg) from the vastus lateralis. Muscle biopsy samples were dissected free of adipose and connective tissue, rinsed in saline, blotted dry and then frozen in liquid nitrogen. Muscle samples were stored at -80ºC until biochemical analysis. At 0800h we began a constant-rate infusion of [1-^{13}C]-palmitate (0.04 µmol/kg/min continuous infusion). After 45 min of the [1-^{13}C]-palmitate isotope infusion, three arterialized blood samples were obtained from a heated hand vein in 5 min intervals for determination of fatty acid rate of appearance (R_a) and disappearance (R_d) to/from the circulation [fatty acid mobilization and uptake, respectively], as well as determination of basal hepatic glucose production via isotope dilution of the constant rate infusion of [6,6 d_2]glucose. These blood samples were also analyzed for plasma concentrations of triacylglycerol, fatty acids, glucose, and insulin. At 0900h we began a hyperinsulinemic-euglycemic clamp to assess insulin sensitivity, as described previously

(44). Briefly, the clamp was performed using a constant 2h insulin infusion at a rate of 100 mU/m^2/min (21). Plasma glucose concentration was monitored every 5 minutes during the clamp using a glucose autoanalyzer (Yellow Springs Instruments; Yellow Springs, OH), while glucose (D20 dextrose solution) was infused at a variable rate to maintain euglycemia. Importantly, this glucose infusion solution was enriched with [6,6 d_2]glucose (2.5% enriched) to limit changes in glucose tracer enrichment in plasma during the clamp procedure (32). In addition to the small blood samples collected every ~5 minutes to assess plasma glucose concentration, additional plasma samples were collected for assessment of insulin and plasma enrichment of [6,6 d_2]glucose every 20 minutes throughout the clamp, as well as in 5 min intervals during the final 20 min of the 2h clamp. Subjects also received an intravenous infusion of potassium (KCl) during the clamp to prevent hypokalemia.

Study diets

During the first day of each trial (Day 1), the total energy content of diet matched estimated daily energy expended (18). Because the duration of exercise was different between trials, daily energy expenditure for each trial was estimated as: ([((VO$_2$ during exercise in L/min x 3.941) + (VCO2 during exercise in L/min x 1.11)) x (duration of exercise in min)] + [(1.5 x RMR) x (time not exercising in min)]). 1.5 x RMR has been estimated as the daily energy expenditure for healthy sedentary adults (9, 60). Therefore, the subjects were in "energy balance" (i.e., energy intake = energy expenditure) during all trials, avoiding the confounding influence of a negative energy balance on insulin sensitivity (5). Breakfast (0930h), lunch (1300h), and the post-exercise meal (1700h) each contained ~20% of daily energy intake. ~30% of total daily energy requirement was provided at dinner (2000h), and ~10% of daily energy requirement was provided in the evening snack (2200h). Although the daily energy requirements differed between the exercise trials compared with the no-exercise control trial, subjects ingested an identical snack at 2200h in all trials (i.e., identical in both absolute energy content and macronutrient composition). This identical snack was designed to avoid the confounding influence of differences in the last meal ingested on metabolic measurements the next day (52). After the snack, subjects did not eat anything until completion of the clamp

procedure the next day. The relative macronutrient content of the diets was 55% carbohydrate, 30% fat, and 15% protein (expressed as percent of total kcals), which represents the macronutrient content of a “typical” western diet (8).

Analytical procedures

Plasma substrate and hormone concentrations

Plasma concentrations of glucose, fatty acid, and triacylglycerol were assessed using commercially available colorimetric assays (Glucose Oxidase: Thermo Fisher Scientific Inc., Waltham, MA; HR Series NEFA: Wako Chemicals USA, Richmond, VA; and Triglyceride Reagent: Sigma Aldrich Inc., St. Louis, MO). Plasma insulin concentration was measured using a commercially available radioimmunoassay kit (Human Insulin-specific RIA; Millipore, Billerica, MA).

Plasma fatty acid kinetics and endogenous glucose production

The tracer-to-tracee ratio (TTR) for plasma palmitate and glucose were determined by gas chromatography-mass spectrometry (GC/MS; Agilent 5973Networks, Mass Selective Detector; Agilent Technologies, Palo Alto, CA) with capillary column (50). Proteins were precipitated from plasma samples with acetone, and hexane was used to extract plasma lipids. For palmitate analysis, fatty acids in the organic phase of the extraction sample were converted to their methyl esters with iodomethane and isolated using solid phase extraction cartridges (Suplerclean LC-Si Silica gel SPE tubes; Sigma Aldrich Inc., St. Louis, MO). Electron impact ionization was used, and the mass-to-charge ratios (m/z) 270 and 271 were selectively monitored. For glucose analysis, the aqueous phase of the deproteinized plasma samples was dried overnight under vacuum. Hydroxylamine/pyridine solution (100 µl) was added to dried samples and heated at 100°C for 30 min. Acetic anhydride (75 µl) was added and samples were incubated at 100°C for 1h, then dried under vacuum. Samples were re-suspended in ethyl acetate (100 µl) and masses of 187 and 189 of the penta-acetate derivative of glucose was assessed using selective ion monitoring.

Muscle glycogen concentration

Muscle biopsy samples were lyophilized at –60°C for 48h, and aliquots were weighed to the nearest 0.1 mg. Muscle glycogen was determined from the measurement of glucose after acid hydrolysis as previously described (49). Briefly, samples were homogenized and then hydrolyzed in 2N HCl, and heated at 100^{o}C for 2h. Samples were then neutralized using 1N NaOH to pH 6.5-7.5 and the free glucose concentration was determined fluorometrically.

Muscle triacylglycerol and diacylglycerol concentrations

Frozen muscle (30-40 mg) was rapidly homogenized in 1.0 ml ice-cold 0.9% saline, and lipids then extracted overnight at 4°C in a single-phase mixture of chloroform-methanol-aqueous homogenate (1:2:0.8, v/v/v) (10). Internal lipid markers for triacylglycerol (TAG), DAG, monoacylglycerol, non-esterified fatty acid (NEFA), phospholipid (PL), and cholesterol ester having fatty acid moieties of odd carbon number were added at the start of extraction, for subsequent purity and recovery determinations (Nu-Chek Prep Inc., Elysian, MN; Avanti Polar Lipids Inc., Alabaster, AL). TAG, DAG and PL markers were each homogenous in fatty acid content (e.g., $[C23:0]_3$-TAG). Extraction was ended by addition of sufficient chloroform and saline to form two phases (2:2:1.8). After vortexing and brief centrifugation, the lower chloroform phase containing lipids was transferred to a clean tube and dried under vacuum. The residue was reconstituted in 100 µl chloroform and applied to a hexane-equilibrated, aminopropyl solid phase extraction column. The small chloroform volume did not significantly alter the hexane equilibrium, permitting virtually complete adsorption of glycerolipid and NEFA. Individual lipid species were eluted using previously described solvent mixtures (11). Column fractions were dried, and those containing either purified TAG or DAG were reconstituted in 100 µl toluene. Fatty acid methyl esters (FAMEs) were then generated via alkaline methanolysis, a transesterification process (11), by addition of 1.0 ml 0.2N NaOH in methanol having ultra-low H_2O content, in order to exclude hydrolysis. After 1h incubation at room temperature, the reaction was neutralized using 0.9 ml 1.0 M Na-acetate (pH 4.75). Chloroform (1.0 ml) was added for two-phase formation, which was unaffected by the small volume of toluene present. The lower chloroform phase

containing FAMEs from TAG or DAG was transferred, dried and reconstituted in heptane. Individual FAMEs were purified by gas chromatography with capillary column (Agilent Technologies). FAMEs were detected by electron-impact mass spectrometry with selective ion monitoring, and quantified using FAME standards.

Muscle ceramide concentration

Analysis of skeletal muscle ceramide concentration was performed after lipid extraction via liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ; Agilent Technologies 6410 Triple Quadrupole Mass Spectrometer). In brief, samples were extracted by a mixture of methanol, chloroform, and water (10) supplemented with internal ceramide standards. The lipid extract was dried under nitrogen gas and reconstituted in a 60:40 mixture of acetonitrile and isoproponal alcohol. The re-constituted lipid extract was analyzed by electrospray ionization LC-MS/MS on a tandem quadrupole instrument operating in multiple reaction monitoring mode (37). Ceramides were identified by retention time and MS/MS fragmentation parameters, and were quantified relative to the closest-matching internal standard.

Western blotting

An aliquot of the initial muscle sample homogenate in 0.9% saline was taken immediately after homogenization (described above in *Muscle triacylglycerol and diacylglycerol concentrations*), and supplemented to achieve the final buffer solution (20mM Tris-HCl pH 7.5, 150mM NaCl, 2mM Na_2EDTA pH 8.0, 20mM NaF, 10% (v/v) glycerol, 1% (v/v) NP-40, 2.5mM NaPP, 20mM β-glycerophosphate, and a mixture of protease inhibitors). Samples were vortexed vigorously, then centrifuged at 20,000 g for 10 min at 4^oC. Supernatants were collected and tested for protein concentration. 30µg protein was separated by SDS-PAGE (8% gels) and transferred to nitrocellulose membranes. Membranes were exposed to primary antibodies against glycerol-3-phosphate acyltransferase (GPAT1; 4613; ProSci Incorporated, Poway, CA), diacylglycerol acyltransferase (DGAT1; NB110-41487; Novus Biologicals, Littleton, CO), and DGAT2 (sc-66859; Santa Cruz Biotechnology Inc., Santa Cruz, CA) to assess the abundance of TAG synthesis enzymes, c-jun N-terminal kinase (JNK; 9251; Cell

Signaling Technology, Danvers, MA), pJNK^{Thr183Tyr185} (4671; Cell Signaling Technology), phosphorylated protein kinase C (pPKCβ^{Thr641}; 07-873; EMD Millipore, Billerica, MA), and IκBβ (9248; Cell Signaling Technology) to assess proinflammatory stress activation, phosphorylated insulin receptor substrate (pIRS1^{Ser312}; 2381; Cell Signaling Technology) to assess inhibition of insulin signaling, and nicotinamide phosphoribosyltransferase (NAMPT; A300-372A; Bethyl Laboratories, Inc., Montgomery, TX) to assess regulation of NAD-synthesis. Membranes were then incubated with appropriate secondary antibodies and developed using enhanced chemiluminescence (GE Healthcare). Bands of interest were imaged and then quantified via densitometry (AlphaEaseFC; Protein Simple, Santa Clara, CA). All within-subject comparisons were made using the same blot.

Calculations

Energy expenditure and fat oxidation

Energy expenditure during rest and exercise was calculated from VO_2 and VCO_2 measurements using the Weir equation (43). Whole body fat/triacylglycerol oxidation (g/min) was calculated from VO_2 and VCO_2 measurements using the equations of Frayn (25). Whole body fatty acid oxidation was calculated by dividing triacylglycerol oxidation by an estimated molecular weight of triacylglycerol (860 g/mol) and multiplying by 3.

Plasma glucose and insulin area under the curve

Area under the curve (AUC) for plasma glucose and insulin concentration curves during time 0-120min of the meal tolerance test was calculated using the trapezoidal rule.

Hepatic glucose production

Steady-state glucose concentration and TTR were achieved during isotope infusion; therefore plasma glucose $R_a = R_d$ and could be calculated using Steele's equation for steady-state conditions (61). Exogenous glucose infusion rates were subtracted from glucose R_a calculated during steady-state of the hyperinsulinemic-euglycemic clamp.

Insulin sensitivity

Insulin sensitivity was calculated as steady-state whole-body glucose R_d (μmol/min) over steady-state plasma insulin concentration (μU/mL) during the final 20 min of the clamp procedure. Whole-body glucose R_d was calculated as the sum of the total glucose infusion rate (both labeled and unlabeled glucose) and hepatic glucose production during the final 20min of the clamp. Hepatic insulin sensitivity was calculated from the insulin-induced suppression of endogenous glucose production as:

$$Hepatic\ insulin\ sensitivity = \left(1 - \frac{HGP_{clamp}}{HGP_{basal}}\right) \times 100$$

Where HGP_{basal} and HGP_{clamp} (μmol/min) refer to hepatic glucose production (HGP) measured immediately before the clamp procedure and during the final 20 min of the clamp, respectively.

Fatty acid mobilization and uptake

Steady-state fatty acid concentration and TTR was achieved during isotope infusion; therefore plasma palmitate $R_a = R_d$ and could be calculated using Steele's equation for steady-state conditions (61). Because palmitate is a reasonable marker for systemic fatty acid kinetics (44), total plasma fatty acid R_a/R_d were calculated by dividing plasma palmitate R_a/R_d by the percent contribution of palmitate to the total plasma fatty acid pool.

Statistical analysis

A repeated measures two-way (*treatment x time*) analysis of variance (ANOVA) was used to test for significant differences in plasma glucose and insulin concentrations during the meal tolerance test. A repeated measures one-way ANOVA was used to test for significant differences in all other outcome variables between trials. Tukey's post hoc pair-wise comparisons were used to examine differences in factor means when significant *F* values were detected during ANOVA analyses. Pearson Product Moment Correlation analysis was used to examine the relationship between outcome variables selected *a priori*. Statistical significance was defined as $P \leq 0.05$.

Results

Exercise intensity and energy expenditure

Exercise intensity and the energy expended during exercise were successfully controlled in all exercise sessions as planned (Table 4-2), and all participants were able to complete the exercise sessions. Because exercise intensity was greater during EX65 than EX50 ($P<0.001$), subjects exercised for about 15 min longer during EX50 than EX65 ($P<0.001$) in order to successfully match exercise energy expenditure between these trials (Table 4-2). Compared with remaining sedentary (CON), neither resting energy expenditure (main effect $P=0.28$) nor resting fatty acid oxidation (main effect $P=0.51$) was different the morning after exercise (data not shown).

Insulin sensitivity

During the meal tolerance test performed 1h after exercise we found strong trends for blunted glucose and insulin responses to the meals after exercise compared with CON (Figure 4-2), but these attenuated responses were not statistically significant (main effects $P=0.09$ and $P=0.07$ for glucose AUC and insulin AUC, respectively). Insulin sensitivity measured the morning after exercise using the clamp procedure was significantly elevated (~35%) above control levels when measured the morning after EX50 ($P=0.01$; Figure 4-3), however, the 20% increase above CON the morning after EX65 did not reach statistical significance ($P=0.17$). There was no statistical difference in insulin sensitivity between EX50 and EX65 ($P=0.39$). Importantly, the ability of insulin to suppress hepatic glucose output during the hyperinsulinemic-euglycemic clamp was nearly identical among trials (83±5, 88±5, and 84±7% for CON, EX50, EX65, respectively, main effect $P=0.48$), indicating that enhanced peripheral glucose metabolism was responsible for the exercise-induced improvement in insulin sensitivity.

Plasma substrates and insulin concentration

Exercise did not alter fasting plasma glucose, insulin, or triacylglycerol concentration the next morning compared with CON (Table 4-3). However, fasting glucose R_a was slightly, but significantly elevated the morning after exercise during EX50 compared with CON ($P=0.04$; Figure 4-4a). In contrast, plasma fatty acid concentration tended to be

lower the morning after exercise during EX50 compared with CON (main effect *P*=0.09; Table 4-3). Accompanying this lower plasma concentration of fatty acid, fatty acid R_d was also ~15% lower the morning after EX50 compared with CON (Figure 4-4b), but this suppression in fatty acid R_a did not quite reach statistical significance (*P*=0.07). Interestingly, these effects of exercise on plasma glucose and plasma fatty acid kinetics were not found the morning after exercise during EX65 (Figure 4-4).

Skeletal muscle glycogen and lipid content

As expected, skeletal muscle glycogen content was lower the morning after exercise compared with remaining sedentary. Glycogen concentration was significantly lower the morning after EX65 compared with CON (343±33 vs. 440±39 mmol/kg dry muscle; *P*=0.007). Although muscle glycogen concentration was also relatively low the morning after EX50 (377±33 *vs.* mmol/kg dry muscle), this reduction below CON was not quite statistically significant (*P*=0.09). There was no difference in muscle glycogen concentration between EX50 and EX65 (*P*=0.46). In contrast to the effect of exercise on muscle glycogen storage, neither EX50 nor EX65 altered TAG, DAG, or ceramide concentrations in muscle the next morning (Figure 4-5). Skeletal muscle protein expression of key TAG-synthesis enzymes GPAT1, DGAT1, and DGAT2 were also not different among trials (Table 4-4).

Insulin signaling, pro-inflammatory markers, and NAMPT in skeletal muscle

Although prior exercise enhanced peripheral insulin sensitivity the morning after exercise, phosphorylation of $IRS1^{Ser312}$ in skeletal muscle, which is an indicator of impaired insulin signaling, was not affected by the exercise stimulus (Figure 4-6a). In addition, skeletal muscle markers of proinflammatory stress including phosphorylation of $PKC\beta^{Thr641}$, and abundance of the NF-κB inhibitor protein IκBβ were not different among trials (Figure 4-6b and 4-6c). Surprisingly, there was a strong trend for exercise to *increase* phosphorylation/activation of JNK (main effect *P*=0.08; Figure 4-6d), which is a known insulin signaling antagonist. Finally, skeletal muscle abundance of NAMPT tended to increase the morning after exercise (main effect *P*=0.09; Figure 4-6e).

Correlation with insulin sensitivity

We found the exercise-induced improvement in insulin sensitivity the morning after exercise compared with control, to be correlated with the reduction in fatty acid uptake (r=-0.60, *P*=0.003). In addition, using data from all trials, insulin sensitivity was negatively correlated with fasting plasma insulin concentration (r=-0.79, *P*<0.001) and fasting plasma triacylglycerol concentration (r=-0.69, *P*<0.001).

Discussion

The main finding of this study was that expending only a modest amount of energy (350kcals) during a single session of exercise at a rather mild intensity (50% VO_2peak) was sufficient to significantly improve insulin sensitivity at least into the next day (~19h after exercise) in obese adults who are at risk for developing type 2 diabetes. Importantly, this improvement in whole-body insulin sensitivity was due to enhanced peripheral glucose uptake, rather than altered hepatic glucose metabolism. Although insulin sensitivity was similar the morning after exercise performed at 50%VO_2peak and 65% VO_2peak, the insulin sensitizing effects of exercise of the higher intensity exercise session did not achieve statistical significance. We also found that the exercise-induced improvement in insulin sensitivity correlated with the change in plasma fatty acid disappearance from plasma, suggesting that exercise-mediated alterations in fatty acid delivery and uptake may contribute to the improvement in insulin sensitivity after exercise. A single session of exercise also tended to improve meal tolerance measured 1h after exercise; however, the trends for lowered plasma glucose and insulin concentrations after the meal did not achieve statistical significance.

One of the overarching goals of this study was to determine whether a relatively modest exercise stimulus could have a persistent effect on insulin sensitivity into the next day in adults at risk for developing type 2 diabetes. Several previous studies (23, 67) including some from our group (46, 58) have reported improvement in insulin sensitivity after a single session of vigorous and/or prolonged exercise. However, the exercise protocols in all of these previous studies were very rigorous (e.g., >65% VO_2peak, >1.5 h duration) and do not reflect realistic expectations for an exercise prescription for most sedentary

obese adults. Furthermore, many of these previous studies examining the insulin sensitizing effect of a single session of exercise have been limited to indirect measures of insulin sensitivity (42, 67). Using the "gold-standard" for assessing insulin sensitivity in our study (i.e., the hyperinsulinemic-euglycemic clamp), our findings indicate that even fairly modest exercise can significantly enhance insulin sensitivity in obese adults. The exercise stimuli used in our study are generally within the current recommendations for physical activity provided by the American College of Sports Medicine (ACSM) (26), the American Heart Association (AHA) (1), and the Centers for Disease Control and Prevention (CDC) (2). It is important to note that the physical activity/exercise recommendations from the aforementioned societies/agencies were derived with the primary objective of enhancing cardiovascular "fitness" accrued after weeks and months of regular activity. Our findings establish that obese adults can incur metabolic benefits after ***each*** session of exercise, even when the exercise is relatively modest (like that recommended by the ACSM, AHA, and CDC), and importantly, these beneficial metabolic effects can clearly be attained prior to any improvement in "fitness." Still, it remains possible that even less of an exercise stimulus than that used in our study (e.g., lower energy expenditure/duration, etc.) may be sufficient to significantly improve insulin sensitivity after only a single session of exercise in sedentary obese individuals, and may thus present an even more attractive exercise prescription for the prevention and/or treatment of insulin resistance. We are currently pursuing this exciting possibility.

Moderate-to-high intensity exercise training (i.e., 65-85% VO_2peak) is classically associated with enhanced beneficial cardiovascular and metabolic adaptations compared with lower intensity exercise (i.e., 40-50% VO_2peak) (24). Based on findings from exercise training studies, it may be logical to presume that a higher intensity of a single session of exercise may also evoke more potent and persistent metabolic effects in the hours after exercise. Along these lines, it has been reported that 1h of exercise at 60% and 70% of VO_2peak significantly reduced insulin resistance the next day (as measured by the Homeostatic Model Assessment of Insulin Resistance [HOMA-IR]), while exercise at 40% VO_2peak did not (67). However, these findings must be interpreted with caution, in part because the researchers (67) did not control for energy balance in their

study (i.e., the higher the exercise intensity, the greater the exercise energy expenditure and subsequent negative energy balance). Without controlling for energy balance, it is impossible to distinguish the effects of exercise intensity on insulin sensitivity, from the potent insulin sensitizing effects of a negative energy balance (5). Interestingly, there have been some recent reports suggesting that lower exercise intensity may actually be superior to higher intensity exercise in terms of glucose regulation/control (6, 42). For example, compared with remaining sedentary, 24h glycemia in type 2 diabetics was found to be lower (i.e., improved) during the day after 1h of low intensity exercise (35% of cycle ergometry W_{max}), but when these same participants exercised for 30 min at 70% W_{max}, no significant improvement in 24h glycemia was found. It is important to note that 24h glycemia is not an assessment of insulin sensitivity, and may be determined by a number of different factors (e.g., plasma glucagon, insulin, and catecholamine concentrations, dietary intake, etc.). Nonetheless, this finding lends support to the argument that lower intensity exercise may induce metabolic benefit in the context of glucose control that is at least equal to, if not possibly greater than higher intensity exercise designed to elicit the same energy expenditure. In a separate study, an eight month exercise training program requiring relatively low intensity exercise (40-50% VO_2peak) enhanced insulin sensitivity in overweight and obese adults to a greater degree than participants who trained at higher exercise intensities (65-80% VO_2peak) (6). However, in their study (6), it was impossible to distinguish possible effects stemming from adaptations accrued during eight months of training from the effects of the most recent session(s) of exercise. Our findings also provide support for the notion that the insulin sensitizing effect of a lower intensity exercise stimulus (EX50) is at least equal to an isoenergetic session of exercise performed at a higher intensity (EX65). Mechanisms underlying the possibility of a *more robust* beneficial metabolic response after mild compared with higher exercise intensity have not been well studied. Because our study was designed to match the energy expended during exercise, this required the participants to exercise ~15 minutes longer during EX50 compared with EX65. Therefore, we cannot rule out the possibility that something associated with a longer duration of exercise may have influenced the metabolic responses (even though energy expenditure was identical), but what may cause a possible beneficial effect of exercise duration is not clear. We

must also reemphasize that despite the fact that the improvement in insulin sensitivity after EX65 did not achieve statistical significance compared with remaining sedentary, we are confident that the insulin sensitizing effect of this exercise stimulus is fairly similar to that of EX50. As shown in Figure 4-7, insulin sensitivity values measured in response to both exercise stimuli were highly correlated and yield a linear regression equation with a slope near 1 and a y-intercept near 0, indicating that insulin sensitivity values measured the morning after EX50 and EX65 were indeed similar. Furthermore, it is noteworthy that insulin sensitivity values measured in response to exercise were similar between women and men.

Because hepatic glucose production during steady-state of the hyperinsulinemic-euglycemic clamp was nearly identical among all of our experimental trials, the improvement in whole-body insulin sensitivity after exercise was not due to changes in the liver, but instead due to enhanced peripheral glucose metabolism. It is likely that much if not all of this effect was driven by improved glucose metabolism within skeletal muscle (20). Muscle glycogen is known to be a key mediator of the improvement in insulin sensitivity after exercise (14, 46). The magnitude of the improvement in insulin sensitivity in the hours after exercise has generally been found to be inversely-related to the exercise-induced reduction in muscle glycogen content (12). As anticipated, muscle glycogen concentration was lowest the day after exercise performed at our higher exercise intensity (EX65). However, it was unexpected that the exercise-induced improvement in insulin sensitivity did not more closely parallel changes in skeletal muscle glycogen content (i.e., the lowest muscle glycogen after EX65 was not accompanied by the greatest insulin sensitivity). The reason for this is not known, but one possibility may be that higher intensity exercise may have acutely activated a greater pro-inflammatory/stress response in skeletal muscle (4), which may counteract the insulin sensitizing effects of a lower muscle glycogen concentration in our participants (39, 47, 51). Indeed, we found a strong trend for exercise to increase the phosphorylation of the highly responsive stress-related kinase JNK in skeletal muscle, and this effect appeared to be greatest during EX65. The trend for an increase in JNK phosphorylation after exercise is somewhat in contrast to previous work from our laboratory, where we reported that a

single session of exercise attenuated JNK phosphorylation in lean subjects who were exposed to an overnight lipid infusion (58). However, in our earlier study (58) we only measured JNK phosphorylation while our subjects were being infused with an exogenous lipid emulsion for several hours, which may have been confounding. Our present observation that exercise tended to increase skeletal muscle NAMPT abundance during EX50 may have also contributed to the significant increase in insulin sensitivity we found after exercise at this rather mild intensity. NAMPT catalyzes a critical step in the biosynthesis of nicotinamide adenine dinucleotide (NAD), which is a key activator of Sirtuin1 (Sirt1); a deacetylase considered to be a putative regulator of skeletal muscle insulin sensitivity (59). It remains to be determined whether augmented skeletal muscle NAMPT abundance and/or increased Sirtuin activity are important for the insulin sensitizing effect of exercise, but the topic appears to be gaining attention (17, 48, 54).

We (57) and others (55, 66) have previously reported systemic fatty acid mobilization and uptake to be a primary determinant of whole-body insulin sensitivity, particularly in obesity-related insulin resistance. In keeping with this hypothesis, here we found the change in insulin sensitivity after exercise compared with control to be correlated with the change in plasma fatty acid rate of disappearance. Although causality cannot be inferred from this correlation, this finding supports the notion that an exercise-induced reduction in fatty acid uptake may be an important mediator of the insulin sensitizing effect of exercise in obese individuals. One seemingly important beneficial effect of a lower fatty acid uptake is that less intracellular substrate is available for ectopic lipid synthesis in skeletal muscle, thereby limiting the accumulation of lipid intermediates, like diacylglycerol and ceramide that have been linked with suppressed insulin action (15, 30, 41, 64-65). In addition to simply lowering the uptake of fatty acids, previous findings from our lab suggest that exercise-mediated changes within skeletal muscle may alter how the fatty acids are "partitioned" within the myocyte; facilitating storage as triacylglycerol, which may also reduce the available substrate for synthesis of some lipid intermediates known to impair insulin signaling (STUDY 1) and (58). However, unlike our previous work (56, 58), in the present study we were unable to detect any changes in skeletal muscle lipid content or protein abundance of key lipid synthesizing enzymes

after either of the exercise trials compared with control. These discrepancies between our current and previous findings may be partly explained by the difference in subject population used (i.e., obese *vs.* lean). For example, the high skeletal muscle lipid content commonly observed in obese individuals (27, 40), may limit the ability to detect what might be relatively subtle (but potentially important) changes in muscle lipid content after exercise. Quantitative measurement of what is really still a relatively small amount of lipid in skeletal muscle tissue is known to be difficult, with sensitivity being dependent upon the method of measurement used (19). However, mass spectrometry technology (employed in this study) appears to provide a sensitive method for assessing skeletal muscle lipids. It is also noteworthy that the vastus lateralis muscle is not highly recruited during low-speed level treadmill walking activity (representing half of the exercise stimulus in both exercise trials), and thus the relatively low activation requirement of this muscle during the exercise bouts may have also limited our ability to detect changes in the lipid pools of our muscle biopsy samples. As such, given that we did not find significant changes in muscle triacylglycerol, diacylglycerol, or ceramide concentration after exercise, we must acknowledge the possibility that changes in fatty acid partitioning among the main lipid compartments in skeletal muscle may not be critical for the insulin sensitizing effect of exercise in obesity.

In summary, although exercise is a key component in the treatment of obesity-related metabolic complications, including insulin resistance (16), it is astonishing that the "dose" of exercise required to improve insulin sensitivity in obese individuals at risk for the development of type 2 diabetes is not more clearly defined. Due to the transient nature of the exercise-induced improvement in insulin sensitivity, we believe it is very important to develop exercise prescriptions aimed at maximizing the beneficial effects of *each* session of exercise. Here we have demonstrated that expending only a modest amount of energy (350kcals) during a single session of exercise at a rather mild intensity (50% VO_2peak) was sufficient to significantly improve insulin sensitivity at least into the next day in obese adults. In addition to the effects of lowered muscle glycogen content on insulin sensitivity, evidence from this study also indicates that the insulin sensitizing effect of exercise in obesity may be mediated in part by attenuated systemic fatty acid

mobilization and uptake, though the underlying mechanism(s) for these effects are not known. Finally, the findings from this study carry encouraging clinical implications given that the exercise performed in this investigation represents a substantial reduction in both the energy expenditure and intensity of exercise previously reported to significantly enhance insulin sensitivity in obese adults.

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Table 4-1. Participant characteristics

Age (y)	28 ± 2
Body mass index (kg/m^2)	37 ± 1
Body mass (kg)	102 ± 3
Body fat (%)	48 ± 2
Fat mass (kg)	50 ± 3
Fat free mass (kg)	53 ± 2
VO_2peak (ml/kg/min)	20 ± 1

Values are mean ± SEM. VO_2peak, peak oxygen consumption.

Table 4-2. Intensity, duration, and energy expended during the exercise sessions

	EX50	**EX65**	***P*-value**
Intensity (% VO_2peak)	51 ± 0	66 ± 0	< 0.001
Duration (min)	70 ± 3	54 ± 2	< 0.001
Energy Expended (kcal)	356 ± 3	355 ± 2	0.511

Values are mean ± SEM. *P*-values are from paired Student's *t*-tests.

Table 4-3. Fasting plasma insulin and substrate concentrations the day after exercise

	CON	EX50	EX65	*P*-value
Insulin (μU/ml)	27.5 ± 4.4	28.0 ± 4.1	26.7 ± 3.7	0.79
Glucose (mmol/L)	5.0 ± 0.1	5.2 ± 0.2	5.0 ± 0.1	0.26
Fatty acid (mmol/L)	0.48 ± 0.05	0.40 ± 0.04	0.45 ± 0.02	0.09
Triacylglycerol (mmol/L)	1.29 ± 0.18	1.40 ± 0.24	1.25 ± 0.19	0.24

Values are mean ± SEM. *P*-values are main effects from one-way, repeated measures ANOVA tests.

Table 4-4. Skeletal muscle immunoblot analysis in skeletal muscle samples collected the day after exercise

	CON	EX50	EX65	*P*-value
GPAT1	4.4 ± 0.8	4.2 ± 0.8	4.0 ± 0.7	0.32
DGAT1	2.1 ± 0.4	1.9 ± 0.3	2.0 ± 0.3	0.86
DGAT2	3.5 ± 0.9	3.7 ± 0.9	4.4 ± 1.4	0.22

Values are mean ± SEM, expressed in arbitrary units. *P*-values are main effects from one-way, repeated measures ANOVA tests.

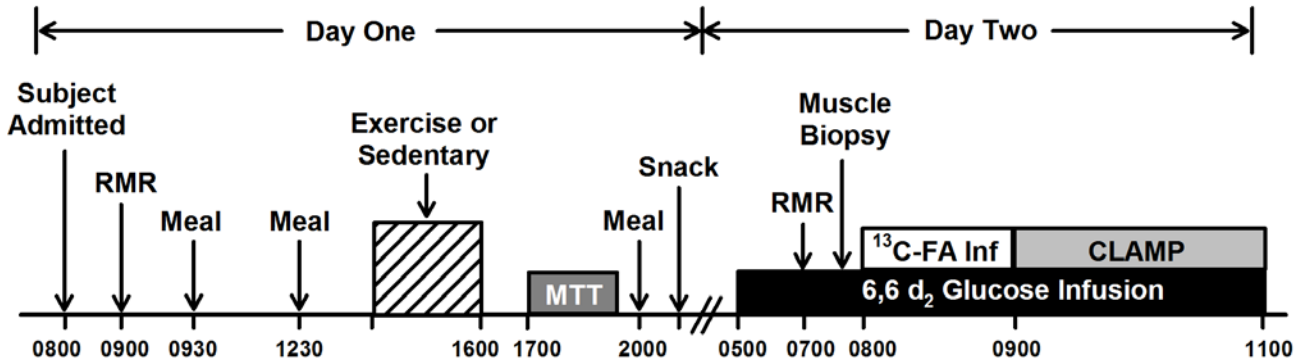
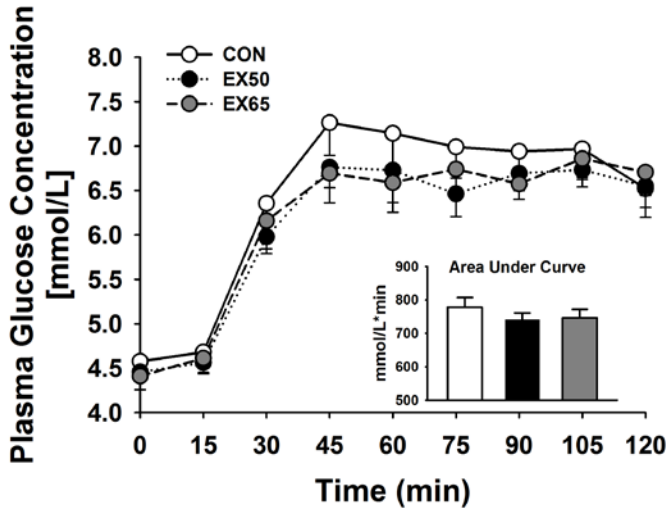


Figure 4-1. Timeline of experimental events. Subjects participated in 3 separate two-day trials. On two occasions subjects expended 350kcals during an exercise session in the afternoon of the first day. These two exercise trials were identical except for the intensity of exercise performed (50% VO_2peak [EX50] and 65% VO_2peak [EX65]). Subjects also completed a control trial [CON] in which they remained sedentary. RMR, resting metabolic rate; MTT, meal tolerance test; ^{13}C-FA Inf, [1-^{13}C]-palmitate isotope infusion; CLAMP, hyperinsulinemic-euglycemic clamp.

A.



B.

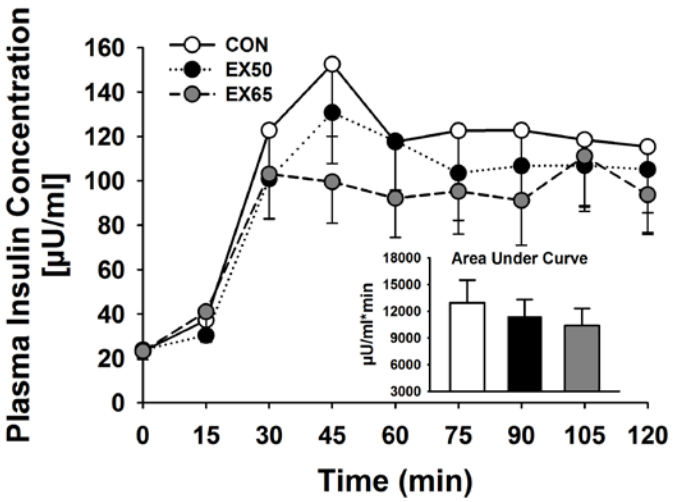


Figure 4-2. Plasma concentrations of: **A**. glucose and **B**. insulin measured during the meal tolerance test conducted 1h after exercise, or remaining sedentary. Inset figures are calculated mean area under the plasma concentration curve for each trial. For plasma glucose concentration (**A**), the two-way (*treatment x time*), repeated measures ANOVA P=0.17, and the main effect area under the curve one-way, repeated measures ANOVA P=0.09. For plasma insulin concentration (**B**), the two-way (*treatment x time*), repeated measures ANOVA P=0.15, and the main effect area under the curve one-way, repeated measures ANOVA P=0.07.

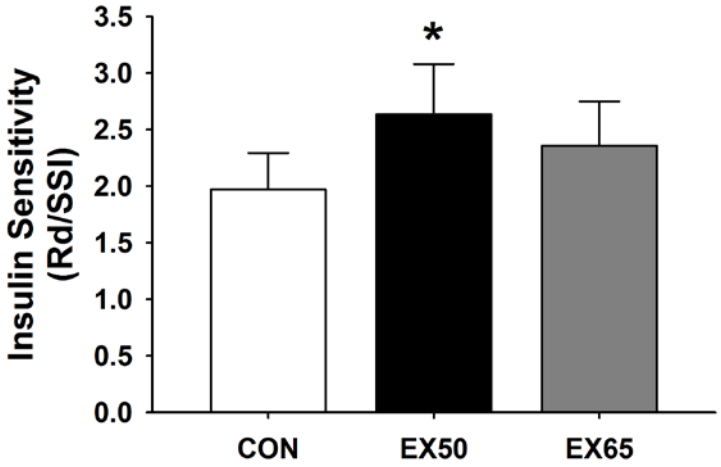
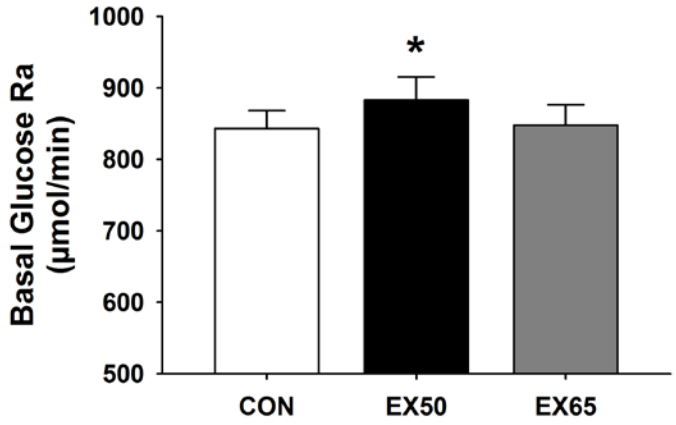


Figure 4-3. Insulin sensitivity measured via hyperinsulinemic-euglycemic clamp the day after exercise. Data are expressed as clamped whole-body glucose disposal (R_d, (µmol/min)) per steady-state plasma insulin concentration (SSI, [µU/ml]). **P*<0.05 EX50 *vs.* CON. (*P*=0.17 for EX65 *vs.* CON)

A.



B.

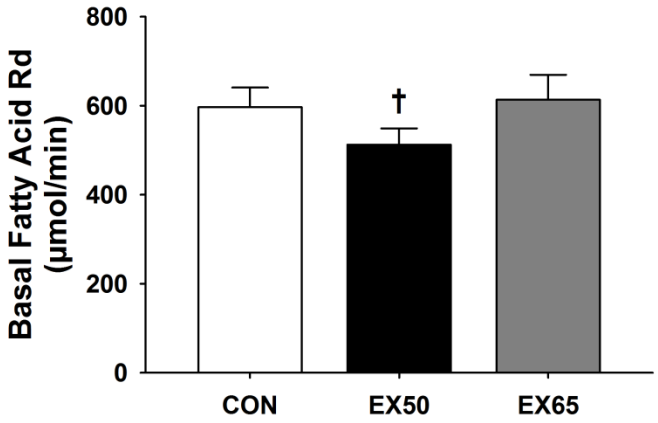


Figure 4-4. A. Basal glucose rate of appearance in plasma (R_a) measured the day after exercise, **P*<0.05 *vs.* CON. **B.** Basal fatty acid rate of disappearance from plasma (R_d) measured the day after exercise, †*P*<0.05 EX50 *vs.* EX65. (*P*=0.07 for EX50 *vs.* CON)

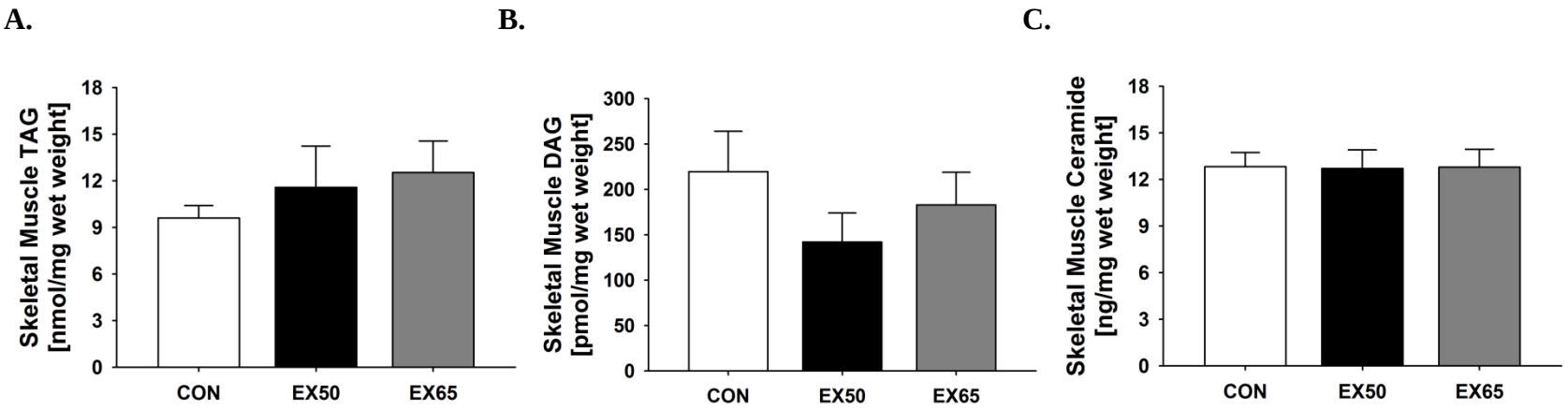


Figure 4-5. Lipid content measured in skeletal muscle collected the day after exercise. **A**. Skeletal muscle triacylglycerol (TAG) concentration. **B**. Skeletal muscle diacylglycerol (DAG) concentration. **C**. Skeletal muscle ceramide concentration.

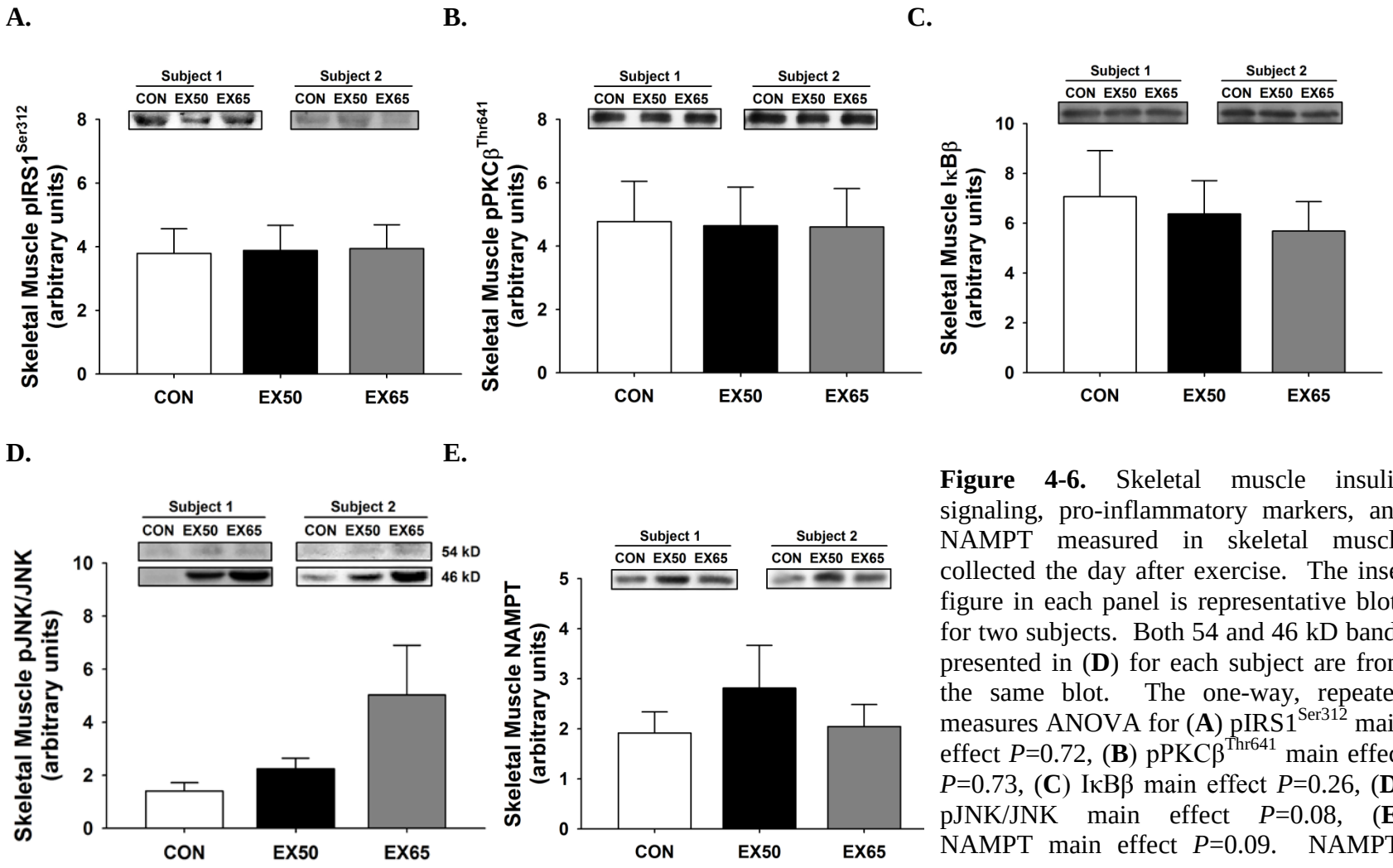


Figure 4-6. Skeletal muscle insulin signaling, pro-inflammatory markers, and NAMPT measured in skeletal muscle collected the day after exercise. The inset figure in each panel is representative blots for two subjects. Both 54 and 46 kD bands presented in (**D**) for each subject are from the same blot. The one-way, repeated measures ANOVA for (**A**) pIRS1^{Ser312} main effect P=0.72, (**B**) pPKCβ^{Thr641} main effect P=0.73, (**C**) IκBβ main effect P=0.26, (**D**) pJNK/JNK main effect P=0.08, (**E**) NAMPT main effect P=0.09. NAMPT,

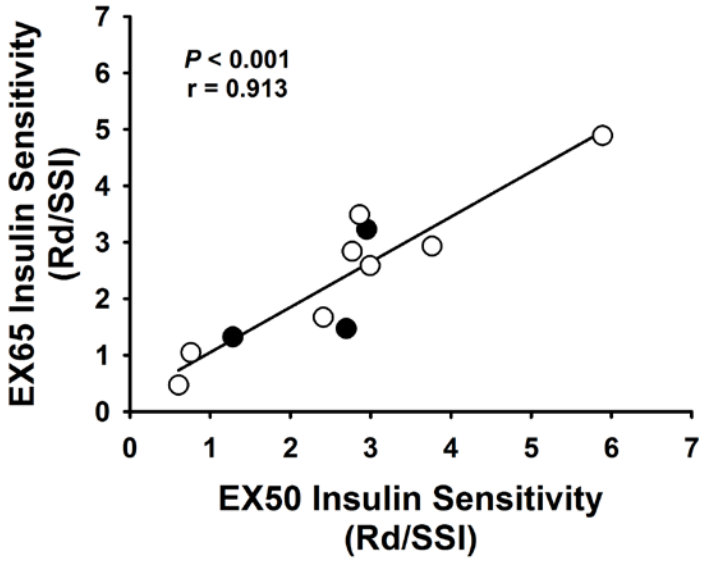


Figure 4-7. Insulin sensitivity measured the day after exercise during EX65 *vs.* EX50. Data are expressed as clamped whole-body glucose disposal (R_d, (μmol/min)) per steady-state plasma insulin concentration (SSI, [μU/ml]). Females (n=8) are represented by open circles, and males (n=3) are represented by filled circles.

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CHAPTER 5

Physiologic mixtures of fatty acids do not impair insulin signaling in cultured muscle cells – even when high in saturated fatty acids

Abstract

In vitro experiments examining the effect of fatty acids on skeletal muscle insulin action are often limited to incubations using only 1 or 2 different fatty acid species, which obviously do not resemble a typical human plasma fatty acid profile. The purpose of this study was to compare graded concentrations of 3 different lipid mixtures: 1) a physiologic fatty acid mixture (NORM; 30% oleate, 25% palmitate, 25% linoleate, 15% stearate, 5% palmitoleate), 2) a physiologic mixture high in saturated fatty acids (HSFA; 20% oleate, 35% palmitate, 15% linoleate, 25% stearate, 5% palmitoleate), and 3) 100% palmitate (PALM) on *in vitro* insulin-stimulated phosphorylation of Akt ($pAkt^{Thr308}$), as a marker of insulin signaling, as well as intracellular lipid accumulation in cultured muscle cells. C2C12 myoblasts differentiated to myotubes were incubated for 12h in serum-free DMEM, 2% BSA, and either 0mM (control), 0.1, 0.2, 0.4, or 0.8mM fatty acid. Insulin (100nM) was added for 15min before harvesting. $pAkt^{Thr308}$ was determined by immunoblot analysis, while diacylglycerol (DAG) and triacylglycerol (TAG) concentrations were measured in muscle lipid extracts using GCMS. As expected, PALM significantly attenuated insulin-stimulated $pAkt^{Thr308}$ compared with 0mM, and this was evident even at the lowest PALM concentration (0.1mM; P=0.001). PALM treatment also markedly increased intracellular DAG content at 0.4 and 0.8mM (2.5- and 5.4-fold compared with 0mM, respectively; both P<0.05). In contrast, NORM resulted in only minimal impairment in insulin-stimulated $pAkt^{Thr308}$ that was not evident at all NORM concentrations (including 0.8mM), and there was no impairment found with

HSFA. Importantly, there was no increase in DAG concentration with NORM or HSFA; however, we found a dose-dependent increase in TAG accumulation with these physiologic fatty acid mixtures. Our findings indicate the robust impairment in insulin signaling and increase in muscle DAG accumulation found with palmitate exposure was not evident with physiologic mixtures of fatty acids, even when the mixture contained a high proportion of saturated fatty acids.

Introduction

Obesity is characterized by excessive adiposity, and a resultant overabundance of fatty acids in the systemic circulation (24, 27, 40). In turn, this elevation in systemic fatty acid availability has been identified as an important factor underlying several of the health complications common in obesity, including insulin resistance and type 2 diabetes (3, 5-6, 46, 49-50, 56). Excessive fatty acid uptake into insulin responsive tissues, like liver and skeletal muscle, has been linked with impaired insulin action in these tissues (5-6, 17, 32, 40, 50). Within skeletal muscle, although several studies have reported strong correlations between the severity of insulin resistance and the accumulation of intramyocellular triacylglycerol (TAG) (21, 30, 41, 43), it is now reasonably well-accepted that increased accumulation of these neutral lipids within the myocyte is a relatively benign reservoir for fatty acid storage. Alternatively, more highly reactive fatty acid metabolites, such as diacylglycerols (DAGs) and ceramides, may be more important for lipid-related impairment in insulin signaling within the muscle cell (1, 11-12, 23, 25, 31, 39, 50, 55). Previous work from our lab (STUDY 1) and (48, 50), and others (31, 34, 44), suggests that when fatty acid availability and uptake into muscle is high (as in obesity), "partitioning" of the excess fatty acid toward TAG synthesis and storage may actually be favorable by limiting substrate available for the formation and accumulation of these more bioactive lipid intermediates within the muscle. Nonetheless, the exact mechanisms underlying the effect of increased fatty acid availability on alterations in skeletal muscle lipid metabolism and the development of insulin resistance are still unclear.

In addition to the effects of a general overabundance of systemic fatty acids on metabolic health, the health impact of the type of fatty acids (i.e., saturated *vs.* unsaturated) has garnered even more attention. Diets high in saturated fatty acids have been linked with the accelerated development of several cardiometabolic abnormalities (for review see (10)). Furthermore, studies performed in vitro have clearly established that saturated fatty acids (e.g., palmitate) induce a marked impairment in insulin action in cultured muscle cells (11-12, 39, 51-52). But this is complicated by more recent findings by several investigators reporting that the addition of oleate (or other unsaturated fatty acids)

to palmitate incubations attenuates or even completely prevents the deleterious effect of palmitate on insulin signaling or insulin action in cultured muscle cells (13, 18, 47, 52). Clearly, incubating muscle cells with only one or two different fatty acids (and often in non-physiologic concentrations or proportions) does not provide an accurate reflection of the elevated fatty acid availability common in obesity. Additionally the mechanisms underlying the putative negative effects of saturated fatty acid on insulin action and the possible salutary effects of unsaturated fatty acids are poorly understood. Moreover, whether the saturation state of the fatty acid impacts the accumulation of different muscle lipids is not known. To address these issues, the primary aim of this study was to determine the effect of increasing availability of physiologic mixtures of the five most abundant fatty acids in human plasma on insulin signaling and lipid accumulation in cultured myotubes. We compared a fatty acid mixture designed to resemble the proportion of fatty acids found in plasma from a healthy human (60% unsaturated *vs.* 40% saturated), with a mixture containing the same fatty acids but with the proportions modified to resemble (or exceed) a diet very high in saturated fatty acids (40% unsaturated *vs.* 60% saturated). The effects of these fatty acid mixtures on insulin signaling and lipid accumulation were compared with the effects of muscle cells incubated with 100% palmitate.

Methods

Cell culture

Mouse C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic solution in 100mM tissue culture treated plates. All cells were passed 3-5 times, and ultimately plated in 6-well, 35mM/well tissue culture treated plates. Upon reaching ~70% confluence, myoblasts were switched to high glucose (~25mM) DMEM supplemented with 2% (v/v) horse serum and 1% (v/v) antibiotic-antimycotic solution to induce differentiation. This media was replaced at 48 h. At 96 h (4 d), differentiated myotubes were used in the experiments described below. All cell culture media and media supplements were

purchased from Gibco-Invitrogen (Grand Island, NY). All other chemicals used in cell culture were purchased from Sigma-Aldrich (St. Louis, MO).

Experimental design

Experimental protocol

Sets of differentiated myotubes were incubated for 12 hours in media containing low glucose (~5mM), serum-free DMEM supplemented with 1% (v/v) antibiotic-antimycotic solution, and 2% (w/v) fatty acid-free bovine serum albumin (BSA) supplemented with one of three different fatty acid mixtures: 1) a normal physiologic mixture of fatty acids generally reflecting their proportion in plasma of a healthy human (**NORM**; 30% oleate [C18:1], 25% linoleate [C18:2], 25% palmitate [C16:0], 15% stearate [C18:0], and 5% palmitoleate [C16:1]), 2) a physiologic mixture of fatty acids designed to resemble a diet very high in saturated fatty acids (**HSFA**; 20% oleate, 15% linoleate, 35% palmitate, 25% stearate, and 5% palmitoleate), or 3) 100% palmitate (**PALM**). The NORM and HSFA fatty acid mixtures were formulated by Nu-Chek Prep Inc (Elysian, MN). We performed incubations at 4 different concentrations of each of the 3 fatty acid mixtures (0.1mM, 0.2mM, 0.4mM, or 0.8mM), and we also included a no fatty acid control (0.0mM). Therefore, we compared 13 different fatty acid conditions (i.e., 3 fatty acid mixtures x 4 concentrations = 12 + control = 13). Importantly, because our concentrated fatty acid supplements were prepared in ethanol, all final incubation media (including 0.0mM) contained 0.5% (v/v) ethanol. After the 12 hr incubation in the different fatty acid treatments, myotubes were treated with or without insulin (100nM) for 15min, and then harvested for later analysis (see details in "*Cell harvest*" section, below). At least n=3 experiments were performed for all conditions.

Experimental outcome variables

To assess insulin action in response to the various treatment conditions, both basal (i.e., no insulin treatment) and insulin-stimulated phosphorylation of key insulin signaling proteins was determined via western blot analysis. Target proteins included phosphorylated (p) Akt^{Thr308}, glycogen synthase kinase-3 ($GSK3\alpha/\beta^{Ser21/9}$), and Akt substrate of 160kD ($AS160^{Thr642}$). Additionally, harvested myotubes were tested for

myocellular diacylglycerol (DAG) and triacylglycerol (TAG) accumulation (see *"Cellular triacylglycerol and diacylglycerol concentrations"* below). Protein abundance of putative regulators of muscle cell lipid accumulation, including of key lipogenic, lipolytic, and lipid transport proteins was examined via western blot analysis. Lipogenic protein targets included the enzymes glycerol-3-phosphate acyltransferase (GPAT1) and diacylglycerol acyltransferase (DGAT1), which catalyze the first and final steps of committed TAG synthesis, respectively. Adipose triacylglycerol lipase (ATGL), its activating co-factor comparative gene identification 58 (CGI-58), hormone sensitive lipase (HSL), and activation phosphorylation of HSL ($pHSL^{Ser563}$) comprised the lipolytic protein targets, while abundance of the fatty acid transporter fatty acid translocase (FAT/CD36) was measured as a marker of myotube fatty acid transport capacity. Finally, markers of proinflammatory/stress activation, including phosphorylation of c-jun N-terminal kinase ($pJNK^{Thr183Tyr185}$), protein kinase C ($pPKC\beta^{Thr641}$), and protein abundance of the NF-κB inhibitor IκBβ, were also determined via western blot analysis. Importantly, all measures of lipid accumulation, lipid metabolism regulatory proteins, and markers of inflammatory pathway activation were made using lysates from basal (i.e., non insulin treated) myotubes.

Analytical procedures

Cell harvest

Cells were rinsed twice with ice-cold Dulbecco's phosphate buffered saline (DPBS), treated with lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM Na_2EDTA pH 8.0, 1mM EGTA pH 8.0, 1% (v/v) Triton X-100, 2.5mM NaPP, 1mM β-glycerophosphate, 1mM Na_3VO_4, and 1x SigmaFAST protease inhibitor cocktail), and scraped on ice into microfuge tubes. Lysates were centrifuged at 20,000 g for 10 min at 4^{o}C. Supernatants were collected and tested for protein concentration (Pierce BCA protein assay, Thermo Scientific, Rockford, IL). For cell lipid content assays (described below), DPBS was used as a harvest solution and lysates were not centrifuged prior to use in cell lipid content assays.

Western blotting

20-30 µg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Various proteins were targeted within whole cell lysates with primary antibodies against: Akt (9272; Cell Signaling Technology, Danvers, MA), pAkt^{Thr308} (9275; Cell Signaling Technology), GSK3β (9315; Cell Signaling Technology), pGSK3α/β$^{Ser21/9}$ (9331; Cell Signaling Technology), AS160 (ABS54; EMD Millipore, Billerica, MA), pAS160^{Thr642} (3028 P1; Symansis, Auckland, New Zealand), GPAT1 (4613; ProSci Incorporated, Poway, CA), DGAT1 (NB110-41487; Novus Biologicals, Littleton, CO), ATGL (2138; Cell Signaling Technology), CGI-58 (NB110-41576; Novus Biologicals), HSL (4107; Cell Signaling Technology), pHSL^{Ser563} (4139; Cell Signaling Technology), FAT/CD36 (sc-9154; Santa Cruz Biotechnology, Santa Cruz, CA), JNK (9251; Cell Signaling Technology), pJNK^{Thr183Tyr185} (4671; Cell Signaling Technology), pPKCβ^{Thr641} (07-873; EMD Millipore), and IκBβ (9248; Cell Signaling Technology). Membranes were incubated with appropriate secondary antibodies and developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Bands were imaged and then quantified via densitometry (AlphaEaseFC, Alpha Innotech Corp., Santa Clara, CA). Data are presented in arbitrary units relative to values obtained for 0.0mM (control) treated myotubes that were normalized to one.

Cellular triacylglycerol and diacylglycerol concentrations

Cells were harvested in ice-cold DPBS, and lipids were extracted overnight at 4°C in a single-phase mixture of chloroform-methanol-aqueous homogenate (1:2:0.8, v/v/v) (4). Internal lipid markers for TAG, DAG, monoacylglycerol, non-esterified fatty acid (NEFA), phospholipid (PL), and cholesterol ester having fatty acid moieties of odd carbon number were added at the start of extraction, for subsequent purity and recovery determinations (Nu-Chek Prep Inc.; Avanti Polar Lipids Inc., Alabaster, AL). TAG, DAG and PL markers were each homogenous in fatty acid content (e.g., $[C23:0]_3$-TAG). Extraction was ended by addition of sufficient chloroform and saline to form two phases (2:2:1.8). After vortexing and brief centrifugation, the lower chloroform phase containing lipids was transferred to a clean tube and dried under vacuum. The residue was reconstituted in 100 µl chloroform and applied to a hexane-equilibrated,

aminopropyl solid phase extraction column. The small chloroform volume did not significantly alter the hexane equilibrium, permitting virtually complete adsorption of glycerolipid and NEFA. Individual lipid species were eluted using previously described solvent mixtures (7). Column fractions were dried, and those containing either purified TAG or DAG were reconstituted in 100 µl toluene. Fatty acid methyl esters (FAMEs) were then generated via alkaline methanolysis, a transesterification process (7), by addition of 1.0 ml 0.2N NaOH in methanol having ultra-low H_2O content, in order to exclude hydrolysis. After 1h incubation at room temperature, the reaction was neutralized using 0.9 ml 1.0 M Na-acetate (pH 4.75). Chloroform (1.0 ml) was added for two-phase formation, which was unaffected by the small volume of toluene present. The lower chloroform phase containing FAMEs from TAG or DAG was transferred, dried and reconstituted in heptane. Individual FAMEs were purified by gas chromatography with capillary column (GC/MS; Agilent 5973Networks, Mass Selective Detector; Agilent Technologies, Palo Alto, CA). FAMEs were detected by electron-impact mass spectrometry with selective ion monitoring, and quantified using FAME standards.

Statistical analysis

A two-way (*dose x treatment type*) analysis of variance (ANOVA) was used to test for significant differences in factor means for all outcome variables. Tukey's post-hoc pair-wise analysis was used to examine significant *F* values detected during ANOVA analyses. Statistical significance was defined as $P \leq 0.05$.

Results

Insulin signaling

As anticipated, incubating the myotubes in PALM suppressed insulin-stimulated $pAkt^{Thr308}$, and this PALM-induced impairment in $pAkt^{Thr308}$ was largely dose-dependent (Figure 5-1a). PALM suppressed $pAkt^{Thr308}$ even at our lowest dose (0.1mM; P=0.001), and at 0.8mM PALM, insulin-stimulated $pAkt^{Thr308}$ was only about one-third of that found when no fatty acids were added to the incubation medium (0.0mM; P<0.001). In contrast, although incubating myotubes in a mixture of fatty acids intended to resemble a "normal" plasma fatty acid profile (NORM) did significantly lower insulin-stimulated

pAkt^{Thr308} (Figure 5-1a), this suppression was relatively modest and was not evident at all treatment doses, including the highest treatment dose (0.8mM). Surprisingly, overnight exposure to our fatty acid mixture containing a high proportion of saturated fatty acids (HSFA) did not significantly attenuate insulin-stimulated pAkt^{Thr308} at any treatment dose (Figure 5-1a), and there were no differences between NORM and HSFA at any dose.

Despite marked attenuation in insulin-stimulated pAkt^{Thr308} in PALM-treated myotubes, we did not find a robust suppression in insulin-stimulated phosphorylation of targets downstream of Akt. Phosphorylation of GSK (pGSK3α/β$^{Ser21/9}$) did tend to decline with increasing doses of PALM (Figure 5-1b), and we did find a significant main effect for pGSK3α/β$^{Ser21/9}$ to be lower in PALM compared with NORM and HSFA (both $P<0.05$). However, there was no apparent effect on pAS160^{Thr642} after exposure to any of the various treatments, at any treatment dose (Figure 5-1c). Importantly, the insulin stimulated increase in pGSK3α/β$^{Ser21/9}$ and pAS160^{Thr642} were rather modest compared with the effect of insulin on pAkt^{Thr308} (Figure 5-2a – lanes 1 and 2). In addition it is important to note that total Akt, GSK3β, and AS160 protein abundance and basal (i.e., non-insulin stimulated) phosphorylation were not altered by exposure to any of the various treatments, at any treatment dose (representative blots are shown in Figure 5-2b).

Lipid accumulation

In parallel with our pAkt^{Thr308} data, PALM incubation increased cellular DAG content in a dose-dependent manner (Figure 5-3a). The increase in DAG content was significantly elevated at the 0.4mM ($P=0.02$) and 0.8mM ($P<0.001$) treatment doses compared with 0.0mM. Conversely, neither NORM nor HSFA increased DAG content at any treatment dose (Figure 5-3a), and cellular DAG content was significantly greater in PALM compared with both NORM and HSFA at 0.8mM ($P<0.001$). Interestingly, the pattern of change in cellular TAG content in response to the different treatments was nearly opposite to that of DAG. Incubating the myotubes in NORM and HSFA increased cellular TAG content markedly, and in a dose-dependent manner. Even the low doses of these lipid mixes induced a significant increase in TAG (Figure 5-3b). PALM treatment did augment TAG content, but this effect was only statistically significant at the higher

doses (0.4 and 0.8mM) and TAG accumulation at these higher doses of PALM was still only about half as great as that measured with NORM and HSFA treatments (both $P<0.01$; Figure 3b). The constituent fatty acids that comprised both the cellular DAG and TAG pools are provided in Tables 5-1 and 5-2, respectively. In general, the fatty acid composition of both DAG and TAG tended to resemble the fatty acid(s) provided in the incubation media, particularly at the higher treatment doses when lipid accumulation was greatest. However, it is noteworthy that oleate (C18:1) tended to be slightly overrepresented whereas stearate (C18:0) tended to be slightly underrepresented (relative to the respective contributions to the incubation mixtures), particularly in the cellular TAG pools.

Lipid metabolism proteins

Despite marked accumulation of TAG with increasing dose of fatty acids, we found no change in protein abundance of the TAG synthesis enzyme GPAT1 (Figure 5-4a). Similarly, DGAT1 abundance was not elevated above 0.0mM in any of our different fatty acid treatments, but we did find DGAT1 protein abundance to be consistently greater in the HSFA treated myotubes compared with both NORM and PALM (main effect for treatment type, both $P<0.01$). In contrast to the limited changes in TAG synthesis enzymes, there was a robust, dose-dependent increase in protein abundance of the TAG lipase ATGL (Figure 5-5a). The increase in ATGL protein abundance was similar for all of our fatty acid treatments and achieved statistical significance at 0.4mM ($P=0.003$) and 0.8mM ($P<0.001$) compared with 0.0mM (Figure 5-5a). This effect of increasing fatty acid concentration on protein abundance was not found for the ATGL co-activator CGI-58, or HSL (Figure 5-5b and 5-5c, respectively). Interestingly, similar to DGAT1, CGI-58 protein abundance was slightly but consistently elevated in the HSFA treated myotubes compared with both NORM and PALM treated myotubes (main effect for treatment type, both $P<0.02$).

Proinflammatory stress markers

Phosphorylation of JNK at threonine 183 and tyrosine 185 residues was consistently elevated in PALM treated myotubes compared with both NORM and HSFA treated

myotubes (Figure 5-6a; main effect for treatment type, both $P<0.01$). Total JNK protein abundance was not affected by any of our fatty acid treatments (data not shown). In contrast to the phosphorylation of JNK, PKCβ phosphorylation at the threonine 641 residue was unaffected by increasing concentrations of any of our fatty acid treatments (Figure 5-6b). IκBβ protein abundance was measured as a marker of NFκB pathway activation, and similar to $pPKC\beta^{Thr641}$, IκBβ was also not significantly altered by any of our lipid treatments (Figure 5-6c).

Discussion

The effects of palmitate exposure on in vitro muscle cell metabolism have been examined for decades (16), and the negative effect of palmitate on insulin signaling in cultured myotubes has been very well established (12, 15, 19, 39, 51-53, 58). More recently, it has been demonstrated that the addition of an unsaturated fatty acid (such as oleate) to palmitate in the incubation media can attenuate, and even completely prevent the deleterious effect(s) of palmitate on insulin signaling and/or insulin action in cultured muscle cells (13, 18, 47, 52). However, exposing muscle cells to media containing only two different fatty acids (and often in non-physiologic concentrations or proportions) does not provide an accurate reflection of the elevated fatty acid availability common in obesity. Our findings expand on the previous studies in this area by demonstrating that when cultured myotubes were exposed to physiologic proportions of the five most abundant fatty acids found in humans, insulin-stimulated phosphorylation of Akt was only minimally impaired, even at concentrations that represent relatively high physiologic levels (0.8mM). Moreover, our novel findings indicate that even a fatty acid mixture containing a relatively high proportion of saturated fatty acids (i.e., resembling or even exceeding the proportion of saturated fatty acids found in a very high saturated fat diet) also failed to induce substantial impairment in insulin-stimulated phosphorylation of Akt. In conjunction with these findings, we also found that incubation in both of these fatty acid mixtures augmented intramyocellular TAG concentration without an increase in DAG accumulation. Not only does this confirm that the fatty acids in our physiologic mixtures were indeed entering the myotubes in our experiments, but also their

preferential storage as neutral lipids may help explain why exposure to even high concentrations of these mixtures did little to suppress insulin signaling.

Although several studies have reported strong correlations between intramyocellular TAG concentration and the severity of insulin resistance (21, 30, 41, 43), it is now reasonably well-accepted that intramyocellular TAGs represent a relatively benign reservoir for fatty acid, and more highly reactive fatty acid metabolites, such as DAGs and ceramides, may impair insulin signaling (1, 11-12, 23, 25, 31, 39, 50, 55). Previous work from our lab STUDY 1 and (48, 50), and others (31, 34, 44), suggests that when fatty acid availability and uptake into muscle is high (as in obesity), “partitioning” of the excess fatty acid toward TAG synthesis and storage may be favorable by limiting substrate available for the formation and accumulation of these more bioactive lipid intermediates within the muscle. In line with this hypothesis, here we found that the highest doses of our NORM and HSFA treatments (0.8mM) resulted in a ~10-fold increase in TAG accumulation, with no increase in DAG concentration, and insulin-stimulated Akt phosphorylation was only minimally suppressed. In contrast, PALM treated myotubes were characterized by attenuated TAG accumulation, robust DAG accumulation, and insulin-stimulated Akt phosphorylation was severely impaired. Thus, as compared with an equivalent dose of palmitate, there clearly was a preferential "partitioning" of the fatty acids in NORM and HSFA treatments to be esterified and stored as TAG. It has been previously suggested that the TAG synthesis pathway (perhaps most importantly DGAT) may have a higher affinity for unsaturated fatty acids compared with saturated fatty acids (12, 39), which may contribute to elevated TAG accumulation when the availability of unsaturated fatty acids is high. However, a high affinity for unsaturated fatty acids may not explain the high TAG accumulation with the lipid mixtures in our study because we found a similar increase in TAG accumulation with our NORM and our HSFA treatments, despite the marked difference in the proportion of unsaturated fatty acids in these mixtures (i.e., 60% *vs.* 40%, respectively). Additionally, if TAG synthesizing enzymes had a greater affinity for the unsaturated fatty acids in our lipid mix, we would expect to find a much higher proportion of unsaturated fatty acids within the TAG fraction of our muscle cells, but instead the fatty acid profiles

within our TAG fraction generally resembled the fatty acid profile in the incubation media. It is not clear why lipid accumulation in our muscle cells was nearly identical between NORM and HSFA, but akin to our observations for insulin signaling, it appears that as long as a generally physiologic mixture of several fatty acids was provided, a relatively high proportion of saturated fatty acids did not abnormally affect fatty acid "partitioning" or lipid accumulation.

Many factors may contribute to the differential accumulation of fatty acids within separate lipid pools (e.g., TAG and DAG), with the most likely targets being the regulation of lipid synthesis, lipolysis, as well as the transport of fatty acids into the myocyte. We (STUDY 1) and (50), and others (35, 44), have previously suggested that altered abundance and/or activity of key lipogenic enzymes may help to facilitate the partitioning of excess fatty acid in muscle cells toward TAG synthesis (and away from DAG and ceramide accumulation), which may in turn improve muscle cell insulin action. However, in general our findings do not support this notion under the present conditions. Our observation that intramyocellular TAG concentration increased up to ~10-fold without a change in protein abundance of the key TAG synthesizing enzymes, GPAT and DGAT, suggests that the basal abundance of these enzymes was sufficient to catalyze this robust increase in TAG synthesis, and is not likely limiting fatty acid accumulation as TAG in our experiments. It is certainly possible that post-translational modifications of these proteins may have altered the enzymatic activity of GPAT1 and/or DGAT1 (not measured in this study), but such modifications have not been well investigated.

Although alterations in the TAG synthesis pathway could certainly contribute to the pattern of intramyocellular lipid accumulation, regulation of lipid hydrolysis may also be very important. It has been hypothesized that the balance between TAG lipase (ATGL) and DAG lipase (HSL) activity could be a key determinant of the accumulation of intramyocellular TAG and DAG (2). Here we found a dose-dependent increase in the abundance of the TAG lipase ATGL for all three of our fatty acid treatments, while there was no change in HSL protein content, or activating phosphorylation at Ser563 (data not shown), with any concentration of any of our treatments. This apparent lipolytic

regulatory enzyme imbalance did not correspond with our DAG accumulation data, which was only elevated in response to PALM treatment. We acknowledge that ATGL protein abundance does not necessarily reflect lipase activity, and note that the abundance of the ATGL co-activator, CGI-58, did *not* increase in parallel with ATGL, which may have helped to mitigate lipolytic activity even when ATGL abundance was very high. Pertaining to the potential mechanism underlying the dose-dependent increase in ATGL that we observed, ATGL expression is known to be mediated by peroxisome proliferator-activated receptor-γ (PPARγ) (29, 36). Because fatty acids are known ligands/activators of PPARs (28) it is likely that the increased availability of fatty acids in our incremental fatty acid exposures augmented ATGL abundance via a PPARγ-dependent mechanism. Importantly, because the upregulation of ATGL abundance was essentially the same for all three of our fatty acid treatments, it appears that the increase in ATGL abundance was responding largely to the quantity of fatty acid(s) rather than the species of fatty acid(s).

We have reported in STUDY 1 and elsewhere (33) that fatty acid transport capacity may be an important determinant for the accumulation of muscle lipids. However, here we found no change in protein abundance of the predominant skeletal muscle fatty acid transporter FAT/CD36 with any treatment. It is possible that altered localization of FAT/CD36, as well as changes in other fatty acid transporters, may have mediated altered fatty acid uptake in the myotubes given that FAT/CD36 has been shown to translocate from intracellular pools to the cell surface membrane in response to several different stimuli (8, 26, 38). Nonetheless, our findings indicate that the basal abundance of FAT/CD36 in these myotubes was likely sufficient to facilitate enough fatty acid transport to induce a ~10-fold increase in TAG content during NORM and HSFA treatments.

Excess DAG is hypothesized to attenuate insulin action via induction of proinflammatory stress signaling pathways that have been shown to negatively regulate insulin signaling proteins and GLUT4 trafficking (14, 25, 52, 57). In agreement with this hypothesis, NORM and HSFA treated cells did not accumulate DAG, we found no change in the phosphorylation of the proinflammatory stress kinase JNK, and subsequently insulin

stimulated Akt phosphorylation was only marginally attenuated compared with when no fatty acids were added to the incubation medium. In contrast, in our PALM treated cells the increase in DAG concentration was accompanied by an increase in pJNK, and impaired insulin stimulated Akt phosphorylation. However, the effect of PALM treatment on JNK phosphorylation appeared to be independent of the dose of palmitate, and thus did not parallel the dose-dependent effect we found with cellular DAG accumulation. While it is possible that the resolution of our assessment of pJNK was not sensitive enough to detect a dose-dependent response, it is more likely that additional factors other than JNK activation during PALM treatment also mediate the impairment in insulin signaling. For example, palmitate has previously been reported to have pronounced cytotoxic (i.e., cell death) effects in muscle cells *in vitro* when provided in high concentrations (e.g., 0.75mM) (53), and this may also contribute to the palmitate-related impairment in insulin-stimulated Akt phosphorylation. Because the novel component of the present study was to examine the effects of physiologic fatty acid mixtures on insulin signaling and lipid metabolism, and most importantly, myotubes exposed to these treatments did *not* show any sign of pronounced insulin signaling impairment, measures of cell viability were not performed in these experiments. Equally important is that skeletal muscles from high fat-fed and *ob/ob* mice (i.e., murine models of obesity-related excessive fatty acid availability) do not exhibit increased pro-apoptotic (i.e., cell death) activation (54), thus indicating that lipid-related cytotoxicity is not likely relevant for *in vivo* skeletal muscle cell physiology and lipid-related insulin resistance.

We must also acknowledge that deleterious effects of palmitate and/or other saturated fatty acids on insulin action and/or proinflammatory stress activation could certainly be due to factors in addition to alterations in lipid accumulation and fatty acid partitioning, *per se*. For example, increased saturated fatty acid availability has been found to alter cell membrane structure and function, and increased saturated fatty acid content in the muscle cell membrane phospholipid pool has been associated with impaired insulin action (9, 42). This association may be mediated in part by impaired membrane fluidity, and a resulting attenuation in insulin receptor content and insulin binding at the cell membrane (20, 22). Differential transport, trafficking, and perhaps oxidation of saturated

vs. unsaturated fatty acids may also have implications for cellular metabolism and insulin action; however, compelling evidence to suggest that the saturation state of the fatty acid influences these properties of cellular lipid metabolism is currently lacking. It is important to reemphasize that despite a relatively large difference in the saturated fatty acid content in our NORM and HSFA lipid mixtures, we observed essentially no difference in our primary outcome measures. This suggests that when muscle cells are exposed to the most abundant fatty acids found in human plasma in generally physiologic proportions and concentrations, the saturation state of the available fatty acids may not be an important factor for the regulation of lipid partitioning/accumulation or insulin signaling within the cell.

Given that both GSK3α/β and AS160 are downstream signaling targets of the serine/threonine kinase Akt, we were surprised that the deleterious effect of PALM treatment on insulin-stimulated phosphorylation of Akt was not paralleled by impaired insulin-stimulated phosphorylation of GSK3α/β and AS160. Upon review of our insulin signaling data, these observations are likely due to a limitation in our ability to detect changes in the phosphorylation of GSK3α/β and AS160 compared with Akt in our model, rather than due to a true disconnect in these well-established signaling cascades. For example, in our muscle cells incubated without fatty acid (0.0 mM), insulin treatment resulted in a >10-fold increase in Akt phosphorylation compared with the basal condition (i.e., non insulin treated), whereas insulin-stimulated phosphorylation of GSK3α/β and AS160 was only ~2-fold and a mere 50% above the basal condition, respectively (see Figure 5-2 for representative blots). This limited range for the change in phosphorylation for these downstream signaling proteins under conditions without fatty acid likely diminished our ability to detect a decrement in the phosphorylation of these proteins in the PALM treated myotubes. We are currently conducting related follow-up studies in human primary skeletal muscle cell culture to combat this unfortunate limitation, and to determine whether our current findings can be extended to a human muscle cell model.

Several unresolved questions remain pertaining to the effects of fatty acids on insulin signaling and insulin action. We recognize that findings in cell culture may not fully

translate to humans *in vivo*, but from our work here and the work of others (13, 18, 47, 52) it appears that as long as there are *some* unsaturated fatty acids present, the negative effects of saturated fatty acids on muscle insulin action are largely abolished. So because more than half of the fatty acids in human plasma are unsaturated (37, 45), even in individuals who eat diets containing a high proportion of saturated fat (45), does a relatively high proportion of plasma saturated fatty acids really present a meaningful health risk in the context of muscle insulin resistance, as is commonly believed? Alternatively, while at relatively low concentrations unsaturated fatty acids may somehow abrogate the negative effects of saturated fatty acids (such as palmitate), but perhaps as the availability of fatty acids increase, the resultant high fatty acid flux into muscle impairs insulin action regardless of the saturation state. This concept is supported by many clinical studies in humans that report marked suppression in insulin sensitivity in response to infusion of lipid emulsion solutions that are comprised almost entirely of unsaturated fatty acids (5-6, 49). Importantly, we recently reported that even a rather modest infusion rate of a lipid emulsion solution containing ~90% unsaturated fatty acids can impair insulin sensitivity in humans (50), so the infusion rates and resultant fatty availability do not need to be supraphysiological in order to observe this phenomenon. Perhaps the balance between the fatty acid flux into muscle and the capacity to sequester the transported fatty acids into neutral lipids within the myocyte plays a key role. Along these lines, despite the relatively high physiologic concentrations of the fatty acid mixtures used in our experiments, this did not appear sufficient to overwhelm the capacity of the myotubes to store these fatty acids as triacylglycerols. As a result, even at the highest concentration of either of our fatty acid mixtures (0.8mM) we found no accumulation of more bioactive lipid intermediates (e.g., DAG), no signs of increased activation of pro-inflammatory pathways (e.g., JNK), and insulin signaling was not impaired. In contrast to our findings, a recent study has demonstrated that higher doses of long-chain polyunsaturated fatty acids did indeed impair insulin action in cultured muscle cells (47). It is very important to consider that the while *in vitro* experiments like ours incubate the muscle cells in elevated fatty acid concentrations for several hours, in human obesity muscles are chronically exposed to elevated fatty acid availability, and it appears that regardless of the proportion of saturated *vs.* unsaturated fatty acids in plasma

this chronic over-exposure may overwhelm the capacity for triacylglycerol synthesis, which may ultimately contribute to impaired insulin sensitivity.

In summary, we found that physiologic mixtures containing the five most abundant plasma fatty acids did not substantially impair muscle cell insulin signaling *in vitro* when provided acutely (12h), and in doses that span the normal-to-high range of physiologic plasma fatty acid concentrations (0.1-0.8mM). This was the case even when the saturated fatty acid content of the mixture was raised to levels that resemble or even exceed the proportion of saturated fatty acids found in a very high saturated fat diet. Importantly, both of our fatty acid mixtures dose-dependently increased triacylglycerol accumulation in the muscle cells, and in turn diacylglycerol content did not increase. Therefore, our findings support the working hypothesis that as long as the availability and flux of fatty acid into muscle does not exceed the capacity to esterify and store these fatty acids as triacylglycerols this may help limit the accumulation of more harmful lipid intermediates, such as diacylglycerol, and thereby "protect" against fatty acid-induced insulin resistance. Moreover, our findings indicate that as long as some unsaturated fatty acids were present, a high proportion of saturated fatty acids did not have an adverse effect on the regulation of lipid accumulation or insulin signaling.

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Table 5-1. Fatty acid composition of cellular diacylglycerol (% of the total diacylglycerol fatty acid pool)

Treatment Type	mM	C16:0	C18:0	C16:1	C18:1	C18:2
No fatty acid (Control)	0.0	24%	3%	17%	49%	4%
PALM incubation media		*100%*	*0%*	*0%*	*0%*	*0%*
PALM	0.1	42%	22%	9%	25%	1%
PALM	0.2	50%	23%	8%	17%	0%
PALM	0.4	54%	18%	9%	17%	1%
PALM	0.8	76%	12%	5%	7%	0%
NORM incubation media		*25%*	*15%*	*5%*	*30%*	*25%*
NORM	0.1	22%	24%	4%	37%	11%
NORM	0.2	21%	25%	6%	34%	13%
NORM	0.4	26%	28%	3%	30%	13%
NORM	0.8	24%	26%	3%	29%	17%
HSFA incubation media		*35%*	*25%*	*5%*	*20%*	*15%*
HSFA	0.1	20%	22%	13%	43%	2%
HSFA	0.2	23%	18%	6%	49%	3%
HSFA	0.4	24%	13%	9%	45%	9%
HSFA	0.8	32%	14%	8%	36%	10%

Values are means for n=3 expressed as a percentage of the total triacylglycerol fatty acid pool. The composition of the different fatty acid treatments in the incubation media is provided in the shaded rows.

Table 5-2. Fatty acid composition of cellular triacylglycerol (% of the total triacylglycerol fatty acid pool)

Treatment Type	mM	C16:0	C18:0	C16:1	C18:1	C18:2
No fatty acid (Control)	0.0	24%	3%	17%	49%	4%
PALM incubation media		*100%*	*0%*	*0%*	*0%*	*0%*
PALM	0.1	50%	1%	14%	31%	2%
PALM	0.2	62%	2%	11%	22%	2%
PALM	0.4	72%	1%	9%	16%	1%
PALM	0.8	83%	2%	5%	8%	1%
NORM incubation media		*25%*	*15%*	*5%*	*30%*	*25%*
NORM	0.1	22%	4%	10%	51%	12%
NORM	0.2	21%	5%	7%	49%	17%
NORM	0.4	23%	7%	5%	43%	22%
NORM	0.8	24%	8%	3%	39%	25%
HSFA incubation media		*35%*	*25%*	*5%*	*20%*	*15%*
HSFA	0.1	28%	6%	12%	46%	7%
HSFA	0.2	31%	7%	9%	44%	8%
HSFA	0.4	34%	10%	6%	40%	10%
HSFA	0.8	35%	14%	4%	35%	12%

Values are means for n=3 expressed as a percentage of the total triacylglycerol fatty acid pool. The composition of the different fatty acid treatments in the incubation media is provided in the shaded rows.

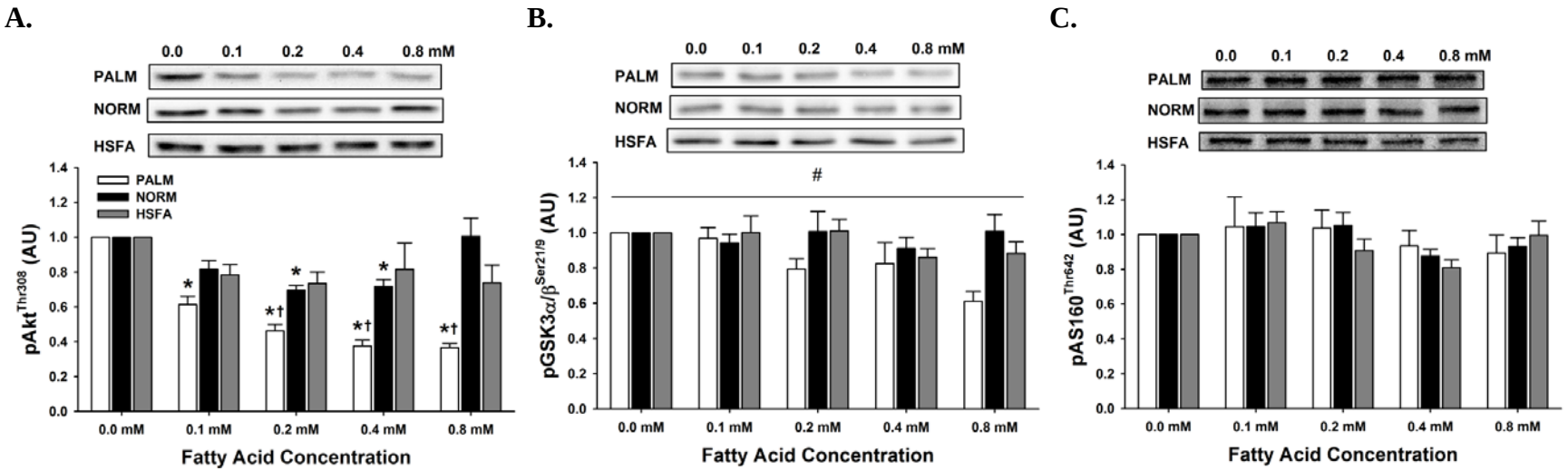
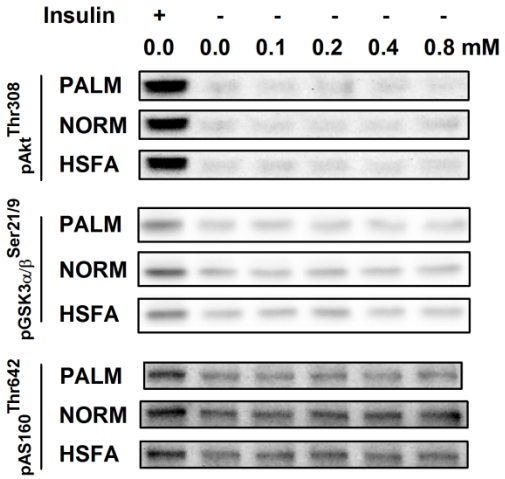


Figure 5-1. Insulin-stimulated phosphorylation of insulin signaling proteins. In all figure panels, pAkt^{Thr308} (A), pGSK3α/β$^{Ser21/9}$ (B), and pAS160^{Thr642} (C), data are expressed relative to a no fatty acid (0.0mM) condition. **P*<0.05 *vs.* 0.0mM. †*P*<0.05 *vs.* NORM and HSFA within treatment dose. #*P*<0.05 for a main effect of PALM *vs.* NORM and HSFA. Representative blots are inset above each figure panel. GSK, glycogen synthase kinase; AS160, Akt substrate of 160 kD; AU, arbitrary units.

A.



B.

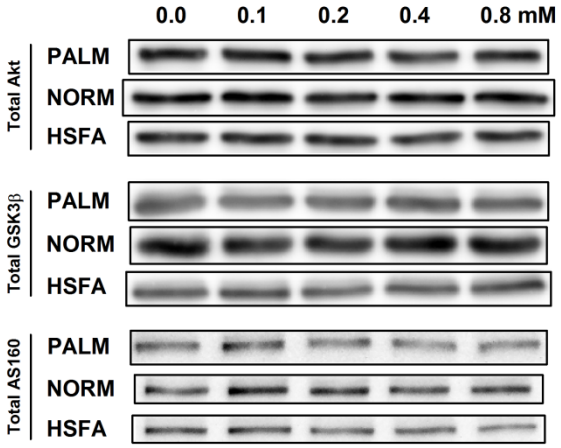
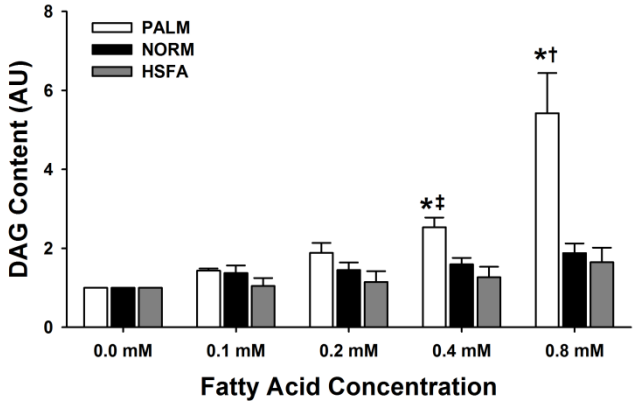


Figure 5-2. Representative blots for basal phosphorylation of insulin signaling proteins (A) and total abundance of insulin signaling proteins (B). In panel (A), insulin stimulated phosphorylation of each insulin signaling protein during no fatty acid (0.0mM) treatment is presented in the far left lane of each representative blot. GSK, glycogen synthase kinase; AS160, Akt substrate of 160 kD.

A.



B.

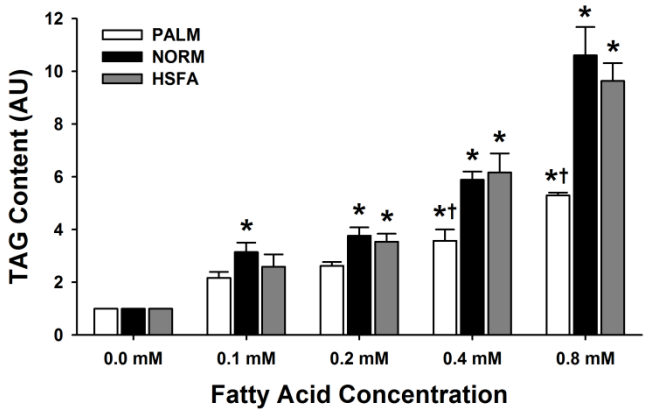
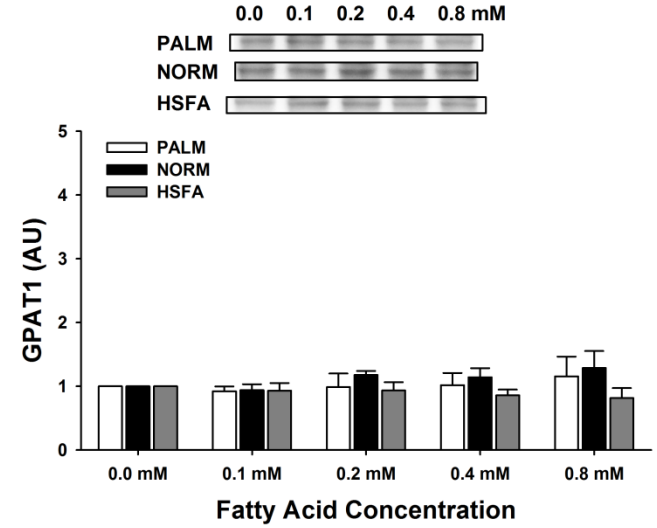


Figure 5-3. Muscle cell lipid accumulation. In both figure panels, DAG (A) and TAG (B), data are expressed relative to a no fatty acid (0.0mM) condition. **P*<0.05 *vs.* 0.0mM. †*P*<0.05 *vs.* NORM and HSFA within treatment dose. ‡*P*<0.05 *vs.* HSFA within treatment dose. DAG, diacylglycerol; TAG, triacylglycerol; AU, arbitrary units.

A.



B.

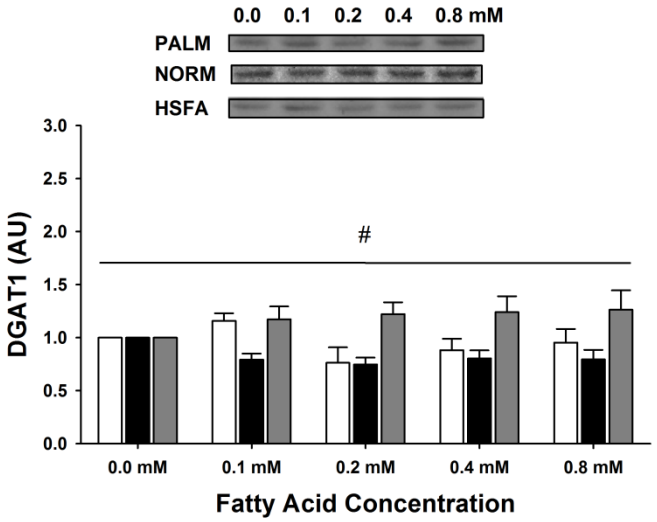


Figure 5-4. Protein abundance of triacylglycerol synthesis enzymes GPAT1 (A) and DGAT1 (B). In both figure panels data are expressed relative to a no fatty acid (0.0mM) condition. #$P<0.05$ for a main effect of HSFA *vs.* PALM and NORM. Representative blots are inset above each figure panel. GPAT, glycerol-3-phosphate acyltransferase; DGAT, diacylglycerol acyltransferase; AU, arbitrary units.

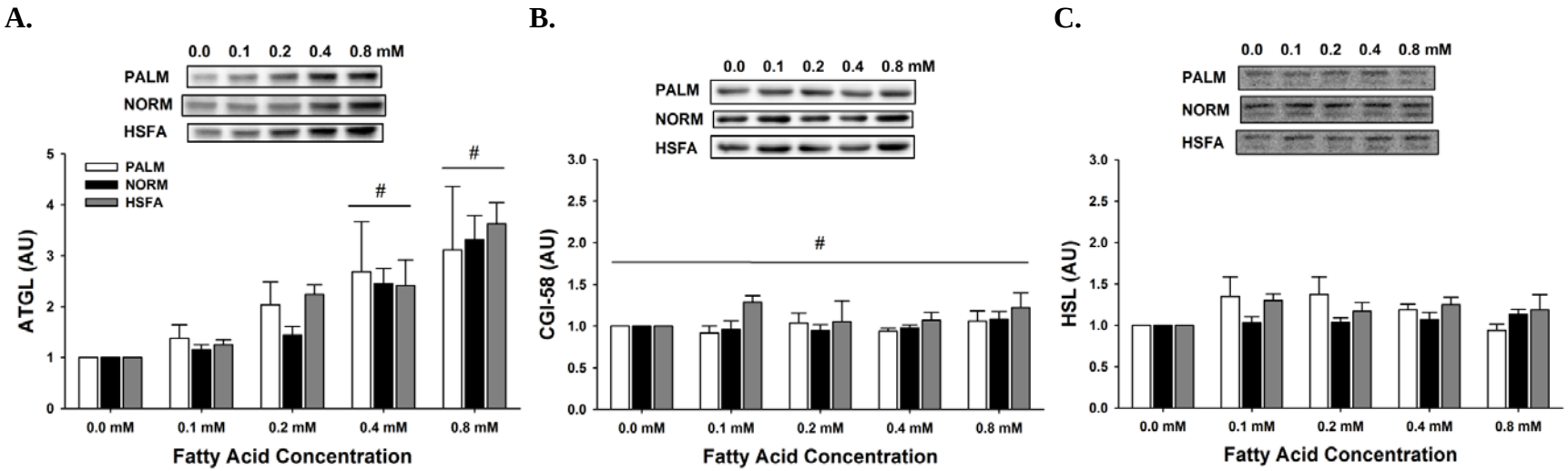


Figure 5-5. Protein abundance of lipolytic regulators ATGL (A), CGI-58 (B), and HSL (C). In all figure panels data are expressed relative to a no fatty acid (0.0mM) condition. In panel (A) #$P<0.05$ for a main effect of treatment dose *vs.* 0.0mM. In panel (B) #$P<0.05$ for a main effect of HSFA *vs.* PALM and NORM. Representative blots are inset above each figure panel. ATGL, adipose triacylglycerol lipase; CGI-58, comparative gene identification 58; HSL, hormone sensitive lipase; AU, arbitrary units.

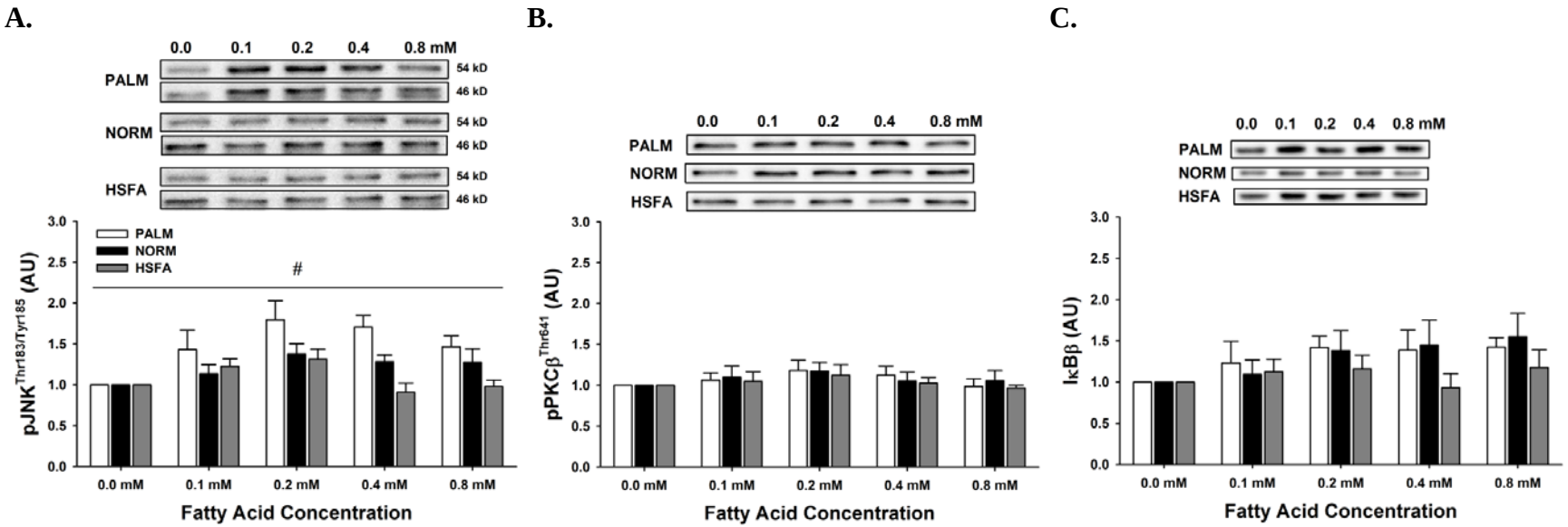


Figure 5-6. Markers of proinflammatory stress activation. In all figure panels, pJNK$^{Thr183/Tyr185}$ (A), pPKCβ^{Thr641} (B), and IκBβ (C), data are expressed relative to a no fatty acid (0.0mM) condition. #$P<0.05$ for a main effect of PALM *vs.* NORM and HSFA. Representative blots are inset above each figure panel. Both 54 and 46 kD bands presented in (A) for each fatty acid treatment type are from the same blot. JNK, c-jun N-terminal kinase; PKC, protein kinase C; IκBβ, inhibitor of κB (β isoform); AU, arbitrary units.

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CHAPTER 6

OVERALL DISCUSSION

It is very well established that both type 2 diabetic and pre-diabetic populations are characterized by insulin resistance, and that over two-thirds of these individuals are overweight or obese. It is equally clear that excess adiposity and the subsequent elevation in systemic availability of fatty acid are causally linked with the development of insulin resistance in obesity. Additionally, although exercise is known to be an important component of lifestyle programs aimed at improving obesity-related diseases, it is surprising that more is not known about the "dose" of exercise required to improve insulin sensitivity or the underlying mechanisms for this improvement. For this reason the overarching purpose of my dissertation was to examine the effects of exercise and elevated fatty acid availability on insulin sensitivity and muscle lipid metabolism, and to determine whether altered muscle lipid metabolism is important for regulation of insulin sensitivity.

Together, the three projects from my dissertation have fulfilled this purpose, and both individually and collectively provided insight regarding the effects of exercise and elevated fatty acid availability on muscle lipid metabolism and insulin sensitivity. Most notably, these studies have: *A*) reported that a rather modest single session of exercise is sufficient to enhance insulin sensitivity in obese individuals, and that low intensity exercise may provide metabolic benefit that is equal (or perhaps enhanced) compared with moderate-to-high intensity exercise resulting in the same total energy expenditure (STUDY 2), *B*) affirmed that enhanced storage of excess fatty acid in muscle as triacylglycerol protects against accumulation of harmful lipid intermediates and impaired insulin sensitivity (STUDY 1 and STUDY 3), and, *C*) found that the saturation state of fatty acids may not be an important factor for the regulation of muscle cell lipid

accumulation/partitioning or insulin signaling when provided a in a mixture of the most abundant fatty acids found in human plasma (STUDY 3). Many other important details of each of my dissertation studies were described in Chapters 3-5, and thus will not be discussed in further detail here. In this overall summary of my dissertation, I will attempt to expand on the findings of my projects, providing an integrative discussion of the collective significance and implications that can be derived from my dissertations studies, particularly as they relate to the working hypothesis of our laboratory.

The key underlying component of all three of my dissertation studies was the examination of the hypothesis that "partitioning" excess fatty acid toward storage as triacylglycerol (TAG) in muscle cells may limit substrate for the accumulation of more harmful lipid intermediates (e.g., diacylglycerol [DAG], ceramide) and thereby protect against lipid-induced insulin resistance. The basis for this hypothesis was largely generated from studies completed by a previous doctoral student in our laboratory, Simon Schenk, Ph.D. Briefly, in his dissertation projects Dr. Schenk reported that a single session of rather vigorous exercise was sufficient to protect against lipid-induced insulin resistance in lean individuals, and that this protective effect of exercise was associated with a robust increase in muscle TAG content and attenuated muscle DAG and ceramide accumulation (compared with remaining sedentary). Both STUDY 1 and STUDY 3 of my dissertation provide further support for this hypothesis. In STUDY 1, I reported that an overnight (16h) lipid/heparin infusion after a single session of exercise resulted in a substantial (~30%) increase in muscle TAG content, but no increase in muscle DAG or ceramide content compared with an overnight saline infusion after exercise. Importantly, despite the well documented negative effects of lipid infusion on insulin sensitivity, these subjects were (previously) shown to have no impairment in insulin sensitivity after the overnight lipid/heparin infusion compared with saline, suggesting that the preferential storage of excess lipid as TAG and limited accumulation of DAG and ceramide in muscle may have been important for this effect. In STUDY 3, I found that, unlike the deleterious effects of 100% palmitate, mixtures of the five most abundant fatty acids in human plasma did not cause substantial impairment in insulin signaling in cultured muscle cells when provided in generally physiologic proportions and concentrations. Because the

failure of these fatty acid mixtures to induce substantial impairment in insulin signaling was paralleled by very robust cellular TAG accumulation (>10-fold) but virtually no DAG accumulation, these findings also support the notion that preferential partitioning of excess fatty acid toward storage as TAG is protective against fatty acid-induced insulin resistance. Conversely, the findings from STUDY 2 neither support nor refute our working hypothesis. In STUDY 2, I reported that a rather modest single session of exercise was sufficient to enhance insulin sensitivity into the next day in obese adults, but did not find any changes in muscle lipid content (TAG, DAG, ceramide) that were associated with this effect. Because I did *not* find an increase in muscle TAG content after exercise in this study, it is therefore *not* possible to evaluate our hypothesis concerning the beneficial effects of enhanced storage of excess fatty acid in muscle as TAG. However, I must also acknowledge the possibility that "partitioning" of fatty acids among the major lipid pools may not be essential for the exercise-induced increase in insulin sensitivity in obesity. This will be discussed in greater detail below. Altogether the findings from my dissertation studies generally support the hypothesis that "partitioning" excess fatty acid toward storage as TAG in muscle cells may limit substrate for the accumulation of more harmful lipid intermediates (e.g., DAG, ceramide) and thereby protect against lipid-induced insulin resistance.

Although my dissertation studies generally reaffirmed the major concepts of our working hypothesis, our previous suggestion that a single session of exercise acts to enhance the storage of fatty acid as intramyocellular TAG, and that an increase in the protein abundance of key TAG synthesis enzymes after exercise may be important for this effect was not as well supported. In STUDY 1, I found that the increase in intramyocellular TAG accumulation after exercise when fatty acid availability was elevated was not accompanied by increased protein abundance of the TAG synthesis enzymes glycerol-3-phosphate acyltransferase (GPAT) or diacylglycerol acyltransferase (DGAT). However, because both of the experimental trials in this study required the participants to exercise (with the only difference between trials being the magnitude of fatty acid availability in the hours after exercise), it is certainly possible that exercise increased the abundance of these lipid synthesizing proteins, and simply that the elevation of fatty acid availability

did not *further* augment their abundance. Nevertheless, in further contrast to our previous findings, I did not even find a significant increase in intramyocellular TAG content the day after either of the relatively modest exercise sessions performed by the participants in STUDY 2 of my dissertation (and similarly GPAT and DGAT protein abundance were also not affected). This apparent discrepancy concerning the effect of exercise on muscle TAG accumulation between my current findings and previous work from our lab may be related to the much lower exercise stimulus used in my dissertation study, perhaps in combination with the fact the participants in my study were obese (Dr. Schenk's study used lean subjects exposed to an overnight lipid/heparin infusion), given that the large intramyocellular lipid pools in obesity may make detecting a relatively small (but potentially important) exercise-induced increase in TAG rather challenging. Nonetheless, even if exercise did induce a non-detectible increase in intramuscular TAG in my study, it was clear that this occurred in absence of an increase in GPAT or DGAT protein abundance. Finally, although STUDY 3 of my dissertation did not incorporate exercise, findings from this study clearly demonstrated that an increase in GPAT and DGAT abundance was not required for cultured muscle cells to markedly increase TAG synthesis, resulting in more than a 10-fold increase in cellular TAG accumulation. At this time, data concerning the notion that a single session of exercise acts to enhance the storage of fatty acid as intramyocellular TAG are somewhat equivocal, but it appears unlikely that an exercise-induced increase in GPAT or DGAT protein abundance is required for this effect.

Much attention has been paid to the effects of different classes of fatty acids (e.g., saturated *vs.* unsaturated) as well as the species of fatty acid (e.g., palmitate *vs.* oleate) on metabolic health outcomes, including insulin resistance. In contrast, our lab has generally placed emphasis on the deleterious consequences resulting from a general overabundance of fatty acid, rather than effects of specific fatty acids, *per se*. Findings from STUDY 3 of my dissertation support the idea that the "amount" of available fatty acid may be a more important determinant of muscle cell lipid accumulation and insulin signaling than the "type" of available fatty acid. In brief, in STUDY 3 I found that exposing cultured muscle cells to a mixture of the five most abundant fatty acids found in

human plasma did not impair insulin signaling, even when the mixture contained a relatively high proportion of saturated fatty acids (60%; thus resembling or even exceeding the proportion of saturated fatty acids found in a very high saturated fat diet). Furthermore, accumulation of cellular TAG in response to the two different physiologic fatty acid mixtures was dose-dependent, but virtually identical between the two mixtures at each specific dose of fatty acid. And even though saturated fatty acids have previously been associated with increased muscle cell DAG accumulation, I found no increase in cellular DAG content in response to either mixture, at any dose of fatty acid exposure (including a fairly high physiologic plasma fatty acid concentration, 0.8mM). These findings suggest that when muscle cells are exposed to the most abundant fatty acids found in human plasma in generally physiologic proportions and concentrations, even a marked difference in the proportion of saturated fatty acids in these mixtures (i.e., 60% *vs.* 40%,) may not be an important factor for determining the regulation of insulin signaling or lipid accumulation/partitioning within the cell. Importantly, this should not be interpreted to suggest that the "type" of fatty acid may not have any influence on metabolic health outcomes (including insulin resistance), but rather that the overall effect of an increased abundance of fatty acids in our cultured C2C12 muscle cell model appeared to outweigh what may be subtle effects of (and deviations in) the various fatty acid classes/species that are typically found in humans.

Several important questions that pertain to the findings from my dissertation studies remain unanswered. Perhaps most intriguing, although my studies do support the hypothesis that that "partitioning" excess fatty acid toward storage as TAG in muscle cells may help to protect against lipid-induced insulin resistance, the mechanism(s) that may underlie enhanced fatty acid storage as TAG are still unknown. Along these lines, though I was able to measure maximal *in vitro* GPAT and DGAT enzyme activity (and reported no changes in the activity of these enzymes) in STUDY 1, it is very important to note that this study did not allow me to capture the possible effect of exercise on the activity of these enzymes, and that these measurements may not reflect *in vivo* activity of these enzymes. Thus, it remains both possible and likely that acute, modifiable regulation of GPAT and DGAT activity *in vivo* may help to explain alterations in muscle

TAG accumulation in my dissertation studies; however, such modifications (e.g., phosphorylation, acetylation) have not been well studied for these enzymes. For this reason I propose conducting proteomic analysis of GPAT and DGAT isolated from the remaining muscle biopsy samples and cell homogenates of each of my dissertation studies. These analyses would allow for detection of multiple forms of post-translational modification to these enzymes in response to exercise and/or elevated fatty acid availability, and even allow for comparisons between lean and obese individuals. Furthermore, if post-translational modifications to either or both of these enzymes were found to associate with changes in intramyocellular TAG accumulation, development of pharmacologic agents capable of modifying the activity of these enzymes may prove to be useful for clinical treatment and/or prevention of skeletal muscle insulin resistance.

In summary, my dissertation studies support the notion that "partitioning" excess fatty acid toward storage as TAG in muscle cells may limit substrate for the accumulation of more harmful lipid intermediates (e.g., DAG, ceramide) and thereby protect against lipid-induced insulin resistance. These studies also reaffirm the importance and efficacy of exercise in the prevention and/or treatment of insulin resistance. Specifically, I have shown that even a rather modest single session of exercise is sufficient to significantly improve insulin sensitivity into the next day in obese adults, and that low intensity exercise may provide metabolic benefit that is equal (or perhaps enhanced) compared with moderate-to-high intensity exercise resulting in the same total energy expenditure. Finally, I have found that the saturation state of fatty acids may not be an important factor for the regulation of muscle cell lipid accumulation/partitioning or insulin signaling, suggesting that the “amount” of available fatty acid may be a more important determinant of muscle cell lipid accumulation and insulin signaling than the “type” of available fatty acid.

APPENDICES

APPENDIX 1

Intramyocellular Lipid Analysis

The following is an outline of the procedure used to analyze intramyocellular lipid content in both STUDY 2 and STUDY 3 (Chapters 4 and 5, respectively). In brief, these procedures describe total lipid extraction, isolation of the appropriate lipid fractions, and transmethylation methods used to ultimately determine triacylglycerol and diacylglycerol content in muscle biopsy samples and cultured C2C12 muscle cells using gas chromatography – mass spectrometry. This method was developed in our laboratory by Alexander Hinko, Ph.D.

A-1. Total Lipid Extraction: Muscle Biopsies

A-2. Total Lipid Extraction: Myocytes

B. Isolation of the Total Lipid Extract

C. Solid Phase Extraction (SPE):

a) Cholesterol Ester (CE)
b) Triacylglycerol (TAG)
c) Diacylglycerol (DAG)
d) Monoacylglycerol (MAG)
e) Non-esterified Fatty Acids (NEFA)
f) Phospholipids (PL)

D. Transmethylation of the Fatty Acids of CE, TAG, DAG, MAG and PL by Alkaline Methanolysis, Yielding Fatty Acid Methyl Esters (FAMES)

E. Methylation of NEFA to Produce FAMES

F. Gas Chromatography - Mass Spectrometry (GCMS) of FAMES

G. Solutions and Reagents

A-1. Total Lipid Extraction: Muscle Biopsies

1) Prepare solutions[a-d] (see appendix) required for total lipid extraction, and make additions to microcentrifuge and extraction tubes (see steps 5 and 8) prior to homogenization.
2) For each sample, rinse an all-glass homogenizer with methanol and air-dry
3) Quickly weigh frozen muscle (~30 mg), then keep in dry ice until homogenization. Homogenize frozen muscle in 1.0 ml of ice-cold 3.5% NaCl[a] for ~30 seconds, with the pestle attached to a motor drive rotating at 800 rpm. Other components of the Western buffer are not used during homogenization due to possible lipid contaminants.
4) Add 0.25 ml cold homogenate to a microcentrifuge tube already containing 0.2 ml of an incomplete Western buffer[b] at room temperature; quickly cap tube, vortex and place in ice.

   Notes:

   a) Components of the buffer may precipitate if the microcentrifuge tube is kept in ice prior to adding sample.
   b) Adding 0.25 ml of 3.5% NaCl (homogenate) completes the Western buffer.
   c) Pipet homogenate immediately after vortexing, before lipids begin to float.
5) From this 0.45 ml volume, transfer 50 µl into an autoanalyzer vial with insert; store vial at -20°C for total protein assay (55.6% of original homogenate protein concentration).
6) Centrifuge the remaining 0.4 ml of diluted homogenate at 15,000 x g for 10 minutes at 4°C, then transfer the supernatant to a clean tube and store at -80°C (for Westerns).
7) Add 0.6 ml of undiluted homogenate to a 16x125 mm glass screw-cap tube containing:
   a. 0.6 ml of a lipid marker mix[c] in chloroform ($CHCl_3$)+10 µg/ml butylhydroxytoluene (BHT)[d]
   b. 1.65 ml additional $CHCl_3$ + BHT
   c. 4.5 ml methanol (MeOH)
   d. 1.2 ml 3.5% NaCl

Also prepare duplicate blanks: omit homogenate; add 0.6 ml 3.5% NaCl in its place.

$CHCl_3$ - MeOH - aqueous ratio is 1 : 2 : 0.8, the single-phase lipid extraction ratio of Bligh & Dyer[1] (i.e. 2.25 ml $CHCl_3$ - 4.5 ml MeOH - 1.8 ml aqueous saline).

If some undiluted homogenate can still be recovered from the homogenizer, store this at -80°C for total protein assay. The diluted homogenate (50 µl) can then be a "backup".

8) Close extraction tube with PTFE-lined cap, vortex ~20 seconds, then allow to stand 1 hour at room temperature (or overnight at 4°C). Proceed to section B.

A-2. Total Lipid Extraction: Myocytes

1) Remove media from the myocyte culture dish, then rinse cells extensively with phosphate-buffered saline[a].
2) Remove the final rinse, then place dish on ice. Add ~2.5 ml ice-cold 3.5% saline (if performing Westerns; see A-1) or a suitable buffer, then scrape cells while the dish rests on ice. Quickly pipet aliquots, e.g. 3 x 0.8 ml, into microcentrifuge tubes kept in ice. Proceed immediately to the next step, or store tubes at -80°C. Thaw cell lyzates in ice just prior to lipid extraction.
3) Add 0.6 ml of lyzate to a 16x125 mm glass screw-cap tube that already contains:
    a. 0.6 ml of 6-lipid marker mix in $CHCl_3$ + BHT
    b. 1.65 ml additional $CHCl_3$ + BHT
    c. 4.5 ml MeOH
    d. 1.2 ml 3.5% NaCl

    Notes: a) Re-freeze remaining lyzate for protein assay
    b) Prepare duplicate blanks (0.6 ml of 3.5% NaCl instead of lyzate).
4) Close extraction tube with PTFE-lined cap, vortex ~20 seconds, then allow to stand 1 hour at room temperature (or overnight at 4°C). Proceed to section B.

B. Isolation of the Total Lipid Extract

1) To separate lipids from polar molecules, first add 2.25 ml $CHCl_3$, cap and vortex 10 seconds, then add 2.25 ml H_2O (order of addition reportedly affects yield).
   Re-cap tube and vortex vigorously ~30 seconds.
   $CHCl_3$ - MeOH - aqueous ratio is now 2:2:1.8 (or 1:1:0.9), forming two phases.
2) Centrifuge at 2,000 rpm (700 x g), for 5 min at room temp in the J-6B centrifuge to sharpen the interface. Slow the rotor gradually (brake setting = 4).
   upper phase: methanol + H_2O + polar compounds, but virtually no lipid
   interface: de-lipidated protein precipitate; forms a compacted disk
   lower phase: chloroform + lipids
3) Using an aspirator flask and a 9" glass pipet as the suction tip, remove and discard nearly all of the upper phase. With practice, the protein interface disk can also be aspirated with little or no loss of lower phase. Residual upper phase forms a ring around exposed chloroform in the center of the surface.
4) Using a 9" glass pipet with manual pump, insert the tip through the central chloroform surface down to the tube bottom. Draw up the lower phase and transfer to a 13x100 mm glass screw-cap tube. Leave behind a droplet of lower phase to keep the tip isolated from residual protein precipitate or upper phase.
   Note: More than one draw is necessary. Watch the level in the pipet to avoid overfilling.
5) Cap the tube containing the total lipid extract, and store at -20^{o}C until lipid separation by SPE (Solid Phase Extraction) (section C).

C. Solid Phase Extraction (SPE):

a) Cholesterol Ester (CE)
b) Triacylglycerol (TAG)
c) Diacylglycerol (DAG)
d) Monoacylglycerol (MAG)
e) Non-esterified Fatty Acids (NEFA)
f) Phospholipids (PL)

1) Begin dry-down of total lipid extracts, using speed-vac without heat (1.5 - 2.0 hours), then prepare elution solvent mixtures[f-i].
2) Clean vacuum chamber ports of polar contaminants:
    a. attach an empty column or syringe barrel to the port inlet, and place a waste tube in the vacuum chamber beneath the outlet, and
    b. add ~3 ml MeOH, then draw this through using vacuum.
3) Equilibrate NH_2-SPE[e] cartridge columns with hexane:
    a. attach NH_2-SPE column to a MeOH-cleaned port, add ~2 ml hexane, and draw through until the hexane surface is just above the upper frit,
    b. add another 2 ml hexane, draw down halfway to the frit, then
    c. allow column to equilibrate in hexane at least 20 minutes.

    Note: Hexane also cleans the ports of non-polar contaminants.
4) Reconstitute each dried lipid extract in 100 µl $CHCl_3$; gently swirl to dissolve lipids.

    Note:-Unlike hexane, $CHCl_3$ is a solvent for lipids of widely varying polarity.
5) Draw down the hexane until surface is just above frit, then replace the waste tube with a 13 x 100 mm glass screw-cap tube (fraction tube #1).
6) Using a gel-loading pipet tip and pipetter adjusted to slightly greater than 100 µl, transfer as much of the lipid sample as possible directly into the hexane above the frit, then draw down the sample slowly until the surface just reaches the frit.

    Notes:
    a. Do not expose the top of the resin bed to air.
    b. Do not increase the $CHCl_3$ addition to the column above 100 µl, or the hexane equilibrium may become altered.
    c. Fraction tubes 2 through 7 are also 13 x 100 mm glass screw-cap.
7) Add 2.0 ml hexane (no splashing), draw down to the frit, then repeat with two additional 2.0 ml hexane volumes.

    Tube #1 now contains purified **CE** in ~6 ml collected hexane.
8) Insert tube #2 and elute with 2 x 2.0 ml of 15.5% ethyl acetate (EtOAc) in hexane[f].

    Tube #2 contains most or all of the **TAG** in 4.0 ml.

9) Insert tube #3 and elute with 2.0 ml of 15.5% EtOAc in hexane.

10) Insert tube #4 and elute with 2.0 ml of 15.5% EtOAc in hexane.

Note: Tubes 3 & 4 contain transition fractions: the tail of TAG and the leading edge of DAG, respectively. Collecting two small, successive volumes reduces the chance of TAG contaminating the bulk of DAG.

11) Insert tube #5 and elute with 2 x 2.0 ml volumes of 15.5% EtOAc in hexane.

Tube #5 contains most/all of the **DAG** in 4.0 ml.

12) Insert tube #6 and elute with 2 x 2.0 ml of 15.5% EtOAc in hexane.

This is an "insurance" tube for DAG if retention is unusually strong.

13) Insert tube #7 and elute with 3 x 2.0 ml of $CHCl_3$ - MeOH (23:1)[g].

Tube #7 contains **MAG** in 6.0 ml.

Note: The following tubes 8, 9 and 10 are 16 x 125 mm glass screw-cap.

14) Insert tube #8 and elute with 3 x 2.0 ml of diisopropyl ether - acetic acid (98:5)[h].

Tube #8 contains **NEFA** in 6.0 ml.

15) Insert tube #9; elute with 2x2.0 ml of $CHCl_3$-MeOH-3.6M ammonium acetate (30:60:8)[i].

Tube #9 contains **PL** in 4.0 ml.

Note:-Do not collect PL in a fraction larger than 4.0 ml, or the solvent volumes subsequently needed for PL extraction may collectively exceed the capacity of the fraction tube (see PL extraction below).

16) Insert tube #10 and elute with 2x2.0 ml of $CHCl_3$-MeOH-3.6M ammonium acetate.

This is the PL "insurance" fraction.

17) Dry down tubes 1, 2, 5,7 and 8 in the speed-vac w/o heat, then reconstitute 1, 2, 5 and 7 (not 8) with 200 µl toluene. Transfer 40 µl to a plain 13 x 100 mm tube for alkaline methanolysis. Re-cap remaining 160 µl and store at -20°C.

The primary purpose of the small aliquot is to determine the purity of each fraction.

If there is contamination indicated by the FAMES from internal standard markers, the remaining 160 µl can be dried, reconstituted in $CHCl_3$, and re-chromatographed on a clean NH_2-SPE cartridge using freshly made elution

solvents. The most likely cause of poor SPE is incorrectly prepared elution solvent mixtures. However, if fractions are well-purified, then results for abundant lipids, e.g. TAG, may be obtainable from the small aliquot. However, scarce lipids such as DAG require processing the remaining 160 µl, and the final FAME prep from this may have to be combined with that from the aliquot, dried, then reconstituted in 40 µl for GCMS.

Note: Do not attempt to dry down tube #9. The large amount of ammonium acetate in this fraction necessitates first extracting PL, to separate it from the ammonium acetate, prior to dry down.

18) Extraction of PL in tube #9:

Since the solvent composition (3 components) of this fraction is $CHCl_3$ = 1.225 ml, MeOH = 2.45 ml, aqeous ammonium acetate = 0.327 ml, then 1.225 ml $CHCl_3$ and 1.878 ml H_2O should be added to obtain $CHCl_3$ = 2.45 ml, MeOH = 2.45 ml, aqeous = 2.205 ml (1 : 1 : 0.9).

This forms two-phases, with [$CHCl_3$ + PL] as the lower phase.

19) Isolate and dry down the lower phase (same procedure as for total lipid extraction).

20) Reconstitute with 200 µl toluene and transfer 40 µl to a plain 13 x 100 mm glass tube for alkaline methanolysis. Re-cap the remaining 160 µl and store at -20°C.

D. Transmethylation of the Fatty Acids of CE, TAG, DAG, MAG and PL by Alkaline Methanolysis, Yielding Fatty Acid Methyl Esters (FAMES)

Alkaline methanolysis only affects ester bonds linking fatty acids to an alcohol, such as glycerol or cholesterol. Under conditions of basic pH, presence of methanol and absence of water, fatty acids are transmethylated, i.e. methanol replaces the original alcohol, to produce FAMES. This procedure neither methylates NEFA, nor transmethylates fatty acids incorporated by amide bonds in sphingolipids, which may be present in some of the SPE fractions.

1) To each aliquot of 1, 2, 5, 7 and 9, add 1.0 ml of 0.2 N sodium hydroxide (NaOH) in MeOH[j]. Cap tube and briefly vortex. Let tube stand 1 hour at room temperature.

2) Neutralize reaction by adding 0.9 ml of 1.0 M sodium acetate buffer, pH 4.75[k] and vortexing. Add 1.0 ml $CHCl_3$ and vortex. Obtain and dry down the lower phase.

   Lower phase = [$CHCl_3$ + lipid-derived FAMES]

3) Reconstitute in 40 µl heptane and transfer to poly-spring inserts in crimp-seal autoanalyzer vials. Tightly cap and store at -20°C until GCMS.

E. Methylation of NEFA to Produce FAMES

An iodomethane (methyl iodide) method is used to methylate NEFA to form FAMES. This method does not transmethylate, so FAMES obtained with iodomethane originate solely from NEFA.

1) Reconstitute tube #8 in 200 µl MeOH, transfer 40 µl to a 16 x 125 mm glass screw-cap tube and dry down. Re-cap the remaining 160 µl and store at -20°C.
2) To the aliquot, add 250 µl of 0.2 M phosphate/TBA buffer, pH 9.0[l], and also 250 µl of 10% iodomethane in dichloromethane[m]. Cap and vortex vigorously for 1 hour at room temperature using a platform shaker.
3) Add 3.0 ml hexane, re-cap tube and vortex vigorously ~30 seconds. Centrifuge in the J-6B as described in section B, to sharpen the interface between the large upper phase (organic solvents + FAMES) and the small, aqueous lower phase.
4) Using a glass pipet and pipet pump, transfer the upper phase to a 13 x 100 mm plain tube. Leave behind a small amount of upper phase to decrease the chance of removing some lower phase. Dry the upper phase in speed-vac without heat.
5) Reconstitute in 200 µl heptane, then transfer 40 µl to a polyspring insert in a crimp-seal vial. Cap and store at -20°C until GCMS. Cap the remaining 160 µl and also store at -20°C. NEFA will be scarce in muscle biopsies, but abundant in myocytes treated with exogenous fatty acids. After verifying purity, the remaining 160 µl will have to be processed for NEFA for biopsies, but not for fatty acid-treated myocytes. However, it may be necessary to process the larger volume for vehicle-treated myocyte controls.

F. Gas Chromatography - Mass Spectrometry (GCMS) of FAMES

Refer to the FAMES program in the Agilent instrument software which controls the GCMS.

G. Solutions and Reagents

a) 3.5% NaCl = 3.5 g NaCl / 100 ml ultrapure H_2O (Fisher cat# W6-4)
(NaCl, SigmaUltra = Sigma cat# S7653)

phosphate-buffered saline (PBS), pH 7.4: 10x solution (Sigma cat# PS493)
dilute to 1x with ultrapure H_2O

b) Western buffer: adapted from Dr. Minghua Li, personal communication

Note: adding 250 µl sample (in 3.5% saline) completes buffer

	per sample	e.g. for "10" samples (actual 8 samples)
	µl	µl
1) NP-40 (density = 1.065 g / ml)	4.5	45
2) 1.0 M tris-HCl, pH 7.4	10	100

Combine 1 and 2: first weigh 4.8 g NP-40 (4.5 ml) into a tared scintillation vial.
Add 10.0 ml of tris-HCl, cap and mix thoroughly end-over-end; let stand until
bubbles dissipate. Slowly take up and dispense 145 µl per 10 samples.

3) 0.5 M sodium fluoride (NaF)	20	200

4) 0.1 M EDTA, pH 8.0	11.2	112
5) 0.1 M sodium pyrophosphate (NaPP)	13	130
6) 1.0 M β-glycero-phosphate	10	100
7) 55.6% glycerol	90	900
8) 60 mM Perfabloc	5	50
9) complete mini	32.3	323
10) PIC 2	2	20
11) PIC 3	2	20
total volume of incomplete buffer	200	2,000
12) sample in 3.5% NaCl	250	
final volume	450	

preparation of buffer components:

1) NP-40, pure	use as shipped	
2) 1.0 M tris-HCl, pH 7.4	mw 157.6	1.0 M = 15.76 g / 100 ml H_2O
3) 0.5 M NaF	mw 41.99	0.5 M = 2.1 g / 100 ml H_2O
4) 0.1 M EDTA, pH 8.0	mw 416.2	0.1 M = 4.162 g / 100 ml H_2O

(tetrasodium dihydrate)		
5) 0.1 M NaPP	mw 446.06	0.1 M = 4.46 g / 100 ml H_2O
(tetrabasic decahydrate)		
6) 1.0 M beta-glycero-	mw 216.04	1.0 M = 21.6 g / 100 ml H_2O
phosphate, disodium		
7) 55.6% glycerol	density of pure glycerol = 1.264 g / ml 55.6% = 70.28 g glycerol + 44.4 ml H_2O	
8) 60 mM Perfabloc	mw 239.5	60 mM = 14.37 mg / ml H_2O
	(Roche; proprietary protease inhibitor)	
9) complete mini	one tablet dissolved in 1.8 ml H_2O; use 4-ml	
(EDTA-free)	glass vial with PTFE-lined cap (Roche; proprietary protease inhibitor)	
10) PIC 2	use as shipped; bring to room temp to melt DMSO (Sigma; phosphatase inhibitor mix)	
11) PIC 3	use as shipped; bring to room temp to melt DMSO (Sigma; phosphatase inhibitor mix)	

c) six odd C# lipid marker standards in CHCl3 + 10 µg / ml BHT (d):

1)	[C13:0] - Cholesteryl Tridecanoate (CE)
	Nu-Chek cat# CH-812 mw 583.08
	3.0 mM stock solution
	0.15 mM working solution (15.0 nmol / 100 µl)
example:	1.0 mM = 583.08 mg / liter = 58.308 mg / 100 ml
	3.0 mM stock = 174.924 mg / 100 ml
	3.0 mM = 129 mg weighed / 73.75 ml solvent
	0.15 mM = 2.0 ml stock +38.0 ml solvent
2)	[C15:0] - Dipentadecanoin (DAG)
	Nu-Chek cat# D-146 mw 540.88
	1.5 mM stock solution
	0.015 mM working solution (1.5 nmol / 100 µl)
example:	1.0 mM = 540.88 mg / liter = 54.088 mg / 100 ml
	1.5 mM stock = 81.132 mg / 100 ml
	1.5 mM = 130 mg weighed / 160.233 ml solvent
	0.015 mM = 0.5 ml stock + 49.5 ml solvent
3)	[C17:0] - Heptadecanoic Acid (NEFA)
	Nu-Chek cat# N-17-A mw 270.48
	3.0 mM stock solution
	0.15 mM working solution (15.0 nmol / 100 µl)
example:	1.0 mM = 270.48 mg / liter = 27.048 mg / 100 ml
	3.0 mM stock = 81.144 mg / 100 ml
	3.0 mM = 115 mg weighed / 141.723 ml solvent
	0.15 mM = 2.0 ml stock + 38.0 ml solvent

4)	[C19:0] - Monononadecanoin (MAG)	
	Nu-Chek cat# M-169	mw 372.51
	3.0 mM stock solution	
	0.03 mM working solution (3.0 nmol / 100 µl)	
example:	1.0 mM = 372.51 mg / liter = 37.251 mg / 100 ml	
	3.0 mM stock = 111.753 mg / 100 ml	
	3.0 mM = 125 mg weighed / 111.85 ml solvent	
	0.03 mM = 0.5 ml stock + 49.5 ml solvent	

5)	[C21:0]-Diheneicosanoyl-glycero-phosphocholine (PL)	
	Avanti cat# 167397	mw 874.32
	1.5 mM stock solution	
	0.75 mM working solution (75.0 nmol / 100 µl)	
example:	1.0 mM = 874.32 mg / liter = 87.432 mg / 100 ml	
	1.5 mM stock = 131.148 mg / 200 ml	
	1.5 mM = 104 mg weighed / 158.60 ml solvent	
	0.75 mM = 25.0 ml stock + 25.0 ml solvent	

6)	[C23:0] - Tritricosanoin (TAG)	
	Nu-Chek cat# T-185	mw 1101.88
	5.0 mM stock solution	
	1.0 mM working solution (100.0 nmol / 100 µl)	
example:	1.0 mM = 1101.88 mg / liter = 110.188 mg / 100 ml	
	5.0 mM stock = 550.94 mg / 100 ml	
	5.0 mM = 296 mg weighed / 53.726 ml solvent	
	1.0 mM = 10.0 ml stock + 40.0 ml solvent	

preparation of the 6-lipid marker mix:

combine 100 µl of each working solution for each sample or blank, e.g. for 10 samples, combine 1.0 ml of each working solution (6 ml total volume)

when 0.6 ml mixture is added to each sample, the following amounts are added:

[C13:0] CE	15.0 nmol
[C15:0] DAG	1.5 nmol
[C17:0] NEFA	15.0 nmol
[C19:0] MAG	3.0 nmol
[C21:0] PL	75.0 nmol
[C23:0] TAG	100.0 nmol

at the end of sample processing, if recovery is 100% for each lipid marker in a pure fraction, then the maximum amounts of the following fatty acid methyl esters (FAMES) derived from the markers would be:

C13:0ME	15.0 nmol
C15:0ME	3.0 nmol
C17:0ME	15.0 nmol
C19:0ME	3.0 nmol
C21:0ME	150.0 nmol
C23:0ME	300.0 nmol

d) BHT = 3,5-Di-tert-butyl-4-hydroxytoluene Supelco cat# 44-2377

(acts as an anti-oxidant; protects double bonds)

BHT stock solution (50x) = 500 µg / ml $CHCl_3$ (0.5 mg / ml)
20 ml stock solution + 980 ml $CHCl_3$ = 10 µg BHT / ml $CHCl_3$

e) aminopropyl SPE tubes, Supelco cat#57014, 500 mg bed, 3 ml volume

f) ethyl acetate - hexane (15.5 : 84.5, v/v) = 124 ml ethyl acetate + 676 ml hexane

g) $CHCl_3$ - MeOH (23 : 1, v/v) = 460 ml chloroform + 20 ml methanol

h) diisopropyl ether - acetic acid (98 : 5, v/v) =
392 ml diisopropyl ether + 20 ml acetic acid

i) $CHCl_3$ - MeOH - 3.6 M ammonium acetate (30 : 60 : 8, v/v/v) =
150 ml chloroform + 300 ml methanol + 40 ml 3.6 M ammonium acetate

Note: Ammonium acetate 7.5 M solution (Sigma cat# A2706).
3.6 M = 96 ml of 7.5 M + 104 ml H_2O
Store 7.5 M and 3.6 M solutions at 4°C.

j) 0.2N NaOH in methanol: NaOH mw = 40

1.0 N NaOH = 40 g / liter
0.2 N NaOH = 8 g / liter = 2 g / 0.25 liter = 2 g / 250 ml MeOH

Note: Some sodium carbonate (fine white particulate) forms when dissolving NaOH in MeOH. Allow the sodium carbonate to settle, then take aliquots from near the top.

k) 1.0 M sodium acetate buffer, pH 4.75:
glacial acetic acid mw = 60.05
sodium acetate trihydrate mw= 36.08

1) dissolve 68 g sodium acetate trihydrate in ~800 ml H_2O in a 1-liter beaker
2) add 30 g glacial acetic acid while stirring (this pure liquid is weighed); rinse the weigh boat into the beaker with several small volumes of H_2O
3) check the pH; it should be approximately 4.75
4) pour into a 1-liter graduated cylinder and rinse beaker into the cylinder
5) bring the volume to 1 liter with H_2O, then carefully drop in a stirring bar and mix until solution is uniform

l) 0.2 M phosphate/TBA buffer, pH 9.0:

potassium phosphate, dibasic mw = 174.18

tetrabutylammonium hydrogen sulfate (TBA) mw = 339.53

dissolve 34.8 g (0.2 mol) K_2HPO_4 and 16.98 g TBA (0.05 mol) in~800 ml H_2O, then adjust pH to 9.0 and bring volume to 1 liter as in (k)

m) 10% (v/v) iodomethane in dichloromethane =

20 ml iodomethane + 180 ml dichloromethane

APPENDIX 2

Cell Culture Methodology

The following is an outline of the basic cell culture methodology used to complete STUDY 3 (Chapter 5). Part A of this appendix serves as a summary of the step-by-step details of the procedures used to grow, plat, split/passage, freeze, and differentiate C2C12 myoblasts. These methods were developed by Sean Alec Newsom with guidance from Christopher L. Mendias, Ph.D. Part B of this appendix provides a brief outline of the protocol used to complete experiments for STUDY 3. This protocol was developed by Sean Alec Newsom.

Part A: Summary of basic cell culture methodology

Growing and Splitting Cells

Growth Media

1. From a volume of 500 mL (total volume) of DMEM High Glucose, remove 55 mL (11% of total volume).
2. Add 5 mL (1% of total volume) of Anti-Biotic, Anti-Mycotic (ABAM-aliquots stored in the -20 °C freezer).
3. Add 50 mL (10% of total volume) of Fetal Bovine Serum (FBS-aliquots stored in the -20 °C freezer).

Plating Cells

1. Remove growth media from the refrigerator and warm to 37 °C in water bath. After media has warmed, remove cryo vials of cells from liquid nitrogen and thaw in water bath at 37 °C.
2. After the cells are thawed, pipette each cryo vial of cells into 15 mL Falcon Tubes.

3. Dilute the cells with at least 10 mL of growth media per Falcon Tube. Rinse cryo vial with growth media.
4. Centrifuge for 5 min at 2000 rpm.
5. Add approximately 4 mL of growth media to each cell dish.
6. Remove the supernatant from each Falcon Tube and dispose in the waste container. Do not hit the cells!
7. Add approximately 4 mL (want a total volume of about 8 mL per dish) of growth media to the cells and mix by rinsing pipette multiple times to break up pellet at the bottom. Make sure that all cells are broken apart from pellet and no clusters of cells can be seen.
8. Add the cell/media mixture to each dish and very gently shake the dish (forward/backward and side/side, do not shake in a circle).
9. Label the plates with your name, date, and pass (this is pass 0 or P_0). Put the cell dishes in the incubator at 37 °C.

Splitting Cells

1. Take growth media and trypsin out of the refrigerator. Place the growth media in the water bath (at 37 °C) and allow the trypsin to warm to room temperature.
2. Remove cells from incubator and check under microscope to ensure that cells grew (confluence $\geq$ 70%).
3. Tip the plate to one side and remove the growth media (which also contains the dead cells). Be sure not to touch the bottom of the plate with the waste pipette.
4. Gently add 4 mL of DPBS to the side of the dish and rinse the cells. Remove DPBS similarly to removing growth media (step 3).
5. Add about 4 mL of Trypsin (.25%). Can be slightly more aggressive since this is used to detach the cells from the bottom of the dish.
6. Put cells with trypsin in incubator for approximately 5 min.
7. Remove the cell dishes from the incubator. Using the microscope, check to see that the cells are detached (should see the cells floating when the plate is moved slightly).

8. Add approximately 9 mL of growth media to the dish and rinse the dish multiple times. To rinse, tip the plate, pipette the media and thoroughly rinse the rest of the plate.
9. Move the media/cell mixture(s) into 15 mL Falcon Tube(s).
10. Centrifuge for 5 min at 2000 rpm.
11. Add approximately 4 mL of growth media to each plate.
12. Remove supernatant of trypsin/media from Falcon Tube(s) and put in waste container. Do not hit the cells!
13. Add approximately 4 mL (want a total volume of about 8 mL per dish) of growth media to each falcon tube (add enough for 4 mL per plate!) and mix by rinsing the pipette multiple times to break up the pellet at the bottom.
14. Take out total volume and evenly distribute between dishes (4mL per plate). When adding to the dish, lightly drop the volume (4mL) evenly throughout the plate.
15. Label the plates with your name, the date and the pass. Incubate the dishes at 37 °C.

Freezing Cells – *Using non-differentiated cells only*

1. Take growth media and trypsin out of the refrigerator. Place the growth media in the water bath (at 37 °C) and allow the trypsin to warm to room temperature.
2. Remove cells from incubator and check under microscope to ensure that cells grew (confluence ≥ 70 °C).
3. Tip the plate to one side and remove the growth media (which also contains the dead cells). Be sure not to touch the bottom of the plate with the waste pipette.
4. Gently add 4 mL of DPBS to the side of the dish and rinse the cells. Remove DPBS similarly to removing growth media (step 3).
5. Add about 4 mL of Trypsin (.25%). Can be slightly more aggressive since this is used to detach the cells from the bottom of the dish.
6. Put cells with trypsin in incubator for approximately 5 min.
7. While waiting for cells to incubate, make freezing media (10 % DMSO in growth media). Make enough for about 1 ½ mL per cryo vial of cells (for one cryo vial add 150µL DMSO to 1350µL growth media)

8. Remove the cell dishes from the incubator. Using the microscope, check to see that the cells are detached (floating).
9. Add approximately 9 mL of growth media and rinse the dish multiple times. To rinse, tip the plate, pipette the media and thoroughly rinse the rest of the plate.
10. Move the media/cell mixture(s) into 15 mL Falcon Tube(s).
11. Centrifuge for 5 min at 2000 rpm.
12. Remove supernatant of trypsin/media from Falcon Tube(s) and put in waste container. Do not hit the cells!
13. Add approximately 1 ½ mL of freezing media (10% DMSO in growth media). Mix by rinsing the pipette multiple times to break up the pellet at the bottom.
14. Move mixture into labeled cryo vial (label with date cells were frozen and name).
15. Put cryo vials of cells into Mr. Frosty for about 24 hours.
16. Move cells from Mr. Frosty to liquid nitrogen.

Differentiating Cells

Differentiating Media

1. From a volume of 500 mL (total volume) of DMEM High Glucose, remove 15 mL (3% of total volume).
2. Add 5 mL (1% of total volume) of Anti-Biotic, Anti-Mycotic (ABAM-aliquots stored in the -20 °C freezer).
3. Add 10 mL (2% of total volume) of Horse Serum (HS-aliquots stored in the -20 °C freezer).

Differentiating Cells

1. Take differentiating media out of the refrigerator and warm in water bath to 37 °C.
2. Remove cells from incubator and check under microscope to ensure that cells grew (confluence $\geq$ 70 °C).
3. Tip the plate to one side and remove the growth media (which also contains the dead cells). Be sure not to touch the bottom of the plate with the waste pipette.

4. If cells have confluence ≥ 90%, remove all growth media and rinse with DPBS. Otherwise, there can be a little bit of growth media in the dish but still remove as much of the growth media as possible.
5. Add differentiating media to each dish/well by tipping the plate and gently adding it to the side.
6. Label the dish with the date the cells were differentiated. Incubate the dishes at 37 °C.

Part B: Example experiment guide

All cells used in these experiments were P3 at point of differentiation!!!

Cell Growth

Media: HG-DMEM + 10% FBS + 1% AbAm

Plastic: 100mm Tissue Culture Treated Plates

Passage: 16-20h

For final passage prior to differentiation, move to 6-well plates:

Plastic: 6x35mm (6-well) Tissue Culture Treated Plates

- One 100mm plate at ~80% confluence suspended in 40mL
- Take 2mL/well (no pre-coating of plate)

Differentiating Cells

Media: HG-DMEM + 2% HS + 1% AbAm, changed every 48h

Plastic: 2x(6x35mm; 6-well) Tissue Culture Treated Plates

- Cells should be ready for differentiation media 20-24h after seeding
- Cells were differentiated for 4.5d before switching to low glucose media

TIMES LISTED BELOW REPRESENT HOURS PRIOR TO INSULIN TREATMENT

12h: Switch to LG-DMEM + 1% AbAm + 2% BSA ± FA

Solutions needed:

- LG-DMEM + 1% AbAm + 2% FAF-BSA + EtOH (CON)
- LG-DMEM + 1% AbAm + 2% FAF-BSA + 0.1mM FA

- LG-DMEM + 1% AbAm + 2% FAF-BSA + 0.2mM FA
- LG-DMEM + 1% AbAm + 2% FAF-BSA + 0.4mM FA
- LG-DMEM + 1% AbAm + 2% FAF-BSA + 0.8mM FA

Directions:

Final FA mixture concentrations desired: 0.1, 0.2, 0.4, 0.8mM

- Stock = 160mM (200x for 0.8mM), therefore serially dilute w/ EtOH for other concentrations
- Will need 10uL/2mL of media
- Be sure to make CON solution with 10uL EtOH/2mL media

0h: 15min incubation ± Insulin

Solutions needed:

- 200x Stock (20uM) is prepared

Directions:

Insulin concentration desired: 100nM

- Add 10uL/2mL media

Cell Lysis for Westerns

1) 2x Rinse w/ cold DPBS
2) Add 125µL cold lysis buffer and lightly rotate in fridge for ≥5min
3) Scrape plate thoroughly, place on ice when not being scraped
4) Repeatedly mix sample using P1000 to ensure homogenization
5) Place homogenate in 1.5mL eppendorf tube
6) Centrifuge at 15k-rpm for 10min at 4C using bench-top centrifuge
7) Collect supernatant, run protein assay and/or place in -80C