Effects of Subcutaneous Adipose Tissue on Metabolic Health in Obesity

by

Douglas Van Pelt

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Dissertation Committee:
       Professor Jeffrey F. Horowitz, Chair
       Professor Charles F. Burant
       Professor Gregory D. Cartee
       Associate Professor Christopher L. Mendias

ORCID ID: 0000-0001-9594-604X

dougvp@umich.edu

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Abstract

Effects of Subcutaneous Adipose Tissue on Metabolic Health in Obesity

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Douglas William Van Pelt Jr.

Chair: Jeffrey F. Horowitz

Adipose tissue metabolic function, structure, and overall adipose tissue "health" are key mediators of obesity-related cardio-metabolic abnormalities, including insulin resistance, which plays a central role in the development of many chronic diseases. Excessive fatty acid release from subcutaneous adipose tissue (SAT) is one of the most important factors underlying insulin resistance. Other major factors in SAT that are linked to abnormalities in metabolic health include adipose tissue inflammation, extracellular matrix composition and structure, adipogenesis, and angiogenesis. However, despite some recent advances, understanding the role of SAT on metabolic health in human obesity is rather limited. In addition, although exercise is often-prescribed in attempt to improve cardio-metabolic health, it remains unclear whether exercise can impart adaptations in adipose tissue that contribute to improvements in whole-body metabolic health in obesity. The overall objectives of this dissertation were to assess how subcutaneous adipose tissue may contribute to whole-body metabolic outcomes in obesity, and whether exercise can modify adipose tissue in a manner that improves markers of metabolic health. In STUDY #1, insulin sensitivity (frequently sampled intravenous tolerance test; FSIVGTT) and systemic fatty acid rate of appearance in plasma (FA Ra; stable isotope dilution methods) were measured in 21 obese adults (BMI = 34 ± 1 kg/m^2; age = 30 ± 1 yrs), and FA Ra was found to be an important determinant of insulin resistance (R^2=0.21; P=0.04). In addition, skeletal muscle samples collected from subjects who were insulin resistant had higher markers of skeletal muscle inflammation known to inhibit insulin signaling (e.g., phosphorylated c-Jun N-terminal kinase) compared with the more insulin sensitive subjects (P<0.05). Given the importance of basal fatty acid mobilization rates on insulin resistance (as reported in STUDY #1), STUDY #2 aimed to identify factors in SAT that may underlie low vs. high rates of fatty

acid mobilization in obese adults. FA Ra (stable isotope dilution methods) and insulin sensitivity (hyperinsulinemic-euglycemic clamp) were measured in 30 obese adults (BMI = 38±1 kg/m^2; age = 30±2 yrs). Confirming results from STUDY #1, insulin sensitivity was inversely proportional to FA Ra (R^2=0.50; P<0.001). In addition, SAT from subjects with low FA Ra had significantly lower (P<0.05) markers of lipase activation and higher abundance (P<0.05) of glycerol-3-phosphate acyltransferase (GPAT), which is a primary enzyme regulating fatty acid esterification. Microarray and pathway analysis also indicated lower fibrosis and lower SAPK/JNK pathway activation in obese adults with low FA Ra compared to those with high FA Ra (P<0.05). Finally, in STUDY #3, overweight-to-obese adults who exercise regularly (ACTIVE: BMI 29±1 kg/m^2; age = 27±1 yr; n=8) or were sedentary (SED: BMI 27±1 kg/m^2; age = 27±2 yr; n=12) were recruited to examine the effects of exercise (chronic and acute) on adipose tissue. In both groups, acute exercise increased SAT mRNA expression of *VEGFA*, an important regulator of angiogenic processes. In line with this finding, SAT from ACTIVE subjects had a higher mRNA expression of the endothelial cell marker, *CD31,* compared with SED, suggesting regular exposure to exercise may increase SAT capillarization. Together, findings from this dissertation provide novel insights into how alterations in SAT structure and metabolic function can greatly impact insulin resistance, and whole-body metabolic health. These data may help lead to development of treatments (lifestyle or pharmaceutical) to target and treat important mediators of insulin resistance and other obesity-related cardio-metabolic diseases.

CHAPTER 1

Statement of the Problem

Approximately one-third of adults in the United States are classified as obese (1). The high prevalence of obesity is alarming since obesity is a leading risk factor for the development of many health complications, such as Type 2 Diabetes Mellitus (T2DM) and cardiovascular disease (CVD), causing obese persons to have increased mortality compared with lean, healthy individuals (2-5). Furthermore, obesity-related diseases and other cardio-metabolic health complications have placed an enormous financial burden on the U.S. healthcare system by accounting for an estimated 5% to 10% of direct medical costs within the US healthcare system (6, 7). Research focusing on the causes and consequences of obesity-related diseases, and optimizing interventions targeting key factors underlying these diseases may alleviate much of the negative health outcomes of obesity.

No single metabolic abnormality is responsible for the elevated cardio-metabolic disease risk in obesity, but the very high prevalence of insulin resistance in obesity is of particular interest (8, 9). Insulin resistance is defined as a subnormal response to a physiological dose of insulin, and can result in impaired skeletal muscle glucose uptake, elevated hepatic glucose production, and reduced inhibition of adipose tissue lipolysis (10, 11). Epidemiological as well as longitudinal studies indicate that insulin resistance is a strong predictor of CVD and mortality (12-14). Conversely, a relatively small subset of obese adults who are not insulin resistant appear to be protected from developing many chronic cardio-metabolic health complications. Data from the National Health and Nutrition Examination Survey (NHANES) indicates that as many as one-third of U.S. obese adults remain metabolically healthy (e.g. not insulin resistant) (15) , and these "insulin sensitive" obese adults often have mortality rates equivalent to lean, healthy individuals (15-17). It is not known why some obese adults remain insulin sensitive while most become insulin resistant, despite being very similar in terms of the magnitude and distribution of their body fat. A greater understanding of the physiological mechanisms that "protect" some obese individuals from insulin resistance may provide valuable insight into potential treatments and interventions that may improve many of the negative health outcomes that plague most obese adults.

The overall health and function of adipose tissue is now recognized an important factor dictating obesity-related metabolic outcomes. Over the past few decades our knowledge about the metabolic function of adipose tissue has expanded from being a rather benign storage site for excess energy to the current understanding that it serves important endocrine and paracrine functions by producing and secreting many different peptides (18). The secretion of adipocyte derived factors has been implicated as an important component of obesity-induced insulin resistance, however, it is important to acknowledge that the primary role of adipose tissue is to store energy, and complications associated with the regulation of lipid storage within adipose tissue can have profound metabolic consequences (18-20). Various elements of adipose tissue structure and function have been postulated to be important mediators of "adipose tissue health" and lipid storage capacity, including cell size (21), adipogenesis (22), angiogenesis(23), extracellular matrix dynamics (24, 25), and inflammation (26, 27). The coordination and response of these adipose tissue biological processes may determine adipose tissue health and function and thus, overall metabolic health in obesity

A major premise of my dissertation is that factors effecting fatty acid flux (such as factors mentioned above) in adipose tissue can play an important role in dictating whole body metabolic health (28-30). In agreement, a major factor underlying insulin resistance in obesity is excessive fatty acid mobilization from adipose tissue (31-34). Lowering fatty acid mobilization in obese adults through pharmacological treatments or weight loss, decreases insulin resistance (35-37). Alternatively, artificially elevating fatty acid mobilization in lean, healthy volunteers (via lipid infusion) profoundly impairs insulin-mediated glucose uptake (38, 39). High rates of fatty acid mobilization into the systemic circulation (referred to here as "fatty acid availability") and a resultant high rate of fatty acid uptake into insulin-sensitive tissues, like skeletal muscle, can directly and indirectly interfere with the insulin signaling cascade (40, 41). Although fatty acid availability is typically very high in obesity (32), there is still considerable variability in fatty acid mobilization from adipose tissue, even among a seemingly homogenous population of obese adults (42). However, the role that this variability in fatty acid availability has on determining the degree of insulin resistance in obese adults is not clear. Moreover, it is not known how some obese adults can maintain a relatively low rate of systemic fatty acid mobilization, while it is

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rather high in most obese adults. Determining whether variations of fatty acid availability dictate skeletal muscle inflammation and insulin resistance – and expanding our understanding about factors underlying the variability in fatty acid mobilization from adipose tissue will provide important insights into potential targets for pharmacological and/or lifestyle interventions. In turn, pharmacological and/or clinical interventions targeting these mechanisms within adipose tissue may help prevent or treat insulin resistance in obesity.

Finally, exercise is often prescribed in attempt to improve metabolic health in obesity, but the mechanisms underlying the insulin sensitizing effects of exercise are still not completely understood. Most of the recognized mechanisms for the improvement in insulin sensitivity after exercise have been isolated to skeletal muscle. Indeed, reductions in muscle glycogen content and alterations in fatty acid partitioning within skeletal muscle are known mechanisms that enhance insulin action after each session of exercise (43-45). Surprisingly, very little is known about the effects of exercise on adipose tissue metabolism and lipid storage. Moreover, it is unclear whether exercise can impact factors regulating inflammation, angiogenesis, extracellular matrix structure and composition, or adipogenesis in the adipose tissue. Available evidence in rodent models suggest exercise may confer beneficial metabolic adaptations by reducing adipose tissue inflammation, fibrosis, as well as fat cell size (46-48), but evidence in human adipose tissue is lacking. Because exercise training is often found not to enhance insulin sensitivity beyond a day or so after the most recent exercise session - it might be assumed that any exercise-induced adaptations in adipose tissue may not be important for long-term improvements in metabolic health in humans. However, exercise-mediated effects on adipose tissue structure and metabolic function may only be apparent during periods of nutrient surplus. The ability to expand adipose tissue during periods of weight gain without excess fibrosis and inflammation may confer long-term protection against insulin resistance. The overall hypothesis for the third study of my dissertation is that each session of exercise "primes" adipose tissue for more effective energy storage through changes in adipose tissue inflammation, extracellular matrix, angiogenic capacity, and/or adipogenic capacity. The key first step in advancing our understanding about the role of exercise on adipose tissue structure and metabolic function is to characterize the effects of each exercise session on pathways regulating these processes.

The studies of my dissertation were designed to address the following specific aims:

1. Determine if the degree of fatty acid mobilization dictates the observed variability in insulin resistance in obesity.
2. Investigate whether obese adults with low rates of fatty acid mobilization exhibit differences in adipose tissue structure and metabolic function compared to obese adults with elevated fatty acid mobilization.
3. Characterize the effects of a single session of aerobic exercise on factors regulating adipose tissue structure, metabolic function, and inflammation.

The overall goal of my dissertation was to investigate the physiological mechanisms within adipose tissue that may protect obese individuals from developing insulin resistance. The information gained from my dissertation studies expands our understanding of adipose tissue physiology in obese adults and highlights its important role for determining metabolic health in obesity.

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CHAPTER 2

Review of Literature

Approximately one-third of adults in the United States are considered obese (1). This has become a major public health concern, as obesity is associated with the development of metabolic, cardiovascular, and other diseases such as Type 2 Diabetes Mellitus (T2DM), cardiovascular disease (CVD), stroke, and cancer (2-4). In addition to the tremendous impact these diseases have on the lives of the patients and their families, the high rates of obesity-induced disease have placed an enormous economic burden on the healthcare system. Increasing body mass index (BMI) is associated with higher direct healthcare costs, with obesity-related medical expenses now accounting for 5% to 10% of U.S. healthcare spending (5, 6). Furthermore, there are indirect costs of obesity due to the reduced workplace productivity and early mortality that impedes workplace function and income (5, 7). The development of excess fat mass in obesity is central to chronic metabolic health complications and most obesity-related diseases can be prevented or even reversed with weight loss. However, rates of successful weight loss in obese adults remain very low (8); thus, research is needed to find alternative therapeutic and preventative options for obesity-related diseases.

Obesity is associated with insulin resistance, which is defined as a subnormal response to a physiological dose of insulin (9, 10). Insulin plays a critical role in the regulation of blood glucose, as well as many other vital metabolic processes (e.g., lipolytic rate, protein synthesis, etc.), and chronic impairments in the ability to respond effectively to insulin can lead to the development of metabolic and cardiovascular disease (11-13). For example, type 2 diabetes is defined as a chronic elevation of blood glucose, caused largely by skeletal muscle and hepatic insulin resistance. Certainly there are other obesity-related abnormalities that contribute to the development of chronic diseases in obesity, but it is clear that strategies aimed at attenuating insulin resistance can play a key role in preserving metabolic health.

Although the vast majority obese adults are insulin resistant with high risk for chronic disease, not all obese adults develop insulin resistance or exhibit metabolic dysfunction. It has been reported that as many as one-third of obese adults can be classified as "metabolically normal" - with insulin sensitivity similar to lean, healthy adults (14-16). Furthermore, obese adults that remain insulin sensitive appear to have mortality rates equivalent to lean, healthy

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adults (17, 18). It should not be overlooked that most obese adults do indeed develop insulin

resistance and metabolic disease, but obese adults who remain insulin sensitive have become a

well-recognized group for investigating factors that may "protect" against obesity-related insulin

resistance. In this review, I will focus my discussion on the current knowledge regarding the role

that adipose tissue structure, function and metabolism may play in the development of obesity-

related insulin resistance and potential adipose tissue characteristics that may "protect" some

obese adults from developing insulin resistance. Specifically, this review will describe the

effects of fatty acid availability as a key mediator of obesity-related insulin resistance and

summarize factors in adipose tissue that may be important for dictating the storage and release of

fatty acids, including adipose tissue components related to inflammation, immune cell function,

and properties of the extracellular matrix. The review will also discuss the current knowledge of

the acute and chronic effects of exercise on adipose tissue lipid metabolism, structure, and

inflammatory processes.

FACTORS REGULATING INSULIN RESISTANCE IN OBESITY

Abnormalities in many different tissues contribute to the development of metabolic

complications in obesity. The liver plays a vital role in blood glucose control through storage

and production of glucose, and dysregulation of hepatic glucose metabolism can lead to

hyperglycemia and impaired overall metabolic health (19, 20). As such, hepatic insulin

resistance is a common feature in the pathology of Type 2 Diabetics, which exacerbates their

chronic hyperglycemia. However, while the liver is certainly important in dictating overall

metabolic health, it has been demonstrated that skeletal muscle is the primary site of insulin

mediated glucose disposal (21). Therefore, impaired insulin action in skeletal muscle is

responsible for most of the systemic insulin resistance commonly found in obesity. The control

of blood glucose uptake in skeletal muscle is regulated by a well-known complex signaling

pathway initiated when insulin binds to its receptor, and ultimately results in the translocation of

the glucose transporter, GLUT 4 to the membrane of the cell (See Figure 2-1A). Binding of

insulin to its receptor leads to tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1)

and subsequent downstream signaling and interaction with phosphatidylinositol 3-kinase (PI3K)

(22-24). This leads to signaling through PKB/Akt and eventually phosphorylates AS160 (Akt

substrate of 160kD), inhibiting the Rab-GTPase-activating protein (Rab-GAP) and releases GLUT4 from its intracellular pool for translocation to the myocyte membrane (25, 26). Disruption of any one or more steps of this signaling pathway can lead to insulin resistance as well as other metabolic abnormalities (27-29).

The insulin resistance that occurs in skeletal muscle and liver is due in part to the excessive systemic fatty acid availability commonly found in obesity. To demonstrate the potent effects of elevated fatty acid availability on insulin resistance, several studies report profound insulin resistance in healthy, lean subjects after just a few hours of a lipid and heparin infusion to mimic the high rates of fatty acid availability found in obesity (30, 31). Conversely, drugs that lower systemic fatty acid availability in obese adults have been found to reverse insulin resistance (32, 33). Much of the excessive fatty acid availability that is common in obesity is due to inadequately sequestering and storing excess fatty acids as triglycerides within subcutaneous adipose tissue (34). This phenomenon is demonstrated in patients with lipodystrophy, which is a genetic disorder characterized by a lack of subcutaneous adipose tissue formation, and an extraordinarily low capacity to store fatty acids as triglycerides (TG) (35). Humans with lipodystrophy appear very lean, yet they develop extreme insulin resistance in conjunction with elevated plasma fatty acids and lipid deposition in the skeletal muscle and liver (36). Lipodystrophic mice develop similar metabolic complications, however, these complications (e.g. insulin resistance, ectopic lipid deposition, etc.) have been found to be reversed with transplantation of functioning subcutaneous adipose tissue (37). Although it may seem counter-intuitive, the ability to **_expand_** subcutaneous adipose tissue to accommodate nutrient oversupply during periods of weight gain may actually help prevent insulin resistance by enhancing the capacity to sequester fatty acids-thereby preventing excess fatty acid release and ectopic lipid deposition in insulin sensitive tissues (34).

High fatty acid availability has been linked to excess skeletal muscle uptake of fatty acids and the accumulation of lipid intermediates within the skeletal muscle, such as diacylglycerol (DAG), ceramides, and long-chain fatty acyl-CoAs (29, 38). DAG, as well as ceramides, and long-chain fatty Acyl-CoAs can impede insulin signaling by interacting with serine kinases protein kinase C-θ, c-Jun N-terminal kinase (JNK), Inhibitor of kB (IkB) kinase (IKK), and nuclear factor κB (NFκB), which can directly and indirectly interfere with the insulin signaling

cascade (29, 39-41) (See Figure 2-1B). The serine kinases PKC- θ, JNK and IKK are related to insulin resistance in obesity and mechanistic studies have revealed their roles in directly inhibiting insulin signaling through phosphorylation of serine residues (Ser307, Ser1101) on IRS-1 (42-44), which inhibits the ability to become phosphorylated at the necessary tyrosine sites required for downstream signaling(27, 28). Moreover, JNK and IKK are involved in many cell signaling pathways and increased phosphorylation and activity of these proteins may decrease the inhibition of NFκB and increase the transcription of inflammatory cytokines (e.g. TNFα, IL-6) that may also interfere with insulin action (45, 46). Ceramides can also inhibit insulin action through interaction with protein phosphatase 2A (PP2A) and protein kinase C-ζ (PKC- ζ), which prevent the stimulation of PKB/Akt- a serine/threonine kinase that is a crucial step in the translation of insulin binding and GLUT 4 translocation to the membrane (47, 48). Interestingly, although intramyocellular triacylglycerol (IMTG) concentration is also commonly found to be elevated in obese insulin resistant adults (49), the accumulation of IMTG does seem to mediate the level of insulin resistance (50). This is demonstrated by the "athlete's paradox", in which athlete's with good metabolic health exhibit elevated levels of IMTG similar to insulin resistant obese adults (51). In all, much of the insulin resistance observed in obesity is a consequence of the excessive fatty acid availability and ectopic lipid deposition that disrupts insulin signaling in peripheral tissues.
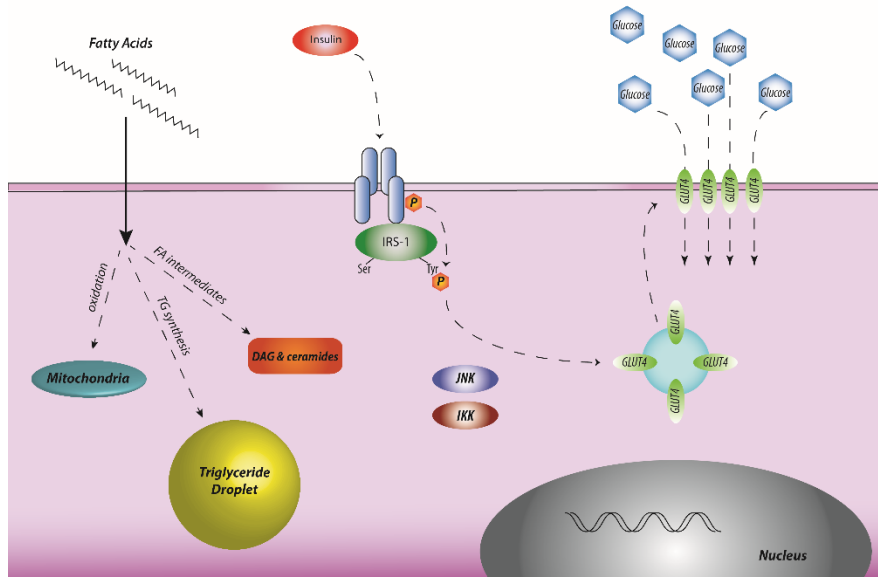
Figure 2-1A: Lipid metabolic fates and insulin signaling. As fatty acids enter the skeletal muscle, most are either oxidized for production of energy in the mitochondria, or synthesized into triacylcerol and/or fatty acid intermediates (i.e. diacylglycerol (DAG), ceramides). Normal insulin signaling involves binding of insulin to its receptor and phosphorylation of insulin receptor substrate (IRS) on tyrosine residues. Downstream signaling events initiate the translocation of GLUT4 to the cell surface membrane and allow for increased glucose uptake.
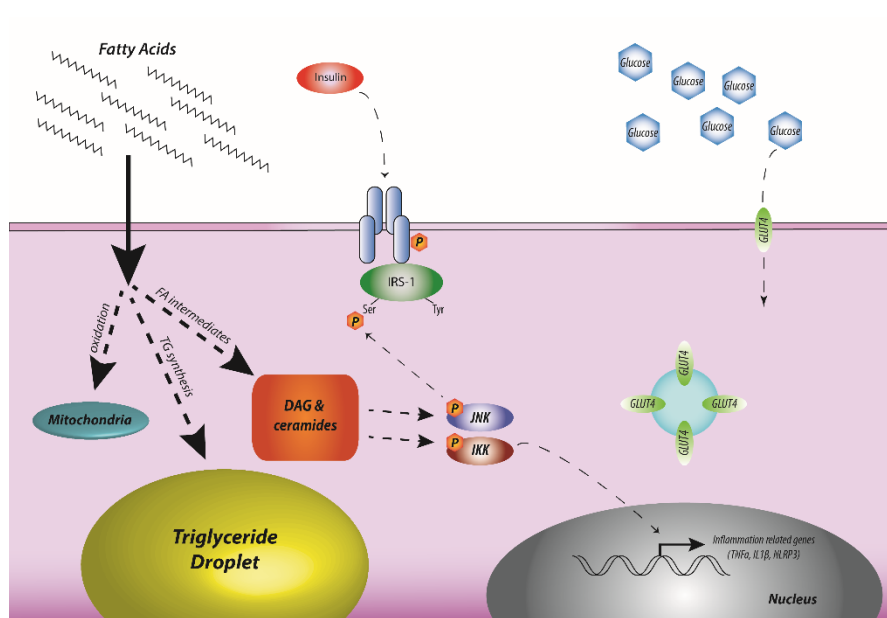
Figure 2-1B: Lipid induced insulin resistance in obesity. Excess fatty acid availability and uptake into skeletal muscle in during obesity increases the abundance of intramuscular triglyceride and fatty acid intermediates. Elevated DAG and ceramides increase the activity of serine kinases (i.e. JNK and IKK) that interfere with normal insulin signaling and reduce GLUT4 translocation to the cell membrane.

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SUBCUTANEOUS ADIPOSE TISSUE IS A KEY MEDIATOR OF FATTY ACID
AVAILABILITY AND INSULIN RESISTANCE IN OBESITY

The ectopic lipid deposition that interferes with insulin signaling is a consequence of the excess release of fatty acids from adipose tissue depots throughout the body. The accumulation of visceral adipose tissue is often linked with the severity of cardio-metabolic disease risk (52), however, the excess accumulation of visceral fat is most likely a result of the subcutaneous adipose tissue's inability to properly store the excess nutrients. Nielsen et al. demonstrated that approximately 90% of all fatty acids in the systemic circulation are derived from subcutaneous adipose tissue, with nearly 70% of these fatty acids coming from abdominal subcutaneous adipose tissue (53). Moreover, despite the anatomical proximity of visceral adipose tissue with the liver, the vast majority of fatty acids in the hepatic circulation are also derived from abdominal subcutaneous adipose tissue (53). These data demonstrate the importance of subcutaneous adipose tissue in the control of fatty acid mobilization and subsequently, the control of lipid deposition and insulin sensitivity in skeletal muscle. Human studies using deuterated water (^2H_2O) to track long-term turnover and storage of TG have found that TG synthesis and storage is impaired in the subcutaneous adipose tissue of insulin-resistant obese individuals compared with obese adults who maintain normal insulin sensitivity (54). Obviously, the regulation of lipolysis and esterification will modulate the degree of fatty acid release from adipose tissue and determine TG storage. There are several other factors within the subcutaneous adipose tissue that contribute to the capacity for fatty acid uptake and storage such as expansion of adipose tissue, fatty acid transport, and adipose tissue blood flow. Additionally, obese adults who remain healthy exhibit adipose tissue characteristics indicative of improved lipid storage and handling, such as increased adipocyte number, lower inflammation, and lower ectopic lipid deposition (14, 55, 56).

Lipolysis, esterification, and fatty acid transport

Regulation of triglyceride storage and release from subcutaneous adipose tissue is multifaceted, involving coordinated activities of factors activating triglyceride hydrolysis, esterification, and fatty acid trafficking. Lipolysis involves the hydrolysis of TG fatty acid-glycerol ester bonds that can ultimately lead to release of non-esterified fatty acids (NEFA) from

the lipid droplet and into the bloodstream for delivery as an energy substrate to peripheral tissues. It is a process activated through multiple mechanisms including catecholamines and other hormones and peptides (e.g. naturetic peptides, growth hormone, and cytokines), and results in the sequential hydrolysis of three fatty acid-glycerol ester bonds (57). In contrast, insulin potently suppresses lipolysis by inhibiting the activity of essential lipases (58, 59). Adipose triglyceride lipase (ATGL) has been identified as a major lipase necessary for the removal of the first fatty acid-glycerol ester bond (60), while hormone sensitive lipase (HSL) and monoglyceride lipase (MGL) predominantly hydrolyze diacylglycerol (DAG) and monoglycerol (MG), respectively (61, 62). Perturbations in the expression, abundance, and activation of these lipases controlling lipolysis may dictate the variability in fatty-acid mobilization and as a result, may impact whole-body insulin resistance. For example, mice lacking ATGL develop larger fat mass compared to wild-type (WT) mice along with increased TG deposition in skeletal muscle, however, the increased storage of fatty acids as neutral lipids and the low rate of fatty acid mobilization improved insulin sensitivity in these mice (63). Similarly, Girousse et al. demonstrated that partial inhibition of lipolysis through pharmacological or genetic manipulation of HSL, without alterations in fat mass, resulted in lower fatty acid mobilization and improved insulin sensitivity compared to WT mice in diet induced obesity (64). Lower markers of lipolysis have also been reported to correlate with reduced indices of insulin resistance, independent of fat mass, in obese humans (64).

Regulation of the rate of triacylglycerol synthesis (i.e., esterification) in adipose tissue can also contribute to the magnitude of systemic fatty acid mobilization. Two main enzymes, mitochondrial glycerol-3-phosphate (mGPAT) and diacylglycerol acyltransferase (DGAT), catalyze the first and final steps in triglyceride synthesis by adding activated fatty acid moieties (i.e., fatty acylCoA) to the glycerol-3-phosphate backbone. In vitro work has also demonstrated an increased TG formation by overexpressing GPAT while mice lacking GPAT are unable to accumulate fat mass (65, 66) However, there is currently little evidence regarding the roles of these two key TG synthesis enzymes in adipose tissue of obese adults that remain insulin sensitive. Lastly, fatty acid availability may also be affected by the adipocytes ability to take up fatty acids from the systemic circulation. CD36 is a transmembrane protein that is highly expressed in adipose tissue, and other tissues, that helps regulate fatty acid uptake, incorporation of fatty acids into TG, and lipolysis (67, 68). Adipose tissue from CD36-null mice exhibit a

significant reduction in fatty acid uptake and synthesis of TG and in humans, CD36 protein abundance is positively correlated to fatty acid storage rates in subcutaneous adipose tissue (69, 70). In addition, obese adults that remain insulin sensitive have a higher abundance of CD36 in subcutaneous adipose tissue in combination with lower intrahepatic lipid content compared to insulin resistant obese adults (71). Together, it can be postulated that rates of lipolysis, esterification, and fatty acid transport may all play an important role in determining the rate of fatty acid mobilization from adipose tissue and subsequently, mediating the degree of skeletal muscle insulin resistance.

Adipogenesis

Adipogenesis is the process of creating new adipocytes and it may also mediate the release and storage of fatty acids in obesity. The expansion of adipose tissue during the development of obesity can be accomplished by increasing the size of the existing cells (hypertrophy) and by creation of new adipocytes from the resident progenitor cells (hyperplasia). Weight gain studies have demonstrated that initial fat mass expansion may occur primarily through hypertrophy of existing adipocytes until a "threshold" in adipocyte hypertrophy and mass are reached; hyperplasia then drives further expansion of fat mass (72). More recent data also suggests that the method of adipose tissue expansion may differ amongst the different regions of the body with lower body subcutaneous adipose tissue having a much greater expansion through hyperplasia versus the abdominal region (73). At first glance, the manner in which the adipose tissue expands its mass may seem trivial since increased fat mass (obesity and overweight) is highly related to insulin resistance. However, hypertrophied or enlarged adipocytes are associated with decreased adipogenic capacity, adipose tissue metabolic dysfunction, and insulin resistance (74-78). In contrast, obese individuals that remain insulin sensitive have been found to have more, smaller fat cells per same volume of fat mass (79). A fat mass with more hypertrophied fat cells may lead to an overall increase in fatty acid mobilization and peripheral insulin resistance. Separation of small and large adipocytes from the same subjects demonstrated an increased lipolytic rate of large adipocytes when stimulated with catecholamines or inhibited with insulin compared with the smaller adipocytes (80).

The metabolic status of the adipose tissue is highly related to the inflammatory response, and hypertrophied adipocytes have a higher secretion of inflammatory cytokines and immune cell chemoattractants, such as MCP-1, that may disrupt insulin signaling (81). Excess hypertrophy of the adipocytes may trigger the increased inflammatory environment seen in insulin resistant obesity, and most observed pro-inflammatory adipose tissue macrophages (ATMs) in human adipose tissue are observed surrounding hypertrophied adipocytes as "crown-like structures" (82). The ability to create new adipocytes that match nutrient excess through adipogenesis may protect against excess lipid mobilization by lessening basal or stimulated lipolysis through lessened inflammatory response; although, the lessened inflammatory response may be secondary to the increased TG storage capacity if the adipose tissue.

Adipogenesis is a complex process that is regulated at multiple levels by numerous transcription factors, along with multiple extracellular,and intracellular signaling cascades (83). The adipogenic process is classically divided into phases: Determination and terminal differentiation. As seen in Figure 2-2, determination involves the commitment of pluripotent mesenchymal stem cells (MSCs) to the adipocyte lineage and results in the stem cell becoming a "pre-adipocyte". Once an MSC is committed to becoming a pre-adipocyte, it unable to differentiate into other cells types. Terminal differentiation occurs when the pre-adipocyte begins to develop characteristics of a mature adipocyte- such as the ability to store lipids in TG droplets, insulin sensitivity, and the ability to secrete adipocyte specific proteins. The "master regulator" of adipocyte differentiation is peroxisome proliferator-activated receptor-γ (PPARγ), which is both necessary and sufficient for adipogenesis (84, 85). In humans, loss of function variants of PPARγ reduce adipogenic capacity and increase the risk for developing diabetes (86). In fact, there has yet to be a pro-adipogenic factor discovered that does not function by at least activating PPARγ expression to a small degree. Another major group of regulators in the adipogenic processes are the CCAAT-enhancer binding proteins (C/EBPs). This family of proteins directly regulates genes involved in lipid metabolism and similar to PPAR, it is considered an important factor in the development of adipose tissue. Multiple knockout models of various members of the protein family (e.g. C/EBPα, C/EBPβ, and C/EBPδ) have demonstrated the importance of C/EBPs in adipose tissue development (87, 88), however C/EBPs are not able to rescue or promote adipogensis in the absence of PPARγ (89). Most extracellular signaling and environmental cues that seem important for adipogenic processes in

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vivo converge on the upregulation of PPARγ and C/EBPα expression and activity. For example, insulin, binding of Wnt proteins to β-catenin-dependent and independent pathways, transforming growth factor- β (TGFβ), fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs) are all implicated in the control of adipogensis through PPARγ and C/EBP pathways. Insulin, FGF, and BMPs, promote formation of adipocytes while Wnt and TGFβ signaling can inhibit adipogenesis (90) (83, 91-93). This simplistic overview of adipogenic processes is a starting point for investigating mechanisms that may allow some obese adults to expand adipose tissue preferentially through hyperplasia. Recent work in mice using adipocyte specific, inducible fluorescent reporters have shed some light on the creation of new adipocytes during weight gain; however, it remains unknown if the response in various fat pads in mice are translatable to the response of human adipose tissue in vivo (94). If it does translate to human adipose tissue, the creation of more cells to store excess fat energy will create smaller fat cells and reduce inflammatory stimuli, which improve adipose tissue and whole-body insulin resistance.
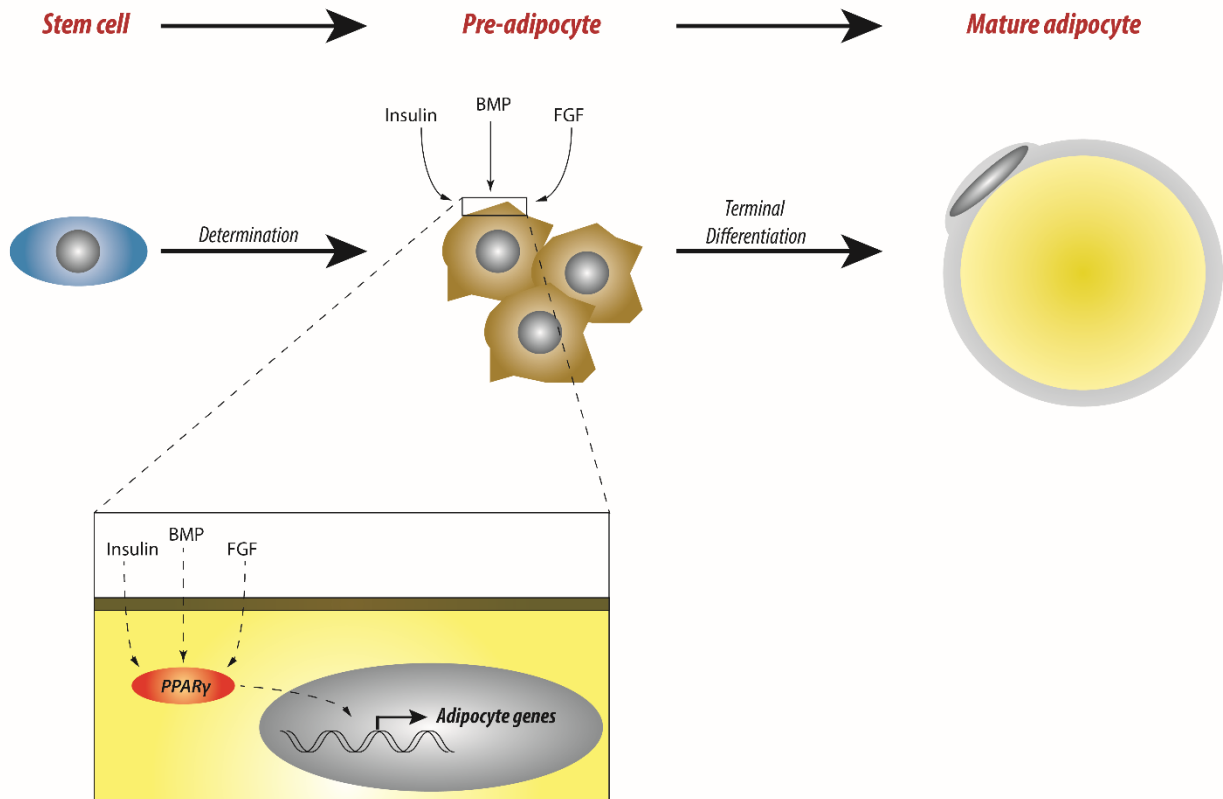
FIGURE 2-2: Adipogenesis. Once adipose stem cells are committed to the adipocyte lineage, a variety of extracellular factors can induce differentiation into a mature adipocyte to increase the fat storage capacity of the adipose tissue. Most signaling events in the adipogenic process converge on PPARγ, which is essential for the expression of necessary lipogenic genes needed for forming a mature adipocyte.

ADIPOSE TISSUE METABOLIC FUNCTION EXTENDS BEYOND THE ADIPOCYTE

Extracellular matrix

An important component of the adipose tissue structure is the extracellular matrix (ECM) that surrounds and encompasses the adipocytes. The ECM provides a scaffolding-like structure to support the structural integrity of the adipocytes as well as participating in many signal transduction events important for adipose tissue function (95). Over the past decade, there has been rising interest in the adipose tissue ECM and its role in adipose tissue remodeling and metabolic function and it is now recognized that the ECM can potently alter fatty acid storage capacity in adipose tissue. Excess accumulation of ECM components causes fibrosis to occur in the adipose tissue of obese individuals and correlates with whole-body insulin resistance (96, 97). Using 3T3-L1 cells, Li et al. demonstrated that plating 3T3-L1 cells on high density ECM surface can inhibit insulin signaling by increasing activity of pro-inflammatory pathways when compared with plating the cells on a lower density ECM (98). In agreement, elevated adipose tissue fibrosis is also highly related to the degree of inflammation in adipose tissue in obesity, which can increase insulin resistance (99). The effects of inflammation in adipose tissue will be discussed later in this review. Collagens, laminins, fibronectin, and proteoglycans all contribute to the composition of the ECM, with the role of collagens being the most well-understood thus far in relation to obesity and metabolic health. Special interest has been placed in collagen VI, a member of the family of collagen proteins that surrounds adipocytes and anchors adipocytes to the basement membrane of the ECM (which contains primarily collagens I, III, and IV). Collagen VI is expressed at far greater levels in adipose tissue than other tissues, making it a putative candidate for understanding the interaction between adipose tissue structure and metabolic function (96). In humans, collagen VI expression is positively correlated with BMI, fat mass, and is upregulated during short-term overfeeding (100). Conversely, mice that are lacking collagen VI are protected from developing insulin resistance on a high fat diet despite gaining an equivalent fat mass as wild type mice (96). The maintenance of insulin sensitivity in these collagen VI-null mice was accompanied by larger, more hypertrophied adipocytes per fat mass indicating that the lack of collagen VI allowed the adipocytes to expand without the constraints seen in the wild type mice and allow for improved fatty acid storage. It seems that collagen VI and fibrosis may play a critical role in mediating the primary function of adipose

tissue: storage of excess nutrients as triacylglycerides in lipid droplets. Obese adults who are able to maintain insulin sensitivity may have lower fibrosis in their subcutaneous adipose tissue resulting in enhanced lipid storage in the adipocyte, lower mobilization of fatty acids, and lower ectopic lipid deposition. However, it remains unclear if the ECM dictates whole-body metabolic health, especially with the close relationship that fibrosis has with inflammation in adipose tissue. Some investigators hypothesize that the immune response may dictate the ECM dynamics, so the fibrosis may be secondary to inflammatory signaling. Moreover, the composition and abundance of the ECM is a complex issue, involving proteolytic enzymes that control the rate of deposition and degradation of the ECM. The expression and relative ratios of the secreted protein, acidic, and rich in cysteine (SPARC) complex, matrix metalloproteinases (MMPs), and tissue inhibitor of metalloproteinases (TIMPs) can all dictate the ECM dynamics (101). While discussing the complex regulation of these very large family of proteins is beyond the scope of this review, data from mouse and in vitro models suggests that modulation of these proteins may allow for enhanced ability to expand adipocytes under conditions of nutrient excess. For example, Sparc-null mice exhibit an enhanced ability to expand adipose tissue on a high fat diet due to less ECM in the adipose tissue (102, 103). The cumulative data suggests ECM plays an important role in dictating the structure, function, and expansion of adipose tissue in obesity and may be important for modulating lipid-induced insulin resistance in obesity.

Inflammation and immune response

It is now well-known that weight gain results in systemic low-grade inflammation and insulin resistance (104, 105). The inflammatory response that occurs as adipose tissue expands with weight gain is postulated to be primary mechanism by which obese humans become insulin resistant, and accordingly, obese individuals that remain insulin sensitive exhibit lower markers of inflammation in their adipose tissue compared with their insulin resistant counterparts (106-108). In addition, weight loss is associated with alleviating adipose tissue inflammation and reducing the degree of insulin resistance (109). While growing evidence also suggests a role of the adipocytes in the induction of inflammation in obesity, many of the inflammatory perturbations are modulated by the resident immune cells that reside within the stromal vascular fraction (SVF) of the adipose tissue. More specifically, adipose tissue macrophages (ATM) are

the most dominant leukocyte population in both the lean and obese state, and obesity is associated with an increase in the abundance of ATM (110, 111). Adipose tissue of lean adults typically consists of approximately 5-10% macrophages and the proportion can increase to as high as 50% in obesity (110). ATMs are a primary source of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF- α) and interleukin-1β (IL-1 β), that can directly or indirectly interfere with insulin signaling in adipocytes (112, 113). Mice lacking gene expression of monocyte-chemoattractant protein-1 (MCP-1), which is important for the recruitment of macrophages to the adipose tissue during obesity, exhibit lower macrophage accumulation in their adipose tissue and remain glucose tolerant despite diet-induced obesity (114). Conversely, mice that overexpress MCP-1 have an elevated infiltration of ATMs in adipose tissue in conjunction with increased insulin resistance compared to wild-type mice (114). However, it is not only the amount of infiltration of ATMs that are important for inflammation in adipose tissue, but the activation state of the ATMs is also crucial. Myeloid specific deletion of c-Jun N-terminal kinase (JNK) or Inhibitor of kB (IkB) kinase (IKK) protected mice from high fat diet-induced insulin resistance (115-118). This suggests that inflammation induced insulin resistance is a combination of both the recruitment and activation of ATMs in the adipose tissue. Macrophages exhibit a vast amount of heterogeneity in function that are affected by the local factors in the adipose tissue, which enhance or inhibit the expression of chemokines, cytokines, and cell surface receptors that can effect metabolic function. ATMs exist on a spectrum classified by the polarization state as M1 or M2 based on the expression of different anti- or pro-inflammatory markers. M1 or "classically activated" macrophages (CD11c$^+$) are marked by increased inflammatory cytokine production (e.g. TNF-α, IL-1 β) and generally are considered detrimental to human adipocyte insulin sensitivity (119). With weight gain, there is a shift towards a larger population of pro-inflammatory M1 ATM within the immune cell population as the fat mass expands, which is related to insulin resistance (120, 121). M2 or "alternatively activated" macrophages (CD11c$^-$) are characterized by expression and secretion of more anti-inflammatory cytokines, such as IL-10 and IL-4, and assist with reducing inflammatory perturbations and stimulating tissue repair. The "classically activated" M1 macrophages can impair insulin signaling in adipocytes in vitro, while M2 alternatively activated macrophages fail to exhibit these effects on the adipocytes (119). In agreement, ablation of only the CD11c$^+$ immune cells (M1) in obese mice eliminates excess markers of inflammation in adipose tissue

and normalizes insulin sensitivity without changes in weight (122), which is most likely driven by reducing the amount of pro-inflammatory cytokine production by the ATMs. Tumor necrosis factor- α (TNF-α), a cytokine primarily released by M1 ATMs and blocks insulin action through serine kinases (JNK, MAPK, IKK) that inhibit IRS-1 phosphorylation by insulin and by altering the expression of critical glucose transport proteins (43, 44, 123). In addition, suppression of insulin action impedes the ability of insulin to suppress lipolysis, thus elevating the release of fatty acids into the circulation and impairing insulin sensitivity in peripheral tissues. The inflammation-induced increase in lipolysis is a potent stimulus for further inflammatory signaling in M1 ATMs (124). This may provide a mechanistic link by which excess fatty acid mobilization from adipocytes may instigate inflammatory processes in the resident immune cells, thus propagating further inflammation, insulin resistance, and adipocyte dysfunction. However, it remains unclear whether the inflammatory stress that occurs in expanded fat mass is a cause or consequence of elevated fatty acid release from adipocytes in obesity since free fatty acids can also induce inflammatory signaling in myeloid cells (125).

Angiogenesis and hypoxia

The expansion of adipose tissue during prolonged periods of nutrient excess requires the proper expansion of the vasculature to provide new cells with the appropriate oxygen and nutrients. The creation of new vasculature, referred to as angiogenesis, has been implicated as one of the primary mediators of adipose tissue metabolic dysfunction and insulin resistance in obesity (126). As adipose tissue expands with overeating, both the hypertrophy of existing adipocytes and the creation of new ones require new vasculature to provide appropriate nutrients and oxygen, otherwise hypoxic conditions (low partial pressure of oxygen) may occur in the tissue. Diet-induced obesity in mice induces a hypoxic environment in the adipose tissue and, in general, obese adults have hypoxic adipose tissue compared to lean persons (127-129). The hypoxic environment may be caused by reduced adipose tissue blood flow (ATBF) and is related to the degree of insulin resistance (130). A common marker used to assess hypoxia, outside of the direct measurement of oxygen tension within the tissue, is the measurement of the expression and abundance of hypoxia-inducible factor-1α (HIF-1α). As the name of the protein implies, HIF-1α is often considered a master regulator of the hypoxic response (131). HIF-1α is elevated

in the adipose tissue of obese individuals and is negatively correlated with insulin sensitivity (132, 133). The severity of the hypoxic conditions may also mediate inflammatory responses and insulin signaling in the adipose tissue (95, 134). In vitro analysis has demonstrated that hypoxic conditions can elicit pro-inflammatory responses (e.g. increased expression and production of cytokines) in adipocytes and immune cells (128, 135-137). This coincides with observations in human adipose tissue, where markers of hypoxia accompany higher inflammation (133). In addition, the expression of HIF-1α decreases with weight loss, although this is paralleled by decreases in many of the inflammatory factors in adipose tissue as well (132). The proposed relationship between fat mass gain, hypoxia, inflammation, and insulin resistance is outlined in Figure 3. One of the genes regulated by the transcription factor HIF-1α is vascular endothelial growth factor (VEGF). The VEGF family of proteins are considered to be one of, if not the most important regulator of endothelial cell growth and HIF-1α upregulates transcription of VEGF proteins (131). Ablation of VEGF in mice disrupts the formation of adipose tissue during a high fat diet and these animals exhibit high levels of inflammation and insulin resistance (138). Conversely, overexpression of VEGF in the adipose tissue of mice increased adipose tissue vascularization and reduced inflammation with weight gain, and protected mice from obesity-induced insulin resistance (138). Although HIF-1α upregulates transcription of VEGF, high levels of HIF-1α have been associated with detrimental metabolic outcomes in adipose tissue, because HIF-1α also acts as a transcription factor for inflammatory pathways, such as NFκB pathways (128, 137). This is supported by the high level of adipose tissue fibrosis, inflammation, and insulin resistance that occurs in mice that overexpress HIF-1α in adipose tissue (139).   Conversely, anti-sense mediated deletion or overexpression of a dominant negative form of HIF-1α in obese mice exhibit reduced insulin resistance (140, 141). Together, this evidence suggests that a hypoxic environment can promote a fibrotic and pro-inflammatory environment in adipose tissue and propagate insulin resistance. However, it remains unknown whether the hypoxic environment occurs first and promotes adipose tissue dysfunction during expansion or if the inability to appropriately expand adipocytes through adipogenesis drives the hypoxia often seen in obesity.
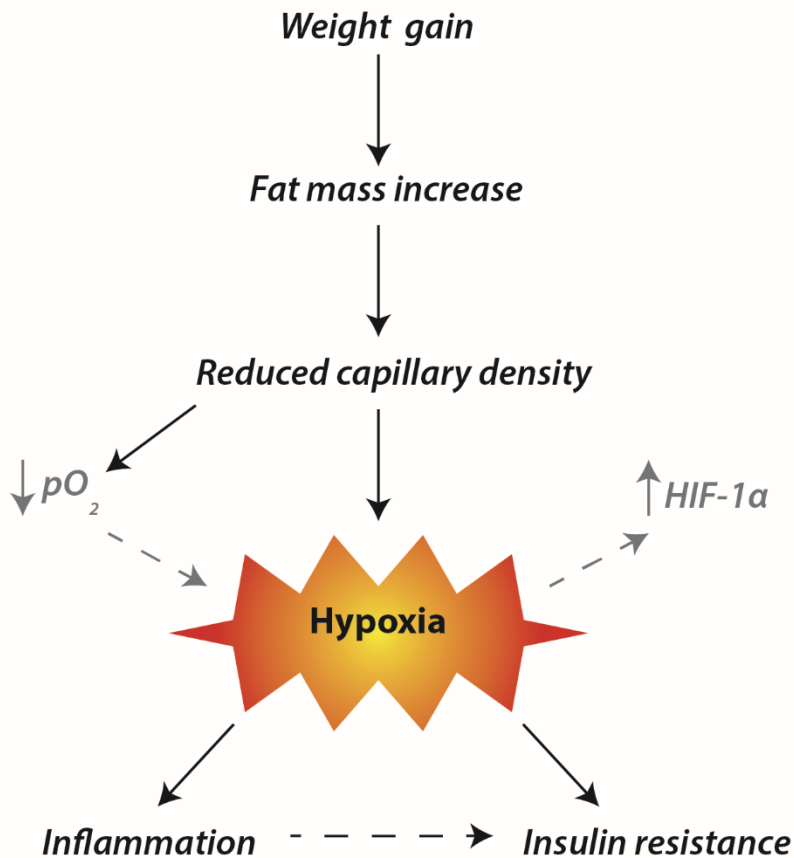
Figure 2-3: Hypoxic conditions induce inflammation and insulin resistance. As adipose tissue expands with weight gain, there is a reduced density of capillarization and a reduced adipose tissue blood flow. This leads to hypoxic conditions within the adipose tissue that can increase inflammation, insulin resistance, and fatty acid handling in adipose tissue.

EXERCISE-INDUCED MODIFICATIONS IN ADIPOSE TISSUE STRUCTURE AND FUNCTION

Exercise is an often-prescribed lifestyle intervention to combat the development of insulin resistance and disease in obesity (142). Elevated cardiorespiratory fitness has been reported to be associated with reduced cardiovascular risk factors, as well as reduced incidence of CVD, and T2DM (143, 144). Even relatively low physical activity levels have been reported to successfully lower incidences of T2DM and CVD (145-147). Physically active adults typically

have an improved glucose tolerance and insulin sensitivity compared with sedentary individuals, which likely contributes to the lower incidence of disease (148, 149). However, these health benefits of exercise may be due largely to the most recent session rather than to adaptations from long-term exercise training, per se. Many studies have distinguished the difference between long-term exercise training adaptation and the metabolic benefits of the last session of exercise, with most evidence clearly showing that the beneficial effects of exercise on insulin sensitivity are transient (~48-72 hours) (150-153). Furthermore, multiple variables associated with exercise such as energy deficit (154), and glycogen depletion (155, 156) can all modulate insulin sensitivity and can alter results and interpretation of not carefully controlled (157).

A large portion of the improvement in insulin sensitivity after a session of exercise is attributed to reductions in intramuscular glycogen concentrations, which can prolong the elevated glucose uptake that is seen after exercise in rodents and humans (155, 158). Importantly, replenishment of glycogen after exercise can quickly reverse the enhanced insulin action caused by the exercise bout (155, 156). Exercise in obese adults may also improve insulin sensitivity into the next day due to improvements in muscle fatty acid metabolism. Schenk et al. demonstrated that a 90-minute bout of aerobic exercise increased the expression of key esterification enzymes such as DGAT and GPAT and increased the capacity of the skeletal muscle to store excess fatty acids as TG (159). In turn, there were reductions in fatty acid intermediates, such as DAG, and lower activation of the serine kinases protein kinase C-θ, c-Jun N-terminal kinase (JNK), Inhibitor of kB (IkB) kinase (IKK), and nuclear factor κB (NFκB), which can directly and indirectly interfere with the insulin signaling cascade (29). Exercise can be an important lifestyle intervention for reducing lipid-induced insulin resistance in obesity if performed regularly, but the exercise training adaptations in skeletal muscle do not appear to provide much long-term protection.

Although much of the focus about the insulin sensitizing effects of exercise has centered on changes in skeletal muscle, exercise-induced changes in other tissues and organs may also contribute to the metabolic health effects of exercise (160). Yet surprisingly, very little is known about the direct effects of exercise on adipose tissue metabolism. Only recently has some interest been generated in understanding the role of physical activity and exercise on adipose tissue due to the recent understanding of adipose tissue's importance in dictating whole-body

health outcomes. Several studies have reported that exercise has beneficial effects on adipose tissue in humans as evidenced by lowered inflammation, fibrosis, and increased adipose tissue insulin sensitivity after exercise training, but these studies are confounded by fat mass loss, which is known to improve adipose tissue and whole body metabolic outcomes (109, 161). However, evidence from some recent animal studies show promising evidence for a beneficial effect of exercise, both acute and chronic, on the structure and metabolic function of adipose tissue (162-165). Oliveira et al. exposed obese rats to an acute bout of exercise and observed a reduction in inflammatory signaling (p-JNK/JNK, p-IKK/IKK) and expression of pro-inflammatory cytokines (TNFα, IL-1β, MCP-1) in adipocytes and the resident immune cells compared to obese rats that did not complete exercise (162). These data suggest that adipose tissue might be altered by a single session of exercise through reductions in inflammatory mechanisms that may translate to improvements in whole-body insulin sensitivity. A more reasonable exercise stimulus of 2 hr of treadmill running was applied to obese mice by Macpherson et al. and also showed decreased indices of inflammation of adipose tissue after the exercise bout (166). More specifically, 2 hours after the exercise session, the inguinal adipose tissue had decreased expression of *CD11c* combined with a lower abundance of F4/80 and CD11c positive cells. Insulin induced activation of Akt^{th308} was also recovered after the exercise bout compared with control mice. But more research needs to be done to verify if these results are translatable to human physiology and with a more reasonable exercise stimulus (i.e. 45-60 min moderate aerobic exercise).

In mice, it was recently demonstrated that short-term exercise training for 11 days, a length of time short enough for the mice to not lose body weight, was very effective for beneficially altering adipose tissue structure and function (165). Histological analysis of the adipose tissue from the exercised trained mice revealed an increased number smaller adipocytes and increased expression of angiogenic markers (e.g. VEGF, PDGF) which may indicate an exercise effect on processes controlling adipose tissue expansion (165). Interestingly, when the investigators transplanted adipose tissue from the exercised mice into sedentary mice, glucose tolerance and whole-body insulin sensitivity was markedly improved (165). If exercise training did promote structural changes to adipose tissue as noted by Stanford et al., then the beneficial metabolic effects of exercise training would be expected to last for many days and weeks after cessation of exercise training, but as described above, the insulin sensitizing effects of exercise are often

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found to be very transient. One possibility is that the beneficial effects of exercise on adipose tissue are only evident during periods of nutrient excess (e.g. weight gain). The primary role of adipose tissue is to store excess energy and exercise may upregulate pathways related to lipid storage and adipose tissue expansion and "prime" the adipose tissue for an increased capacity to store energy in response to exercise. Unfortunately, little is known about the role of exercise with simultaneous weight gain. The few studies that have examined the health impact of exercise while overeating demonstrated that increased levels of exercise and physical activity during overeating may preserve metabolic health (167, 168). But in these studies, comparisons were made by having an overeating groups reduce physical activity levels while overeating, and the metabolic impairments in these groups may be a result of reduced physical activity (167, 168). Furthermore, these studies were only 7 and 14 days long, and exhibited differences in fat gain and caloric balance amongst comparison groups (167, 168). Multiple studies have been produced using mice that have demonstrated beneficial effects of exercise training during weight gain (163, 164). C57BL/6J mice were fed an obesity inducing high fat diet either with or without concurrent exercise training on a treadmill for 60 min/day, 5 days/week for 14 weeks (163, 164). Despite no differences in overall body mass between the groups, the exercise trained obese mice actually gained more epididymal fat mass than the sedentary obese mice, while having vastly smaller liver mass (163, 164). In addition, the adipose tissue from the exercised mice had less infiltration of M1 or CD11c^{+} macrophages and less T cell infiltration as evidenced by histological and mRNA examination. Furthermore, mice that exercised while gaining weight exhibited far less fibrosis of the adipose tissue in conjunction with larger proportion of larger sized fat cells. These studies demonstrate compelling evidence that exercise might enhance the lipid storage capacity of adipose tissue by limiting the development of fibrosis and excessive inflammation. However, these studies did not determine if glucose uptake or whole-body insulin sensitivity was different between the sedentary and exercise group. It is not clear whether these findings in mice translate to humans. The compelling evidence that exercise training may alter adipose tissue structure and function and lead to improved metabolic health outcome is an important avenue for future research in human obesity.

SUMMARY

The high rate of systemic fatty acid mobilization commonly found in obesity is a primary determinant in the development of insulin resistance. The overall working hypothesis of my dissertation is that the structure and metabolic function of adipose tissue of obese adults who are "protected" from developing insulin resistance differs from the adipose tissue from most other obese adults by having greater capacity to expand and store lipid to better match nutrient access (See Figure 4). As a result, the "protected" obese individuals will have a lower release of fatty acids from adipose tissue into systemic circulation which allows them to maintain whole-body insulin sensitivity. To test this hypothesis, I will examine several factors implicated in the determination of fatty acid handling and storage in adipose tissue, such as factors regulating lipolysis and esterification, adipogenesis, the extracellular matrix, and immune cells. Importantly, exercise is an often-prescribed lifestyle intervention to alleviate insulin resistance, yet the impact of exercise on adipose tissue structure and metabolic function is poorly understood. I hypothesize that each session of exercise "primes" adipose tissue for improved lipogenic processes such as adipogenesis, angiogenesis, and lipid transport and storage; all of which would be highly beneficial for improved health outcomes as people gain weight. Together, the projects proposed in this dissertation will identify important factors helping to sequester fatty acids in adipose tissue, thereby, attenuating the magnitude of systemic fatty acid mobilization and protecting against the development of insulin resistance.
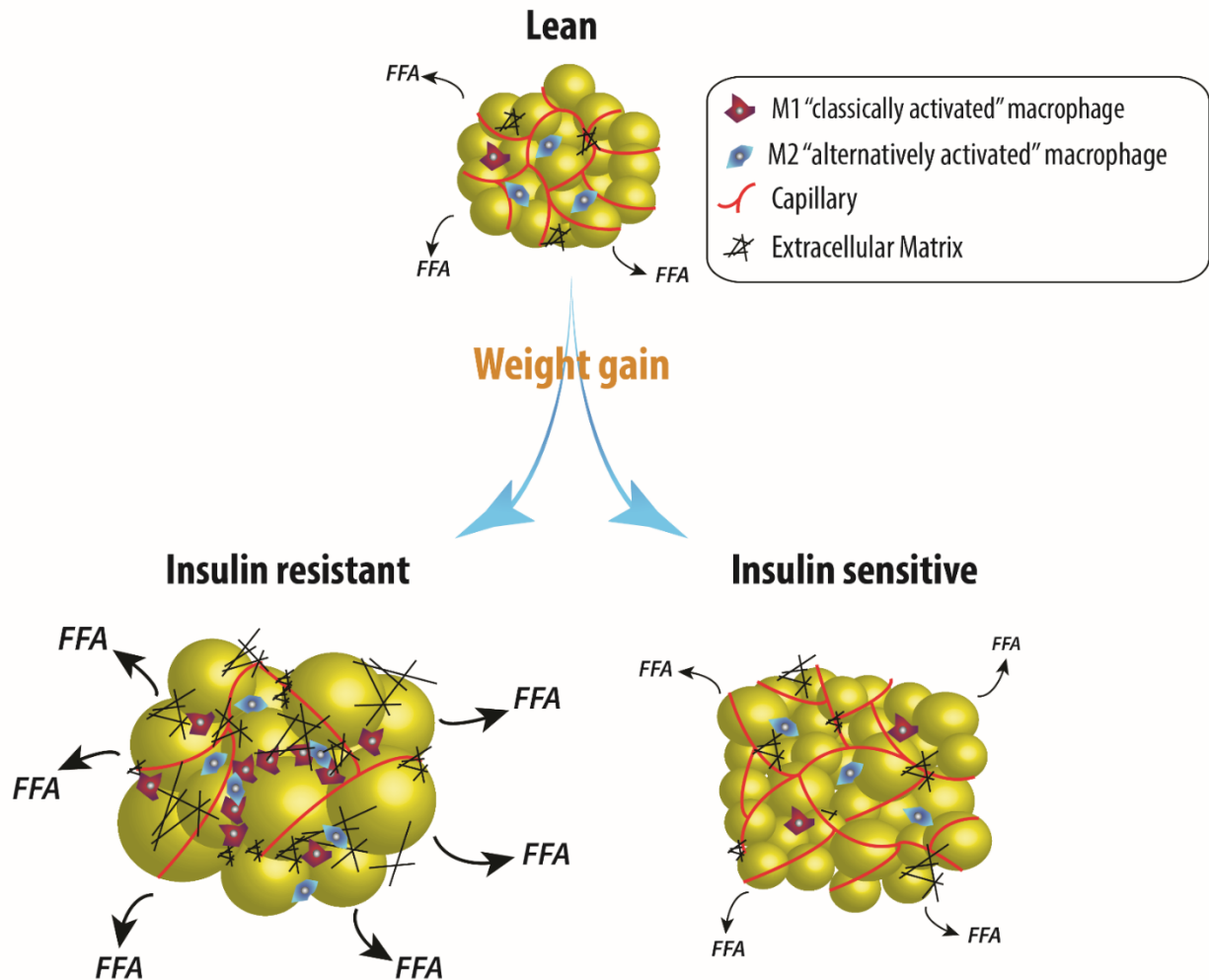
Figure 2-4: How the adipose tissue expands in obesity may dictate whole-body insulin sensitivity. Weight gain and increased fat mass are associated with whole-body insulin resistance due in part to the metabolic responses in adipose tissue. Increased immune cell infiltration and inflammation, increased fibrosis, decreased capillary density, and hypertrophied adipocytes have all been reported to be associated with excess fatty acid release and subsequent whole-body resistance. Obese adults that remain insulin sensitive may have favorable adipose tissue responses to weight gain, such as smaller fat cells per fat mass, reduced immune cell infiltration and inflammation, and higher capillary density when compared to insulin resistant obese adults, which results in lower fatty acid release and maintenance of normal insulin sensitivity.

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CHAPTER 3

Relatively low endogenous fatty acid mobilization and uptake helps preserve insulin sensitivity in obese women

Abstract

Although obesity is commonly linked with metabolic disease risk, there are still some obese adults who remain relatively healthy. The primary aim of this study was to determine if alterations in fatty acid metabolism may underlie differences in insulin sensitivity (S_i) among a seemingly homogeneous cohort of obese women. We measured insulin sensitivity (FSIVGTT), basal fatty acid rate of disappearance from plasma (Rd), whole-body fat oxidation, and performed a muscle biopsy in 21 obese women. We found S_i to be inversely related to fatty acid Rd (R^2=0.21; P=0.04). We then divided our subjects into tertiles based on their S_i, and compared the subset of subjects with the lowest S_i (LOW-S_i; $S_i \leq 2.1$ $(mU/L)^{-1} \cdot min^{-1}$; n=7) with the subset of our subjects with the highest S_i, who exhibited relatively normal insulin sensitivity (NORM-S_i; $S_i \geq 3.4$ $(mU/L)^{-1} \cdot min^{-1}$; n=8). Despite nearly identical physical characteristics in LOW-S_i vs NORM-S_i (BMI: 34±2 vs 34±1 kg/m^2; % body fat: 48±1% vs 47±1%; waist circumference: 104±2 vs 104±2 cm; VO$_{2peak}$: 2.2±0.2 vs 2.3±0.1 L/min), fatty acid Rd was nearly 30% lower in NORM-S_i (P=0.02). Activation of inflammatory pathways known to impair insulin action in skeletal muscle was also lower in NORM-S_i vs. LOW-S_i (i.e. lower phosphorylated JNK, higher IκBα abundance). In contrast, LOW-S_i and NORM-S_i exhibited no differences in systemic markers of inflammation (i.e. TNFα, IL-6, MCP-1), basal whole-body fat oxidation, and intramyocellular lipid concentrations. Our findings suggest that obese adults who maintain relatively low rates of endogenous fatty acid mobilization and uptake may be somewhat "protected" against the development of insulin resistance.

Introduction

Obesity is often associated with metabolic complications, such as insulin resistance, which is a major contributor to the development of several chronic diseases, including type 2 diabetes (1). Despite the common link between obesity and disease risk, there are still many obese adults who appear to be somewhat "protected" against the development of many chronic health issues. In fact, based on data collected as part of the National Health and Nutrition Examination Survey (NHANES) it has been reported that over 30% of obese adults are classified as "metabolically healthy" (2). However, it is still unclear why some obese adults are more prone to develop metabolic complications, like insulin resistance, while others are not, even when they are very similar in terms of the magnitude and distribution of their adiposity.

Abdominally obese individuals typically have elevated fatty acid availability compared to their lean counterparts (3), and this excessive systemic fatty acid availability and uptake have been identified as key factors underlying insulin resistance in obesity. Lipid infusions used to elevate systemic fatty acid availability in healthy, lean participants have been found to profoundly suppress their insulin sensitivity (4, 5). Furthermore, acute and chronic administration of the lipolytic inhibitor acipimox in obese adults decreased fatty acid availability and enhanced insulin sensitivity (6-8). The high rates of fatty acid availability and uptake into insulin responsive tissues like skeletal muscle have been found to induce insulin resistance in part via the intracellular accumulation of lipid intermediates, such as diacylglycerides, ceramides, and fatty acyl-CoA (9, 10). In turn, accumulation of these intermediates is linked with increased activation of pro-inflammatory pathways (e.g.; c-jun N-terminal kinase (JNK), and inhibitor of kB/nuclear factor-kB (IkB/NF-kB)) that disrupt the insulin signaling cascade (11-14). Together, these findings highlight the progression of elevated systemic fatty acid availability and subsequent inflammatory pathway activation toward the development of insulin resistance. Importantly, while lipolytic rate and fatty acid availability are typically elevated in obese compared with lean adults (3), there is still considerable variability among the obese population (15). However, it remains unclear if the magnitude of endogenous fatty acid flux contributes to differences in the severity of insulin resistance in obesity.

The overall objective of this study was to identify factors that may help explain differences in insulin resistance among a seemingly homogeneous cohort of obese sedentary women. More specifically, we sought to determine whether the magnitude of fatty acid uptake

and markers of inflammatory pathway activation in skeletal muscle were related with the degree of insulin resistance in obese adults. We hypothesized that obese adults with the lowest rate of fatty acid flux would exhibit relatively low pro-inflammatory pathway activation in skeletal muscle, and they would be the least insulin resistant among the cohort.

Methods

Subjects

Twenty-one sedentary, obese adults (body mass index (BMI): 30-40 kg/m^2; waist circumference >100cm) were recruited to participate in the study. All women were premenopausal and considered to be in good health after a medical examination. The medical evaluation included a history questionnaire, physical examination, and a resting 12-lead electrocardiogram. The participants were not taking regular medications, except for those women who were taking contraceptive medication. All subjects were nonsmokers, weight stable (±2 kg) for 6 months and had not participated in regular exercise for at least 6 months before participating in the study. Participants with coronary heart disease, type 2 diabetes, hypertension or clinically significant hypertriglyceridemia (plasma triacylglycerol concentration >150 mg/dl) were excluded. 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

Body composition was determined using dual X-ray absorptiometry (Lunar DPX DEXA Scanner, Madison, WI, USA). Subjects performed an incremental peak oxygen uptake test (VO_2peak) on a stationary bicycle ergometer to assess aerobic fitness. The protocol consisted of a 4-min warm-up, followed by a progressive increase in work rate every minute until volitional fatigue.

Experimental protocol

The trial was performed during the first 2 weeks of the female participants' menstrual cycle. Participants were admitted to the Michigan Clinical Research Unit (MCRU) at the University of

Michigan hospital at 1800 h and stayed overnight. A standardized meal was provided at 2000h and the participants remained fasted after the meal until completion of the trial the next day. At 0700 h the next morning, a skeletal muscle biopsy was obtained from the vastus lateralis. We separated any adipose and connective tissue from the muscle sample, rinsed it clean with saline, blotted it dry, and froze it in liquid nitrogen and store at − 80 °C until later analysis. Intravenous catheters were placed in a hand vein for blood sampling and in a forearm vein of the opposite arm for infusion of the stable isotope tracer, [^{13}C]-palmitate (Cambridge Isotope Laboratories, Andover, MA, USA). A blood sample was obtained before the start of the tracer infusion to assess background [^{13}C]-palmitate enrichment and to measure baseline concentrations of plasma pro-inflammatory markers (TNF-✔, interleukin-6 (IL-6) and MCP-1). At 0800 h, a constant infusion of [^{13}C] palmitate (0.04 μmol kg− 1 min−1) bound to human albumin (Baxter, Deerfield, IL, USA) began, and four arterialized blood samples were collected in 5-min intervals from a heated hand vein at minutes 45, 50, 55 and 60 for determination of FA Ra. Resting whole-body fat oxidation was calculated from the rates of oxygen consumption (VO$_2$) and carbon dioxide production (VCO$_2$) using a metabolic cart (Delta Trac, Sensor Medics, Yorba Linda, CA, USA). After all of the fatty acid metabolism measurements, a frequently sampled intravenous glucose tolerance test (FSIVGTT) was conducted to assess insulin sensitivity (S$_i$) using the minimal model technique.

Analytical Procedures

Plasma fatty acid kinetics

The tracer-to-tracee ratio for plasma palmitate was determined by gas chromatography–mass spectrometry with an MSD 5973 system (Agilent Technologies, Wilmington, DE, USA) with capillary column as previously described (16). Plasma palmitate concentration was measured by the internal standard method using gas chromatography/flame ionization detection. Proteins were precipitated from plasma samples with acetone, and hexane will be 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 by using solid phase extraction cartridges (Supelclean LC-Si Silica gel SPE tubes; Sigma Inc). Electron impact ionization was used, and the mass-to-charge ratios (m/z) 270 and 271 was selectively monitored.

Plasma glucose, fatty acid, and insulin concentrations

Plasma glucose (Thermo Scientific, Waltham, MA, USA) and fatty acid (Wako Chemicals USA, Richmond, VA, USA) concentrations were measured using commercially available colorimetric assay kits. Plasma insulin concentration was measured by radioimmunoassay (Linco Research Inc., St Louis, MO, USA).

Intramyocellular triacylglycerol concentration

Frozen muscle samples (~20 mg) were lyophilized at –60°C for 48h, and aliquots will be weighed to the nearest 0.1 mg. IMTG was measured from the liberation of free glycerol (17). Triacylglycerol was extracted from the dried muscle sample using 2:1 chloroform:methanol and saponified in 4% ethanolic KOH. Free glycerol concentration in these samples was determined fluorometrically.

Muscle tissue lysate preparation

Frozen muscle samples were weighed and transferred into prechilled microfuge tubes containing 1 ml ice-cold lysis buffer and a steel ball bearing. Tissue samples were then be homogenized in the microfuge tubes for 1 min using a Qiagen TissueLyser II (Qiagen, Hilden, Germany). The lysis buffer contained T-PER Tissue Protein Extraction Reagent (#78510, Fisher Scientific, Waltham, MA, USA), 1mM EDTA, 1mM EGTA, 2.5mM sodium pyrophosphate, 1mM sodium orthovanadate, 1mM β-glycerophosphate, 1 μgml − 1 leupeptin and 1mM phenylmethylsulfonyl fluoride. This lysis buffer recipe has been used successfully by Dr. Greg Cartee's laboratory for use with muscle tissue multiplex analysis. To remove insoluble material, the homogenates were transferred to new microfuge tubes and rotated for 1 hour at 4 °C and then centrifuged at 15,000 g for 15 min at 4 °C. Protein concentration were determined using the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA).

Multiplex analysis

Plasma cytokines and skeletal muscle proteins associated with inflammation were quantified using a commercially available Multiplex bead assay as per manufacturer recommendations (Luminex L200, Luminex, Austin, TX, USA). Plasma was assayed for IL-6, TNF-α and MCP-1

(#HCVD3-67CK, Milliplex MAP Kit, Millipore, Billerica, MA, USA). Prepared tissue lysates were analyzed for total protein abundance of JNK and IkBα and separately analyzed for the total phosphorylation of $JNK^{Thr185/Tyr185}$ (#48-602, Milliplex MAP Kit, Millipore).

Subject stratification based on degree of insulin resistance

After examining the relationship of FA Ra and insulin resistance across the entire cohort, we divided the group into tertiles based on their magnitude of insulin sensitivity. Obese adults that maintained an insulin sensitivity similar to lean individuals were designated as "NORM-S_i" while those that exhibit the lowest one-third of insulin sensitivity were designated as "LOW-S_i". Importantly, we ensured the groups exhibited homogenous physical characteristics (e.g. body mass, fat mass, cardiorespiratory fitness, etc.) in order to eliminate variables known to affect metabolic outcomes.

Calculations

Fat oxidation

Whole-body fat (i.e., triacyglycerol) oxidation ($g \cdot min^{-1}$) was calculated from VO_2 and VCO_2 measurements using the equations of Frayn (18). Whole-body fatty acid oxidation was calculated by dividing triacylglycerol oxidation by an estimated molecular weight of triacylglycerol (860 $g \cdot mol^{-1}$) and multiplying by 3.

Fatty acid mobilization

Palmitate rate of appearance (Ra) into plasma was calculated using the Steele equation for steady-state conditions (19). Fatty acid Ra was calculated by dividing palmitate Ra by the ratio of plasma palmitate to total plasma fatty acid concentration.

Insulin sensitivity index (S_i)

The insulin sensitivity index (S_i) was calculated from least squares fitting of the insulin and glucose concentration curves from the frequently sampled intravenous glucose tolerance test (FSIVGTT) using the Minimal Model Millenium (version 6.02; MinMod Inc., Pasadena, CA, USA) computer analysis software.

Statistics

Simple linear regression was used to assess significance in the relationship between S_i and fatty acid Ra among all subjects (n=21). Unpaired Student's t-tests was used to test for significant between-group (NORM-S_i vs LOW-S_i) differences in all outcome variables. Simple linear regression was also used to test for significant relationship between FA Ra and markers of inflammation in the circulation (e.g., IL-6, TNF-a, MCP-1) and in skeletal muscle (e.g., IκBα, and phosphorylated JNK) in all participants (n = 21). Statistical significance was defined as P≤0.05.

Results

Insulin sensitivity index and cohort stratification

As anticipated, the subject pool was largely homogeneous in terms of BMI, adiposity, waist circumference, and cardiorespiratory fitness, however, S_i varied widely among the 21 subjects (Figure 3-1), ranging from 4.8 to 0.8 $(mU/L)^{-1}/min$. As noted in Figure 3-1A, subjects with an S_i in the lowest one-third of our overall subject pool (≤ 2.1 $(mU/L)^{-1}/min$) were grouped into our low insulin sensitive cohort ("LOW-S_i"; n=7) and those in the highest one-third (≥ 3.4 $(mU/L)^{-1}/min$) were grouped into our normal insulin sensitivity cohort ("NORM-S_i"; n=8). We used the term "normal" to define the S_i of our most insulin sensitive subjects in this study because these obese subjects had a similar S_i as we previously reported in healthy lean adults (13)(20), and as such we identified this cohort as exhibiting "normal" insulin sensitivity. As designed, the difference in S_i between NORM- S_i and LOW- S_i was significant (Figure 3-1B; P<0.000001), but importantly, these groups were very well matched for BMI, adiposity, waist circumference, and cardiorespiratory fitness (Table 3-1). In order to compare groups with distinct differences in insulin sensitivity, our primary comparisons did not include subjects with S_i values between 2.1 and 3.4 $(mU/L)^{-1}/min$ (grey bars in Figure 3-1). The subjects with intermediate S_i were included in our correlations, which incorporated our entire subject pool.

Fat metabolism

In conjunction with the higher insulin sensitivity in NORM- S_i compared with LOW- S_i, FA uptake was nearly 30% lower in NORM-S_i vs. LOW-S_i (Figure 3-2; $P<0.02$). Despite this lower rate of FA uptake in NORM-S_i vs. LOW-S_i, resting whole-body fatty acid oxidation was not different between groups (3.7 ± 0.2 vs 3.5 ± 0.2 μmol/kg/min, respectively). Interestingly, FA uptake was reasonably well matched to the rate of fatty acid oxidation in the NORM-S_i cohort (3.7 ± 0.2 vs. 3.8 ± 0.5 μmol/kg/min, respectively), but in our LOW-S_i cohort the rate of FA uptake exceeded fatty acid oxidation by nearly 50% (5.2 ± 0.4 vs. 3.5 ± 0.2 μmol/kg/min, respectively). However, this disparity between fatty acid uptake and fat oxidation in LOW-S_i did not translate to a measureable increase in IMTG accumulation, which was similar between groups (63.7 ± 6.7 vs. 68.3 ± 12.7 vs. μmol/g dry weight for NORM- S_i and LOW- S_i, respectively).

Markers of inflammation

Within skeletal muscle, JNK phosphorylation (p-JNK) was significantly lower in NORM-S_i compared with LOW-S_i (Figure 3-3A), suggestive of an attenuated inflammatory pathway activation in muscle from our NORM-S_i subjects. Interestingly, a lower total JNK protein abundance in NORM-S_i vs, LOW-S_i (101 ± 10 vs. 149 ± 20 arbitrary units (AU), respectively; $p\leq0.05$) may underlie much of the difference in p-JNK between the groups. We also found a greater protein abundance in IκB-α in muscle from NORM-Si compared with LOW-S_i (Figure 3B), but this difference between groups did not quite reach statistical significance ($p=0.067$). Because IκB-α suppresses activation of the IKK-NFκB inflammatory pathway (21), the trend we observed for a greater abundance of IκB-α in muscle from NORM-S_i vs LOW-S_i subjects is indicative of reduced inflammatory/stress in NORM-S_i. In contrast to the differences in markers of inflammation we found in skeletal muscle of NORM-S_i vs LOW-S_i, there were no significant differences in fasting plasma concentrations of IL-6 (15.0 ± 8.4 vs. 23.8 ± 10.0 pg/ml), TNF-α (4.3 ± 1.0 vs. 7.9 ± 2.2 pg/ml), or MCP-1 (128.5 ± 12.6 vs. 114.5 ± 32.7 pg/ml).

Relationships between insulin sensitivity, fatty acid uptake, and inflammatory markers

Using our entire subject population in this study (n=21), we found a significant inverse correlation between FA uptake and Si, (Figure 3-4A; R^2=0.21; p = 0.04), further supporting the notion that those with relatively low FA uptake were the most insulin sensitive. Similarly, we found a significant positive relationship between abundance of IκB-α within the skeletal muscle and Si (Figure 3-4B; R2=0.22; p=0.035), as well as a trend for an inverse relationship between pro-inflammatory p-JNK and S_i (Figure 3-4C; R^2 = 0.18; p= 0.06), suggesting that preservation of lower inflammatory/stress in skeletal muscle is related to a greater insulin sensitivity.

Discussion

Clearly there are some obese adults who are generally in good health, while many others have serious metabolic complications (2). We found that just over one-third of our obese cohort exhibited "normal" insulin sensitivity, which matched very well with epidemiological evidence using data collected from the National Health and Nutrition Examination Survey (NHANES) (2). However, it is not clear why some obese adults are prone to develop insulin resistance (as well as other cardio-metabolic complications), while others appear to remain somewhat "protected". In our subjects, differences in cardiorespiratory fitness, resting fat oxidation, or skeletal muscle triglyceride accumulation could not explain the disparity in insulin resistance. Instead, our main findings suggest that a relatively low basal rate of fatty acid uptake may be a key contributor protecting against insulin resistance in obese adults.

An overabundance of fatty acids have long been known to induce insulin resistance (4, 5). Excessive fatty acid uptake into insulin responsive tissues like skeletal muscle is thought to induce insulin resistance in part via the intracellular accumulation of lipid intermediates, such as diacylglycerides, ceramides, and fatty acyl-CoA (9, 10). Theoretically, high rates of fatty acid oxidation may help compensate for high rates of fatty acid uptake, thereby attenuating the accumulation of lipid intermediates, which may help protect against the development of insulin resistance (22). However, the impact that normal variation in resting fatty acid oxidation may have on the development of insulin resistance is debated (23, 24). In our study, rates of fatty acid oxidation were identical in NORM-S_i and LOW-S_i, so differences in the oxidative disposal

of fatty acids were not contributing to the differences in insulin sensitivity observed between these groups. In contrast, a 50% greater rate of fatty acid uptake in the LOW-S$_i$ group was responsible for creating a large mismatch between the rates of fatty acid uptake and oxidation in this group. Although we did not measure the muscle accumulation of diacylglycerol, ceramide, or fatty acyl-CoA in this study (due to limitations in the amount of skeletal muscle tissue available for analysis), it is plausible that a disparity between fatty acid uptake and oxidation of this magnitude may have led to an accumulation of these lipid intermediates. In turn, the accumulation of these lipids within the muscle cell can increase the activation of inflammatory pathways, which may contribute to the impairment in insulin action (25, 26).

It has become evident that increased inflammatory pathway activation in skeletal muscle is an important link between altered fatty acid metabolism and impaired insulin signaling (25). For example, incubating skeletal muscle cells in media containing a high concentration of fatty acids is known to activate cellular inflammatory factors, such as Inhibitor of nuclear factor kappa B kinase (IKK) and c-jun n-terminal kinase (JNK), which mediate the suppression in insulin-stimulated glucose uptake (14, 27, 28). More clinically relevant, the high rates of fatty acid mobilization and uptake commonly found in obesity (3) is often accompanied by an elevated activation of JNK (11, 29) and/or IKK (27, 29) in skeletal muscle. Importantly, pharmacologically induced suppression in fatty acid availability in human subjects was found to suppress the activation of inflammatory pathways in skeletal muscle, and improve insulin action (6-8). Furthermore, previous work in our laboratory demonstrated that the improvement in insulin sensitivity found after weight loss was largely attributed to the weight loss-induced reduction in fatty acid availability, with an accompanying decline in the activation of the inflammatory JNK and IKK-NFκB pathways (30). Our observation that the relatively low rate of fatty acid uptake in our NORM-S$_i$ subjects was accompanied by lower p-JNK and a higher IkBa abundance highlights the importance of the magnitude of fatty acid uptake as a key mediator of inflammation and insulin sensitivity within skeletal muscle.

When examining health disparities among obese adults, there is considerable emphasis on the role that differences in anatomical distribution of body fat (i.e., subcutaneous vs. visceral) may play on determining metabolic health in obesity. Abdominal obesity is most commonly linked with insulin resistance (31, 32), as well as other cardiovascular disease risk factors (33,

34). More specifically, accumulation of fat in the visceral region is typically identified as being an especially potent contributor to the development of metabolic disorders (35-37). We attempted to control for abdominal obesity in our study population by matching waist circumference between our NORM-S_i and LOW-S_i groups. It has been demonstrated that waist circumference can provide a reasonably accurate surrogate measure for visceral adiposity when compared to more precise measurements (e.g. MRI) (33). However, because we did not definitively assess visceral fat mass, we cannot rule out the possibility that potential differences in visceral adiposity may help explain some of the observed difference in insulin sensitivity among our subjects. Some suggest that visceral adiposity contributes to insulin resistance as a consequence of the relatively high lipolytic rate measured in visceral compared with subcutaneous adipocytes (38, 39), which in turn may lead to excessive FFA availability known to impair insulin action. Yet, while high lipolytic rates in visceral vs. subcutaneous adipocytes are commonly found when measured *in vitro*, *in vivo* measurements indicate that about 85% of systemic fatty acids are derived from subcutaneous rather than visceral adipose tissue (40). Similarly, only about 20% of fatty acids delivered to the liver were found to be derived from visceral adipose tissue (40). Therefore, the vast majority of fatty acids delivered to, and taken up by skeletal muscle and liver (i.e., the majority of fatty acids that may enhance inflammatory/stress and insulin resistance in these tissues) likely originate from subcutaneous, rather than visceral adipose tissue.

The rate of fatty acid mobilization from subcutaneous adipose tissue has recently been reported to vary widely among a cohort of obese adults (15), and our findings support this notion. The rate of fatty acid release from adipose tissue is determined in large part by the rate of lipolysis, which liberates fatty acids from triglycerides. Therefore, a relatively low lipolytic rate within adipose tissue would suppress the mobilization of fatty acids into the systemic circulation, which may lower the risk for developing insulin resistance. The abundance and activation of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) largely dictate the rate of lipolysis within adipose tissue (41, 42). Lipolytic rate has recently been positively associated with insulin resistance in humans (43). Furthermore, the same authors also showed that inhibition of HSL and the subsequent reduction in fatty acid mobilization from adipose tissue in mice resulted in improved glucose tolerance without changes in body mass (43). Importantly, the rate of triglyceride synthesis (i.e., fatty acid esterification) can also

modulate the rate fatty acid release, and it has recently been reported that obese individuals with a relatively high capacity for triglyceride synthesis in subcutaneous adipose tissue were largely protected from insulin resistance (15). Triglyceride synthesis within adipose tissue is regulated by a series of enzymes (i.e., glycerol phosphate acyltransferase (GPAT), acylglycerolphosphate acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), and diacylglycerol acyltransferase (DGAT)) that catalyze the esterification of three fatty acyl-CoA moieties to a glycerol backbone. Future studies designed to further examine the abundance and activity of key factors that regulate fatty acid release and triglyceride synthesis in adipose tissue will help identify potential mechanisms mediating the differences in fatty acid mobilization in obesity independent of adiposity.

It is important to acknowledge that we measured fatty acid mobilization in the post-absorptive state, and obviously insulin resistance was assessed during the subsequent IVGTT. Because insulin potently suppresses lipolysis (44), individual variability in the magnitude of the insulin-mediated suppression of fatty acid mobilization may have also contributed to the variability in insulin resistance among our subjects. In fact, Magkos, et al., (45) recently reported that the magnitude of the insulin-induced suppression in fatty acid mobilization was significantly correlated with insulin-mediated glucose uptake. Interestingly, despite also demonstrating a significant relationship between post-absorptive fatty acid availability and insulin resistance (similar to our present findings), the authors challenged the notion that fatty acid mobilization in the post-absorptive state was an important contributor to the development of insulin resistance (45). We agree that if fatty acid availability remains relatively high despite insulin exposure (e.g., after a meal, during an IVGTT, or hyperinsulinemic clamp) – this certainly could contribute to the development of insulin resistance. However, even among their most insulin resistant subjects, Magkos, et al (45) found insulin suppressed fatty acid mobilization at least 35% below post-absorptive levels, and the absolute fatty acid availability was relatively low in response to insulin in all subjects. Moreover, if the accumulation of lipid intermediates within the muscle cell does indeed play an important role in the development of insulin resistance (9, 10), these intracellular lipids likely accumulate in large part in the post-absorptive state, when fatty acid availability is highest. Therefore, we contend that the exposure to high availability of fatty acids that is often found in the post-absorptive state of many obese adults, can have a potent impact on insulin resistance.

Interestingly, we did not find any differences in plasma cytokines known to be involved with systemic inflammation between the NORM-S_i and LOW-S_i groups. There were no significant correlations of the measured cytokines among the entire cohort as well (data not shown). The chronic low grade inflammation observed in obesity is partly attributed to elevated systemically circulating inflammatory cytokines such as Tumor Necrosis Factor (TNF-α) and Interleukin 6 (IL-6) (46, 47). We can possibly attribute these results to a smaller sample size and the inherent variability typically observed in these circulating cytokines. Alternatively, it may be indicative that the inflammatory response is primarily mediated within the peripheral tissues as opposed to the systemically circulating cytokines.

In conclusion, our findings indicate that variability in systemic fatty acid availability and tissue uptake are key mediators of the observed differences in insulin resistance among a seemingly homogenous cohort of obese women. More specifically, obese subjects who had a relatively low rate of fatty acid uptake appear to be somewhat "protected" against the development of insulin resistance. This lower rate of fatty acid uptake was accompanied by a lower activation of markers for inflammatory pathways within the skeletal muscle, and consequently, a higher insulin sensitivity when compared with our subjects with a relatively high rate of fatty acid uptake. Importantly, the observed variability in insulin resistance among our obese subjects could not be attributed to differences in basal fat oxidation, common markers of systemic inflammation (e.g., IL-6, TNF-α, MCP-1), skeletal muscle triglyceride accumulation, or cardiorespiratory fitness. It is likely that differences in the regulation of fatty acid storage and release from adipose tissue mediates a large portion of this observed variability in fatty acid availability and uptake. Therefore, it is now very important to identify the factors underlying the relatively low fatty acid availability and uptake among obese individuals who are not insulin resistant (and/or factors underlying the high fatty acid availability among those who are) in order to develop preventative, and/or therapeutic approaches in the treatment of insulin resistance and related diseases.

Acknowledgments

	All subjects	NORM-S_i	LOW-S_i
Age (y)	30 ± 1	32 ± 2	29 ± 3
Body Mass (kg)	93 ± 2	92 ± 2	91 ± 5
BMI (kg/m^2)	34 ± 1	34 ± 1	34 ± 2
Waist circumference (cm)	104 ± 1	104 ± 2	104 ± 2
Fat mass (kg)	44 ± 2	44 ± 1	44 ± 4
Fat free mass (kg)	49 ± 1	49 ± 1	48 ± 2
Body fat (%)	48 ± 1	47 ± 1	48 ± 1
VO$_{2peak}$ (ml/kg/min)	24 ± 1	25 ± 1	23 ± 1

Data presented as Mean ± SE.

Table 3-1. Physical characteristics
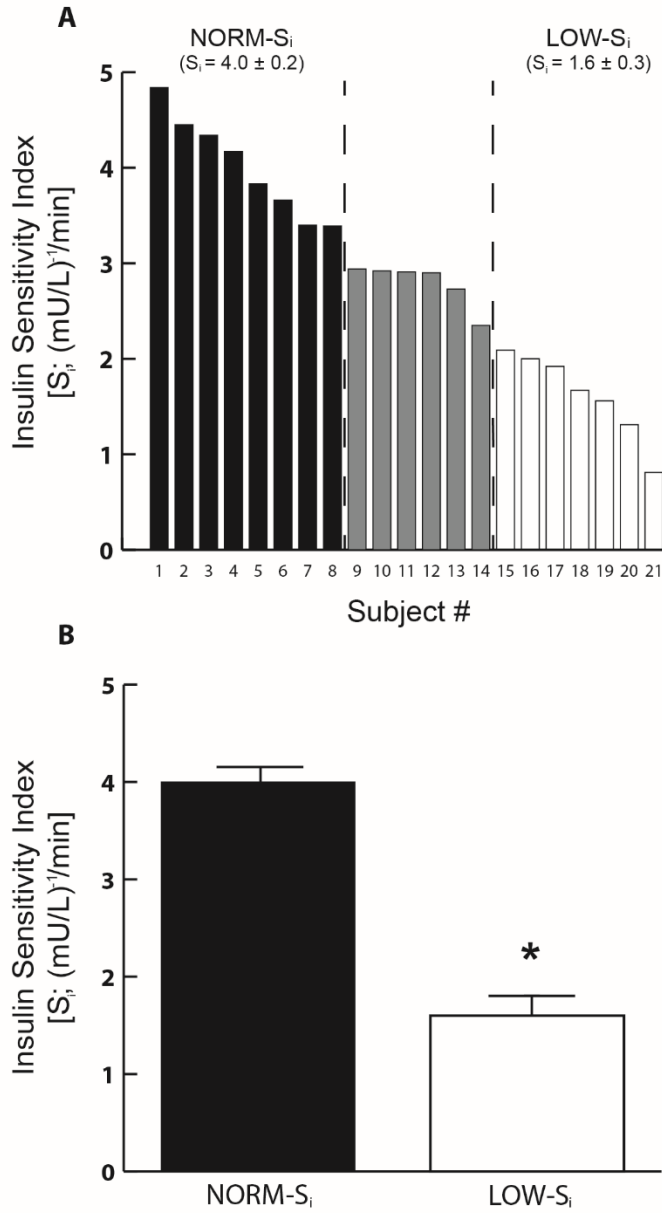
Figure 3-1: Insulin sensitivity. (a) Insulin sensitivity index (S_i) measured in the morning after an overnight fast in all subjects (n= 21). Subjects were ordered from highest to lowest S_i, and stratified into tertiles to identify a low insulin sensitivity cohort (LOW-S_i; $S_i \leqslant 2.1$ (mU/l) $^{-1}$ min^{-1}; n=7) and a normal insulin sensitivity cohort (NORM-S_i; $S_i \geqslant 3.4$ (mU/l) $^{-1}$ min^{-1}). (b) Mean S_i in NORM-S_i and LOW-S_i cohorts. *P<0.000001 vs NORM-S_i. Values presented as MEAN ± SE
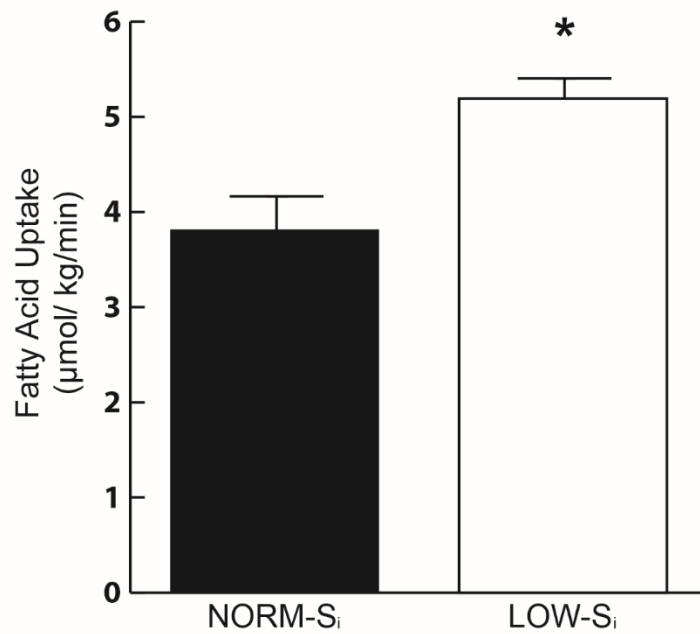
Figure 3-2: Fatty acid uptake. Fatty acid rate of disappearance from plasma (Rd) in NORM-S_i and LOW-S_i cohorts, measured in the morning after an overnight fast. *$P<0.05$ vs NORM-S_i. Values presented as MEAN ± SE
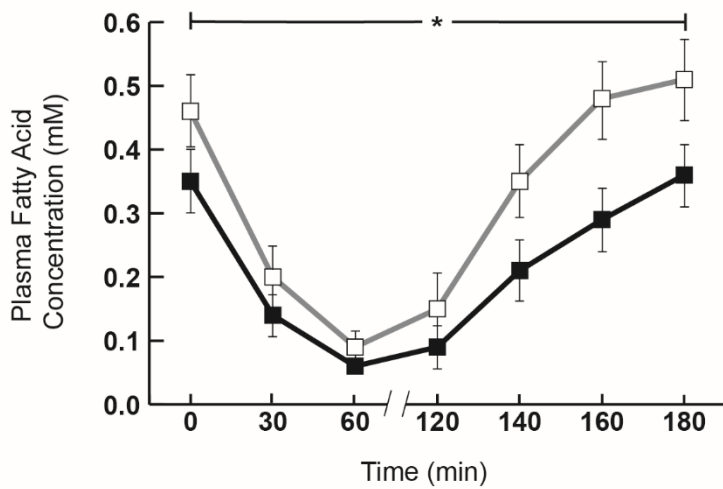
Figure 3-3: Insulin-induced suppression of plasma fatty acid concentration. Plasma fatty acid concentration during the 3 h IVGTT in LOW-S_i (white square □) and NORM-S_i (black square ■).Values presented as MEAN ± SE. *Significant difference in area under the curve between the groups, p=0.02.
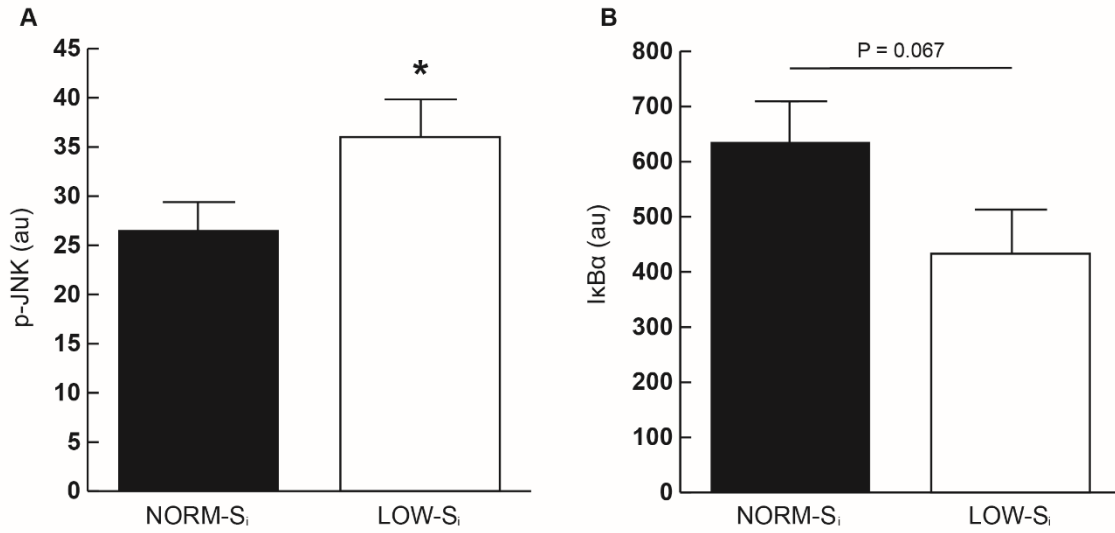
Figure 3-4: Markers of inflammation. (a) Total protein abundance of phosphorylated c-Jun N-terminal kinase (p-JNK). (b) Total protein abundance of inhibitor of NF-κBα (IκB-α). *$P<0.05$ for NORM-S_i vs LOW-S_i. au, arbitrary units. Values presented as MEAN ± SE.
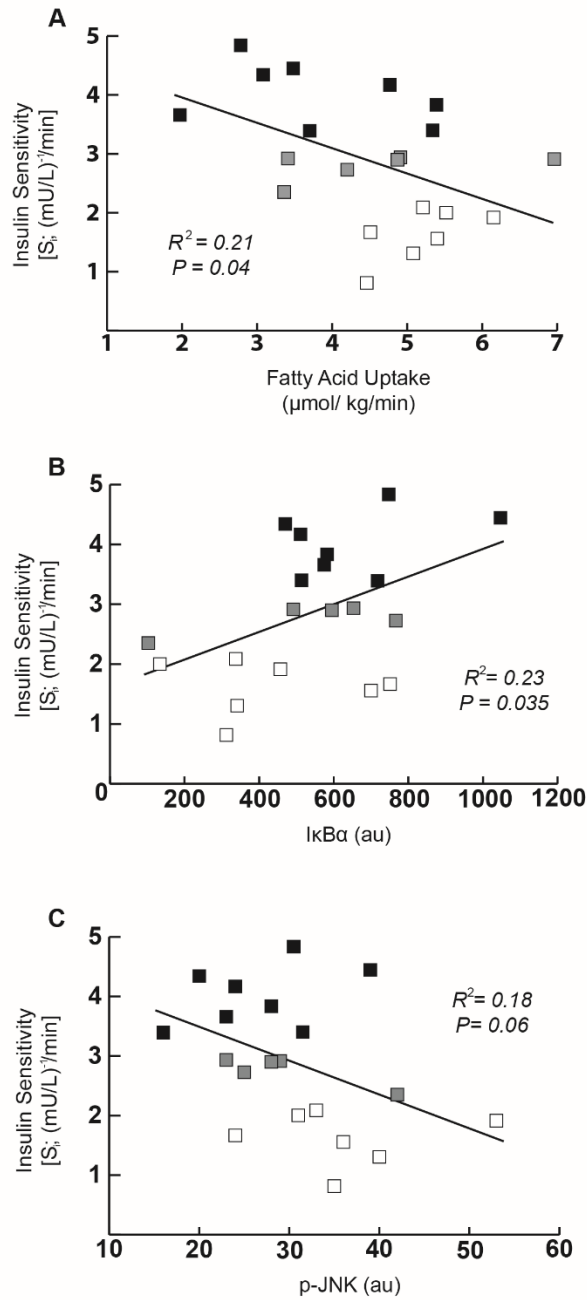
Figure 3-5: Correlational analyses for insulin sensitivity with fatty acid uptake and inflammatory markers in skeletal muscle. (a) Correlation between insulin sensitivity index (S_i) and fatty acid uptake. (b) Correlation between S_i and IκB-α abundance. (c) Correlation between S_i and p-JNK abundance. All correlational analyses were performed using the entire subject cohort (n =21) (LOW-S_i (n=7; white square □), NORM-S_i (n =8; black square ■) and participants who did not fall into either category (n=6; gray square ■)).

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CHAPTER 4

Factors regulating subcutaneous adipose tissue storage, fibrosis, and inflammation may underlie low systemic rates of fatty acid mobilization in insulin sensitive obese adults.

Abstract

Although the rate of fatty acid release from adipose tissue into the systemic circulation is very high in most obese adults, some obese adults maintain relatively low rates of fatty acid release, which helps protect them against the development of systemic insulin resistance. The primary aim of this study was to identify factors in adipose tissue that may underlie low vs. high rates of fatty acid mobilization in a relatively homogeneous cohort of obese adults. We measured systemic fatty acid rate of appearance (FA Ra) via ^{13}C-palmitate isotope dilution, and we obtained subcutaneous abdominal adipose tissue samples from 30 obese adults (BMI: 38±1 kg/m^2, age: 30±2 yr) after an overnight fast. We then measured insulin sensitivity using a hyperinsulinemic-euglycemic clamp. Confirming our previous work, insulin sensitivity was inversely proportional to FA Ra (R^2=0.50; p<0.001). Immunoblot analysis of subcutaneous adipose tissue samples revealed that, compared with obese adults with high FA Ra, those with low FA Ra had lower markers of lipase activation and higher abundance of glycerol-3-phosphate acyltransferase (GPAT), which is a primary enzyme for fatty acid esterification. Microarray and pathway analysis provided evidence of lower fibrosis and lower SAPK/JNK pathway activation in obese adults with low FA Ra compared to those with high FA Ra. Our findings suggest that alterations in factors regulating triglyceride storage in adipose tissue, along with lower fibrosis and inflammatory pathway activation, may underlie maintenance of a relatively low FA Ra in obesity, which may help protect against the development of insulin resistance.

Introduction

Obesity is associated with insulin resistance, which is central to the development of many cardio-metabolic diseases. However, up to one-third of obese adults remain metabolically healthy (i.e., not insulin resistant) (1); these "insulin sensitive" obese adults have fewer metabolic health complications, and their mortality rates are similar to lean, healthy individuals (2, 3). While it remains unclear why some obese adults remain insulin sensitive, mounting evidence suggests adipose tissue plays an important role.

Much of the insulin resistance observed in obesity is a consequence of excessive release of fatty acids into systemic circulation (fatty acid mobilization [FA Ra]) and the resultant ectopic lipid deposition that disrupts insulin signaling in peripheral tissues. In fact, several studies report profound insulin resistance in healthy, lean subjects after short-term lipid and heparin infusion to mimic the high FA Ra found in obesity (4, 5). Conversely, drugs that lower systemic fatty acid availability in obese adults can reverse insulin resistance (6, 7). Our lab and others have demonstrated that the degree of FA Ra dictates the degree of insulin resistance in obesity and that obese adults who maintain a relatively low basal FA Ra remain insulin sensitive (8, 9). However, while it is clear that the rate of FA Ra from adipose tissue can greatly impact insulin resistance, little is known about factors that may help sequester excess fatty acids in adipose tissue, and thereby limit ectopic lipid deposition.

The accumulation of visceral adipose tissue is often linked with the severity of cardio-metabolic disease risk (10); however, the excess accumulation of visceral fat most likely results from the subcutaneous adipose tissue's inability to effectively store excess nutrients. Nielsen et al. (11) demonstrated that approximately 90% of circulating fatty acids are derived from subcutaneous adipose tissue, with nearly 70% of these fatty acids coming from abdominal subcutaneous adipose tissue. Moreover, despite the anatomical proximity of visceral adipose tissue to the liver, the vast majority of fatty acids in the hepatic circulation are also derived from abdominal subcutaneous adipose tissue (11). Human studies using deuterated water to track long-term turnover and storage of triacylglycerol (TG) found that TG synthesis and storage are impaired in the subcutaneous adipose tissue of insulin-resistant obese individuals compared with obese adults who maintain normal insulin sensitivity (12). These data demonstrate the importance of subcutaneous adipose tissue in the control of FA Ra and subsequently, the control of ectopic lipid deposition and insulin sensitivity.

The factors responsible for differences in FA Ra in obesity are not clear, and although the enzymes regulating TG hydrolysis and esterification are well described, the regulation of fatty acid release from adipose tissue is far more complex. Various adipose tissue factors have been postulated to be important mediators of adipose tissue function, including cell size (13), lipid storage capacity (14), adipogenesis (15), angiogenesis (16), extracellular matrix (ECM) dynamics (17), and inflammation (18). Advancing our understanding about what dictates high vs. low FA Ra in obesity could lead to targeted approaches to attenuate elevated FA Ra, which could markedly improve metabolic health. The primary purpose of this study was to determine factors that protect some obese adults from developing high FA Ra from adipose tissue.

Methods

Subjects

Thirty sedentary, premenopausal women (n=25) and obese men (n=5) (BMI: 30-45 kg/m^2) ages 18-40 years were recruited for this study. Participants with coronary heart disease, type 2 diabetes, hypertension, or clinically significant hypertriacylglycerolemia (plasma TG >150 mg/dL) were excluded. Participants were not taking regular medications known to effect metabolic processes, and some women were taking contraceptive medication. All subjects were non-smokers, weight stable (±2 kg) for 6 months, and had not participated in any regularly planned exercise or physical activity for at least 6 months. Body composition was assessed using dual energy X-ray absorptiometry (Lunar DPX DEXA Scanner). Written, informed consent was obtained from all subjects before initiating participation and all study procedures were approved by the University of Michigan Institutional Review Board.

Experimental Procedures

Subjects were admitted to the Michigan Clinical Research Unit at 0700h after an overnight fast. We obtained a baseline blood sample and an ~100-200 mg subcutaneous adipose tissue sample from the abdominal region approximately 6 cm lateral to the umbilicus. The sample was cleaned with saline, blotted dry, and quickly frozen in liquid nitrogen. At ~0900h, we began a constant-rate infusion of [1-^{13}C]-palmitate (0.04 µmol/kg/min); after 45 min of infusion, three arterialized blood samples were obtained from a heated hand vein in 5-min intervals for

determination of FA Ra. At ~1000h, we began a hyperinsulinemic-euglycemic clamp to assess peripheral insulin sensitivity using a primed 2-h insulin infusion at a rate of 100 mU/m^2/min (19). This insulin infusion rate was selected to inhibit hepatic glucose production, even in insulin resistant subjects (20), allowing us to assess insulin sensitivity largely independent of the liver. Plasma glucose concentration was monitored every 5 minutes during the clamp study with a glucose autoanalyzer (Yellow Springs Instruments, Yellow Springs, OH), and glucose was infused at a variable rate to maintain plasma glucose concentration at each participant's baseline fasting glucose concentration. Insulin sensitivity was defined as the glucose infusion rate (GIR: mg/min) during the last 20 minutes of the hyperinsulinemic-euglycemic clamp (steady-state) divided by fat-free mass (FFM, kg).

Analytical Procedures

Plasma fatty acid kinetics

The tracer-to-tracee ratio for plasma palmitate was determined by gas chromatography–mass spectrometry (MSD 5973, Agilent Technologies, Wilmington, DE) as previously described (21). Palmitate rate of appearance (Ra) into plasma was calculated using the Steele equation for steady-state conditions (22). FA Ra was calculated by dividing palmitate Ra by the ratio of plasma palmitate to total plasma fatty acid concentration.

Subject stratification

Subjects were divided into tertiles based on the magnitude of their FA Ra. For our main comparisons, we examined differences between subjects that maintained a relatively low FA Ra despite being obese (LOW-FA; n=10) to those with a high FA Ra (HIGH-FA; n=10).

Plasma measurements

Plasma glucose (Thermo Fisher Scientific, Waltham, MA), fatty acids (Wako Chemicals, Richmond, VA), TG (Sigma Aldrich, St. Louis, MO), and total cholesterol (Wako Chemicals, Richmond, VA) concentrations were measured using commercially available colorimetric assay kits. Plasma insulin concentration was measured by radioimmunoassay (EMD Millipore, St. Charles, MO).

mRNA expression

RNA was isolated from subcutaneous adipose tissue (~50mg) using a commercially available kit (Aurum total RNA fatty and fibrous tissue kit, Bio-Rad, Hercules, CA), quantified spectrophotometrically, and reverse transcribed (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific). Predesigned PrimeTime qPCR Assays (Integrated DNA Technologies, San Diego, CA) were used for mRNA analyses. Real-time quantitative PCR data was normalized to peptidylprolyl isomerase A (*PPIA*) and beta-2-microglobulin (*B2M*) expression using the -ΔCt method (23).

Microarray Analysis

Microarray analysis of adipose tissue gene expression was performed by the University of Michigan DNA Sequencing Core following manufacturer recommendations. RNA from the 5 subjects with highest and lowest FA RA was hybridized to Human Gene ST 2.1 strips (Affymetrix, Santa Clara, CA). The fold difference in gene expression between LOW-FA and HIGH-FA was determined with ArrayStar version 12.1 (DNASTAR, Rockville, MD). The Upstream Regulator Module of Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA) was used to identify upstream transcriptional regulators potentially explaining differences in microarray gene expression. We performed gene set enrichment analysis with Pathway Analysis using Logistic Regression (LRPath: http://lrpath.ncibi.org/)(24) to test for predefined biologically relevant gene sets containing more significant genes than expected by chance between LOW-FA and HIGH-FA. The data discussed in this study have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE95777.

Western blotting

Adipose tissue was homogenized in tissue lysis buffer (Cellytic MT cell lysis reagent, Sigma-Aldrich) with commercially available proteinase and phosphatase inhibitors (P8340, P5726, and P0044, Sigma-Aldrich). Protein concentration was determined using the bicinchoninic acid method (Thermo Fisher Scientific). Fifteen micrograms of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were Ponceau stained to confirm equal loading. Membranes were blocked, incubated in primary antibodies (Table 4-1) overnight at 4°C, washed, and incubated with appropriate secondary antibodies for 1 h. Blots were developed

using enhanced chemiluminescence (Clarity western ECL substrate, Bio-Rad), imaged, and quantified via densitometry (Image Lab software, Bio-Rad).

Statistical Analysis

Simple linear regression was used to examine the relationship between FA Ra and insulin sensitivity. Unpaired student's t-tests were used to test for between group differences (LOW-FA vs. HIGH-FA) in all measured outcome variables. The linear regression and student's t-tests were processed using Sigmaplot 13.0. Statistical significance was defined as $p < 0.05$. Gene expression differences in the microarray data were determined in ArrayStar version 12.1 using a moderated t-test (25). The fold difference values and p-values generated from ArrayStar were then used for directional tests in LRpath gene ontology analysis. For all immunoblot and gene expression analyses, we used Grubb's outlier test (26) to detect outliers in the data sets using GraphPad statistical software (https://graphpad.com/quickcalcs/). Using this test, we found one outlier value in each of the immunoblot analyses of pHSL/HSL, HSL, and CD36 and removed these data points. We also found and removed one outlier from each of the mRNA expression analyses for *SORL1* and *SPP1*. All values are presented as Mean ± SD.

Results

FA Ra and cohort stratification

As expected, FA Ra varied widely among our subjects (Figure 4-1A). Subjects were stratified into FA Ra tertiles (Figure 4-1A), allowing for direct comparisons between subjects with the highest FA Ra values ("HIGH-FA"; ≥12.8 μmol/min/kg FM; n=10) vs. subjects with the lowest FA Ra values ("LOW-FA"; ≤8.7 μmol/min/kg FM; n=10). Subjects with intermediate/moderate FA Ra values ("MOD-FA"; 8.8-12.7 μmol/min/kg FM; n=10) were excluded from our primary analyses (i.e., HIGH-FA vs. LOW-FA; Figure 4-1B), but were included in our correlational analyses across the entire cohort. Expressing FA Ra relative to fat mass allowed us to evaluate differences in FA Ra independently of adipose tissue mass. Importantly, however, FA Ra remained significantly different ($p < 0.05$) between HIGH-FA and LOW-FA even when not normalized (Figure 4-1C) or when expressed relative to FFM (Figure 4-1D).

Subject characteristics

Age, body mass, and BMI were not different between groups (Table 4-2). However, LOW-FA had a significantly higher percentage of body fat (p<0.01) and lower FFM (p<0.01) compared with HIGH-FA (Table 4-2). Although the HIGH-FA group contained 3 males and 7 females, while the LOW-FA contained only females (n=10), between-group differences in body composition were still evident even when comparing only female subjects in each group (Table 4-3). There were no significant differences in fasting plasma concentrations of insulin, glucose, fatty acids, TG, or total cholesterol (Table 4-4).

FA Ra and insulin sensitivity

Subjects exhibited a wide range of insulin-mediated glucose uptake (i.e., insulin sensitivity) during the hyperinsulinemic-euglycemic clamp. In agreement with our previous findings (8), we found a highly significant negative correlation between insulin sensitivity and FA Ra across the entire cohort (p<0.001; Figure 4-2A), and 50% of the variability in insulin sensitivity among subjects was explained by the magnitude of FA Ra (R^2=0.50). In addition, mean insulin sensitivity was nearly 85% greater in LOW-FA vs. HIGH-FA (p=0.003; Figure 2B). While the insulin sensitivity data presented in Figure 2 was normalized per FFM, it is important to note that insulin sensitivity was also significantly different between LOW-FA and HIGH-FA when not normalized to FFM (Table 4-5). Furthermore, because the steady-state insulin concentration in plasma (SSI) during the clamp was not different between LOW-FA and HIGH-FA (Table 4-5), insulin sensitivity also remained significantly different (p=0.005) between the groups when normalized to SSI (GIR/SSI; Table 4-5). To assess whether differences in the sex distribution in our cohorts (HIGH-FA: 7 women and 3 men; LOW-FA: 10 women and 0 men) influenced our findings, we also compared insulin sensitivity between only the female subjects in our groups, and found the differences in insulin sensitivity remained significant when the 3 men were removed from the HIGH-FA group (Table 4-5); Therefore, it appears that the inclusion of men in the HIGH-FA group was not responsible for the observed differences in insulin sensitivity between the HIGH-FA and LOW-FA groups.

Adipose tissue

Lipolysis and esterification markers

We found a lower abundance (p=0.02) of phosphorylated hormone sensitive lipase (HSL) at serine 660 (HSL^{ser660}), a marker of HSL activity, and a trend (p=0.07) for a lower total HSL protein abundance in LOW-FA compared with HIGH-FA (Figure 5-3A and 5-3C). However, there was no difference in HSL mRNA expression between groups (Figure 5-3D). In contrast to HSL, neither protein abundance nor mRNA expression of adipose triglyceride lipase (ATGL) were different between groups (Figures 5-3A, 5-3C, and 5-3D). Extracellular signal-regulated kinase (ERK) pathway activation, which increases lipolysis, was also significantly lower in LOW-FA vs. HIGH-FA (p-ERK$^{Thr202/Tyr204}$/ERK; p=0.05; Figures 5-3A and 5-3C). We also found 3-fold greater protein abundance of glycerol-3-phosphate acyltransferase (GPAT) in LOW-FA compared with HIGH-FA (p=0.02; Figures 5-3B and 5-3C), despite no difference in *GPAT1* mRNA expression (Figure 5-3D). There were no differences in protein abundance or mRNA expression of diacylglycerol acyltransferase (DGAT) (Figures 5-3A-D).

Markers of lipogenesis, adipogenesis, lipid storage, and transport

mRNA expression of factors involved in lipid droplet storage (*PLIN1* and *CIDEA*) was not different between LOW-FA and HIGH-FA (Figure 5-3D). mRNA expression of factors involved in lipogenic and adipogenic processes (i.e., *PPARG*, *CEBPA*, and *SREBP1C*) was also similar between LOW-FA and HIGH-FA (Figure 5-3D). Lastly, we found no differences between LOW-FA and HIGH-FA in protein abundance of CD36, a primary fatty acid transporter (Figure 5-3B and 5-3C).

LOW-FA vs. HIGH-FA gene expression profile

We performed gene ontology (GO) analyses on the microarray data from LOW-FA and HIGH-FA to identify novel alterations in biological pathways that may underlie the observed differences in FA Ra between LOW-FA and HIGH-FA. Table 6 summarizes the three most highly upregulated and downregulated GO terms in LOW-FA compared to HIGH-FA that were statistically significant and biologically relevant. LOW-FA had lower enrichment of pathways related to ECM structure, organization, and disassembly. Surprisingly, the most highly

upregulated GO processes in LOW-FA were antigen receptor-mediated signaling, defense response, and lymphocyte differentiation, suggesting greater immune activity in LOW-FA adipose tissue. Of the 413 statistically significant differentially (>2-fold) expressed genes in LOW-FA compared with HIGH-FA (Figure 5-4A), 210 genes were upregulated and 203 were downregulated in LOW-FA vs. HIGH-FA. We examined the top genes (47 genes; Table 5-7that we thought may have an importantnovel involvement in adipose tissue metabolism and whole body metabolic outcomes. Out of 47 genes, 40 were related to the adipose tissue immune response, while other candidate genes were related to fatty acid metabolism, ECM, and mitochondria. Follow-up qPCR validation analyses performed for *SORL1*, *SPP1*, *MEFV*, *BTLA*, *KLRK1*, and *CXCR1* (Table 5-7) confirmed significant differences in mRNA expression between HIGH-FA and LOW-FA for these 6 genes (p<0.05; Figure 5-4B).

Inflammation and fibrosis

IPA analysis predicted significant (p=0.04) downregulation of the stress/inflammatory SAPK/JNK pathway in LOW-FA vs. HIGH-FA (Figure 5-5A). Follow-up western blot analysis confirmed significantly lower JNK pathway activation (p-JNK$^{Thr183/Tyr185}$/JNK) in LOW-FA compared with HIGH-FA (p=0.04; Figures 5-5B and 5-5C). We found no differences between groups for other stress-related signaling pathways (i.e., p38 MAPK or STAT3; Figures 5-5B and 5-5C). There were also no differences in protein expression of MCP-1 or MAC2, a crude marker of macrophage abundance (Figures 5-5B and 5-5C). qPCR analysis revealed no differences in mRNA expression of some canonical markers of inflammation (*TNFA* and *MCP1*) or *COL1A1* between LOW-FA and HIGH-FA (Figure 5-6A). However, in agreement with the GO analysis, *COL6A1* mRNA expression was lower in LOW-FA vs. HIGH-FA (p=0.009; Figure 5-6A), and *COL6A1* expression was positively correlated with FA Ra (R^2=0.29; p=0.003; Figure 5-6B).

Discussion

Our observation that FA Ra from subcutaneous adipose tissue is associated with insulin resistance in obesity is consistent with previous work from our lab (8) and others (9). Our findings suggest lower lipase activation, greater fatty acid esterification capacity, and alterations in ECM organization and fibrosis in subcutaneous adipose tissue may all contribute to the attenuated fatty acid release in our LOW-FA cohort, perhaps through an enhanced ability to sequester and store

fatty acids as TG within adipocytes. In contrast to our hypothesis, our microarray data revealed upregulated markers of immune activity in subcutaneous adipose tissue from LOW-FA vs. HIGH-FA. Because insulin sensitivity was relatively high in our LOW-FA subjects, these findings from our microarray conflict with the overly simplistic notion that upregulated adipose tissue immune activity directly underlies the development of insulin resistance. Furthermore, other novel mechanisms, perhaps involving SORL1, may be enhancing the ability of adipose tissue to suppress the release of fatty acids into circulation.

Excessive FA Ra from subcutaneous adipose tissue is causally linked with a host of cardio-metabolic complications, including insulin resistance (27). This is demonstrated clinically in patients with lipodystrophy, a condition characterized by an extraordinarily low capacity to store fatty acids in subcutaneous adipose tissue, leading to extreme insulin resistance (28). Lipodystrophic mice develop similar metabolic complications; however, these complications (e.g., insulin resistance, ectopic lipid deposition, etc.) can be reversed with transplantation of well-functioning subcutaneous adipose tissue (29). Although it may seem counter-intuitive, the ability to expand subcutaneous adipose tissue to accommodate nutrient oversupply may actually help prevent insulin resistance by enhancing the capacity to sequester fatty acids – thereby preventing excess fatty acid release and ectopic lipid deposition in insulin sensitive tissues (30). Our data suggest that LOW-FA subjects may have an enhanced ability to expand and store excess nutrients as TG in subcutaneous adipose tissue. While our data captures only a snapshot of the fat storage capacity in our subjects, other human studies that tracked long-term TG turnover and storage using deuterated water reported impaired TG synthesis and storage in subcutaneous adipose tissue of insulin-resistant obese adults (12). Our data further support the importance of subcutaneous adipose tissue in the control of FA Ra and, subsequently, peripheral tissue insulin sensitivity.

Differences in the expression, abundance, and activation of enzymes controlling lipolysis and esterification among our subjects may contribute to the variability in their FA Ra and may impact whole-body insulin resistance. Partial inhibition of lipolysis in obese mice through pharmacological or genetic manipulation of HSL resulted in lower fatty acid availability and improved insulin sensitivity without affecting fat mass (31). Lower markers of lipolysis are also correlated with reduced indices of insulin resistance in obese humans, independently of fat mass (31). In agreement, our LOW-FA group had lower markers of HSL activation (p-HSL^{ser660}/HSL) and ERK (p-ERK$^{Thr202/Tyr204}$/ERK) signaling, which are indicative of lower lipolytic activation (32,

33). Systemic fatty acid release from subcutaneous adipose tissue can also be attenuated by upregulated esterification, which can synthesize TGs from locally released fatty acids before they enter the circulation. Our finding that GPAT protein abundance (i.e., the enzyme catalyzing the first committed step of the esterification pathway (34)) was 3-fold greater in LOW-FA than HIGH-FA suggests greater esterification capacity may have also contributed to a lower systemic FA release. Together, our findings suggest that obese adults who maintain relatively low FA Ra (LOW-FA) may have reduced activation of basal lipolysis and an increased capacity for fatty acid esterification compared with obese adults with high FA Ra.

Our finding that markers of ECM organization and assembly were lower in LOW-FA vs. HIGH-FA is very intriguing and among the most novel findings of our study. Furthermore, to our knowledge, this is the first study demonstrating a strong direct correlation between FA Ra and collagen VI expression in human adipose tissue. Therefore, ECM-mediated alterations in adipose tissue fatty acid metabolism may be an important contributor to the well-described relationship between excess accumulation of certain adipose tissue ECM components (including collagen VI) and insulin resistance in obesity (35). Subcutaneous adipose tissue collagen VI expression has been found to be positively correlated with BMI, fat mass, and insulin resistance, and is upregulated during short-term overfeeding (36). In agreement, mice lacking collagen VI were protected from high-fat diet induced insulin resistance (37). The maintenance of insulin sensitivity in these mice was accompanied by larger, more hypertrophied adipocytes despite similar fat mass, indicating the lack of collagen VI may have allowed the adipocytes to expand with less constraint, providing improved capacity for fatty acid storage. Having less subcutaneous adipose tissue fibrosis, resulting in enhanced adipocyte lipid storage capacity, lower mobilization of fatty acids, and a resultant lower ectopic lipid deposition is likely advantageous for maintaining insulin sensitivity in obese humans as well.

Fibrotic adipose tissue is also often accompanied by an elevated inflammatory profile (18, 38), which is a key contributor to metabolic dysfunction in obesity (39). However, despite evidence for higher adipose tissue fibrosis and lower insulin sensitivity in HIGH-FA vs. LOW-FA, our finding that adipose tissue immune activity was lower in HIGH-FA compared with LOW-FA conflicts with this notion. The adipose tissue immune response is very complex, and inflammatory status clearly cannot be classified simply as "pro-inflammatory" and "anti-inflammatory". The regulation of macrophages, neutrophils, lymphocytes, and T cell activity has

many redundant, overlapping mechanisms controlling whether these cells confer pathogenic or protective functions. While macrophages are commonly linked to the development and maintenance of fibrosis, they are also involved in the inhibition and reversal of fibrosis (40, 41), which corroborates our data. Although overall immune activity may be elevated, there could be a compensatory rise in mechanisms that mitigate the detrimental effects of increased immune activity on adipose tissue function, such as *MEFV* and *BTLA*. MEFV plays a role in the degradation of several inflammasome components (42), while BTLA is involved in inhibiting the Th1 "pro-inflammatory" T cell response (43). Furthermore, our LOW-FA group had higher mRNA expression of *CXCR1*, a powerful chemoattractant factor that activates neutrophils (44), and lower expression of *SPP1*, which is related to induction of the inflammatory response in obesity (45). Unfortunately, without specific sorting and analysis of the adipose tissue immune cells, we can only speculate on the "protective" vs. "pathogenic" immune cell abundance and activity in the LOW-FA group. Nevertheless, these data suggest an uncoupling of the immune inflammatory response and the development of insulin resistance in obese, yet otherwise healthy, individuals.

Despite evidence of higher overall adipose tissue immune activity, SAP/JNK pathway activation was lower in adipose tissue from LOW-FA compared with HIGH-FA. The SAPK/JNK pathway is activated in multiple tissues in obesity, including adipose tissue, and is implicated in orchestrating the relationship between inflammation and poor metabolic outcomes (46). While the SAPK/JNK pathway can interfere with insulin signaling and promote insulin resistance (47), SAPK/JNK pathway activation in macrophages may also be important for controlling basal fatty acid release from adipose tissue. For example, reducing JNK pathway activity in macrophages alters macrophage polarization and lowers the expression and release of pro-inflammatory cytokines that may cause excess FA Ra (48, 49). Therefore, the lower SAPK/JNK pathway activation in LOW-FA may explain why this group can maintain a lower FA Ra despite elevated overall immune activity. Our findings demonstrate that preserving low JNK pathway activation in adipose tissue in obesity may be important for maintaining low FA Ra and preserving whole body insulin sensitivity in humans.

Another novel finding was the greater mRNA expression of sortilin-related receptor (*SORL1*) in adipose tissue from LOW-FA vs. HIGH-FA. SORL1 is best known for its role in the neurodegenerative processes involved in Alzheimer's disease (50). However, Schmidt et al.

demonstrated an important role for SORL1 in modulating adipose tissue metabolic processes (51). For example, *SORL1* overexpression in mice reduced TG hydrolysis, while inactivation of SORL1 increased TG breakdown and fatty acid release from adipose tissue (51). Perhaps the elevated *SORL1* expression we found in subcutaneous adipose tissue from our LOW-FA subjects may be beneficial for sequestering fatty acids, reducing their release into the systemic circulation.

While our data provide important insight into possible mechanisms protecting some obese adults from developing insulin resistance, there are some limitations to this study. First, although our findings identify several factors that may underlie differences in FA Ra among our cohort of obese subjects, our study does not directly address causation, which is always very challenging in human studies. Second, because our measurements of FA Ra, gene expression, and protein abundance were performed on samples collected during the post-absorptive state, our conclusions do not address alterations in adipose tissue in the post-prandial state (i.e., insulin-stimulated). Relevant to this, we acknowledge that varying degrees of adipose tissue resistance to the anti-lipolytic effects of insulin could also contribute to differences in FA Ra among our subjects. However, because our measurements were made in the post-absorptive state (i.e., when plasma insulin concentration was at its lowest point), the contribution of differences in adipose tissue insulin resistance on FA Ra was likely rather small in our study. More importantly, we contend that ectopic lipid deposition (and its negative impact on whole-body insulin resistance) is primarily affected by the much higher FA Ra that occurs in the post-absorptive state, which is why we focused on assessing differences in FA Ra among our subjects after an overnight fast. Unfortunately, because we could not measure plasma catecholamines due to inadequate sample availability, we do not know if differences in circulating catecholamines may have also contributed to the differences in FA Ra between our groups. Finally, we did not have adequate adipose tissue samples to perform histological measurements, which could provide more information about adipose tissue fibrosis and inflammation. These measures will be incorporated in follow-up studies.

In conclusion, our findings support the importance of FA Ra from adipose tissue in modulating the degree of insulin resistance in obese adults. Obese adults who can maintain low FA Ra may do so via reduced activation of lipolytic pathways (ERK and HSL) and enhanced fatty acid esterification. The reduced activation of lipolytic pathways may also be mediated, in part, by increased *SORL1* expression and/or reduced SAPK/JNK pathway activation. Furthermore, obese

adults who maintain low FA Ra have lower markers of fibrosis and ECM deposition, potentially enhancing their ability to store fatty acids as TG within adipocytes and protecting them from ectopic lipid deposition. Expanding our understanding about what dictates systemic fatty acid release from adipose tissue may lead to the development of treatments or therapeutic interventions that can reduce and/or prevent insulin resistance in obesity.

Table 1: Primary antibodies

α- phosphorylated extracellular signal-regulated kinase (p-ERK1/2$^{Thr202/Tyr204}$) (Cell Signaling #9101)
α- extracellular signal-regulated kinase (p-ERK1/2$^{Thr202/Tyr204}$) (Cell Signaling #9102)
α- phosphorylated hormone sensitive lipase (p-HSL^{ser660}) (Cell Signaling #4126)
α- hormone sensitive lipase (HSL) (Cell Signaling #4107)
α- adipose triglyceride lipase (ATGL) (Cell Signaling #2138)
α- glycerol-3-phosphate acyltransferase 1 (GPAT1) (Sigma-Aldrich, PRS4613)
α- diacylglycerol acyltransferase (DGAT) (Novus Biologicals, NB110-41487)
α- phospho-signal transducer and activator of transcription 3 (p-Stat3^{Tyr705}) (Cell Signaling, #9131)
α- signal transducer and activator of transcription 3 (Stat3) (Cell Signaling, #4904)
α- phospho-p38 MAPK$^{Thr180/Tyr182}$ (Cell Signaling #9211)
α- p38 MAPK (Cell Signaling #9212)
α- monocyte chemotactant protein 1 (MCP1) (Cell Signaling #2720)
α- Galectin-3 (MAC2) (Abcam Inc., ab2785)
α- fatty acid translocase (FAT/CD36) (Abcam Inc., EPR6573)

Table 4-1: Primary antibodies

	HIGH-FA	LOW-FA
Sex (M/F)	3/7	0/10
Age (y)	28 ± 2	34 ± 3
BMI (kg/m^2)	36 ± 1	37 ± 1
Mass (kg)	106 ± 6	99 ± 3
Fat mass (kg)	45 ± 5	52 ± 2
Fat free mass (kg)	61 ± 4	47 ± 1*
Body Fat (%)	44 ± 2	53 ± 1*

BMI: Body Mass Index. * significant difference compared to HIGH-FA, p<0.05. Data presented as Mean ± SD.

Table 4-2: Physical characteristics

	HIGH-FA	LOW-FA
Sex (M/F)	0/7	0/10
Age (y)	30 ± 2	34 ± 3
BMI (kg/m^2)	37 ± 1	37 ± 1
Mass (kg)	102 ± 7	99 ± 3
Fat mass (kg)	48 ± 5	52 ± 2
Fat free mass (kg)	54 ± 3	47 ± 1*
Body Fat (%)	47 ± 2	53 ± 1*

BMI: Body Mass Index. * significant difference compared to HIGH-FA, p<0.05. Data presented as Mean ± SD.

Table 4-3: Physical Characteristics of only women

	HIGH-FA	LOW-FA
Glucose (mM)	4.9 ± 0.2	4.6 ± 0.1
Insulin (μU/mL)	21.3 ± 3.8	18.7 ± 2.3
NEFA (μM)	606 ± 61	493 ± 54
Triglycerides (mg/dL)	73 ± 13	49 ± 8
Total cholesterol (mg/dL)	161 ± 7	146 ± 11

Data presented as Mean ± SD.

Table 4-4: Blood measurements from fasting condition

A	HIGH-FA (n=10)	LOW-FA (n=10)	P value
Glucose infusion rate (GIR: mg/min)	446 ± 182	610 ± 128*	0.04
Steady-state insulin (SSI: μU/ml)	324 ± 78	287 ± 64	0.28
GIR/SSI	1.4 ± 0.5	2.2 ± 0.5*	0.005

B	HIGH-FA (n=7; all women)	LOW-FA (n=10; all women)	P value
Glucose infusion rate (GIR: mg/min)	429 ± 145	610 ± 128*	0.02
Steady-state insulin (SSI: μU/ml)	321 ± 87	287 ± 64	0.38
GIR/SSI	1.3 ± 0.4	2.2 ± 0.5*	0.002

Values are Means ± SD. GIR: Glucose infusion rate; SSI: steady-state insulin concentration in plasma.
* Significantly greater than HIGH –FA, P<0.05.

Table 4-5: Insulin sensitivity data

GO term	GO description	Q value	Genes	LOW-FA vs HIGH-FA Direction
GO:0022617	extracellular matrix disassembly	6.04E-06	*FN1, TIMP1, COL1A2, COL6A1, LAMB1, COL5A2, COL16A1*	down
GO:0030198	extracellular matrix organization	6.04E-06	*APP1, FN1, TIMP1, COL1A2, ITGA3, CD151, SERPINH1, COL6A1, LAMB1, MUSK, COL5A1, APLP1, COL16A1, CCDC80*	down
GO:0043062	extracellular structure organization	6.04E-06	*APP1, FN1, TIMP1, COL1A2, ITGA3, CD151, SERPINH1, COL6A1, LAMB1, MUSK, COL5A1, APLP1, COL16A1, CCDC80*	down
GO:0050851	antigen receptor-mediated signaling pathway	4.57E-04	*LCK, CD38, UBA52*	up
GO:0098542	defense response to other organism	0.001437458	*LCK, DDX58, ISG15, DMBT1, BCL3, IFITM1, HERC5, BNIP3L, HIST1H2BJ*	up
GO:0030098	lymphocyte differentiation	0.00292656	*LCK, ATP7A, POU1F1, CD27, BCL3, SLC46A2*	up

Table 4-6: Gene ontology analysis of adipose tissue genes in LOW-FA compared with HIGH-FA

			LOW-FA relative to HIGH-FA	
		Gene		**Fold change**
Fatty acid metabolism		AADAC	Arylacetamide Deacetylase	**0.24**
		FFAR3	Free Fatty Acid Receptor 3	**0.40**
		SORL1	Sortilin-related receptor	**2.71**
Extracellular Matrix		MMP9	Matrix Metalloproteinase 9	**0.41**
		MMP7	Matrix Metalloproteinase 7	**0.45**
		MXRA5	Matrix-Remodelling Associated 5	**0.45**
Immune response	*Antigen presentation*	HLA-DRB1	Major Histocompatibility Complex, Class II, DR Beta 1	**0.35**
		HLA-DQB1	Major Histocompatibility Complex, Class II, DQ Beta 1	**2.88**
		LILRA	Leukocyte Immunoglobulin Like Receptor A1	**2.40**
	T cell	CCL19	Chemokine (C-C Motif) Ligand 19	**0.48**
		TRBV11	T Cell Receptor Beta Variable 11-1	**0.46**
		TRGJP2	T Cell Receptor Gamma Joining P2	**3.80**
		TRGJ2	T cell receptor gamma joining 2	**3.03**
		TAGAP	T-Cell Activation RhoGTPase Activating Protein	**2.79**
		TRAJ17	T cell receptor alpha joining 17	**2.39**
		TRAT1	T Cell Receptor Associated Transmembrane Adaptor 1	**2.35**
		TRAJ49	T cell receptor	**2.35**
		TRAJ14	T cell receptor	**2.19**
		TRAJ42	T cell receptor	**2.19**
		TRAJ47	T cell receptor	**2.18**
	T cell activation	NKG7	Natural Killer Cell Granule Protein 7	**2.77**
		BTLA	B and T Lymphocyte Associated	**2.73**
		TXK	Non-receptor tyrosine kinase	**2.31**
		KLRK1	killer cell lectin like receptor K1	**2.20**
		GZMA	Granzyme A	**3.15**
		CD3D	CD3	**2.16**
	"Anti" inflammatory	MEFV	Mediterranean Fever	**2.53**
		SPP1	Secreted Phosphoprotein 1	**0.35**
		LBP	Lipopolysaccharide Binding Protein	**0.43**
		FPR3	Formyl Peptide Receptor 3	**0.54**
	Neutrophils	CSF3R	Colony stimulating factor 3	**2.36**

	CXCR1	Chemokine (C-X-C Motif) Ligand 1	**4.20**
	VNN2	Vanin 2	**3.60**
Lymphocyte related	SELL	Selectin L	**3.40**
	FPR1	Formyl Peptide Receptor 1	**2.73**
	S1PR4	Shpingosine-1-phosphate receptor 4	**2.44**
	MS4A1	Membrane Spanning Protein 4 A1	**2.55**
Cytokines / other	IL23A	Interleukin 23 Subunit Alpha	**3.15**
	IL18	Interleukin 18 receptor accessory protein	**2.33**
	CLEC1B	C-Type Lectin Domain Family 1 Member B	**3.01**
	RNASE2	Ribonuclease A Family Member 2	**2.75**
	GBP5	Guanylate Binding Protein 5	**2.75**
	IFIT1B	Interferon Induced Protein	**2.47**
	DEFA	Defensin	**2.42**
	EMR3	EGF-like module-containing mucin-like hormone receptor-like 3	**2.38**
	MNDA	Myeloid Cell Nuclear Differentiation Antigen	**2.36**
Mitochondria	YME1L1	YME1 Like 1 ATPase	**3.15**

Table 4-7: Genes of interest over 2-fold different in LOW-FA compared with HIGH-FA
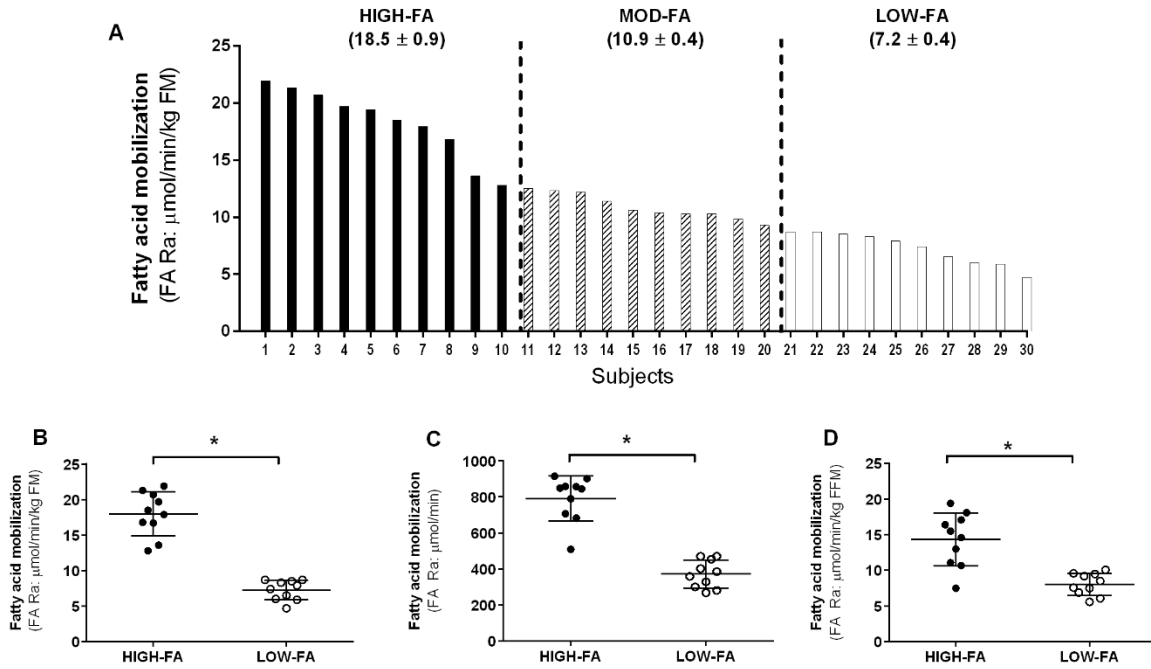
Figure 4-1: FA Ra variability across all subjects. (A) FA Ra across the entire cohort (n=30) normalized to FM (B) in HIGH-FA (black circles) vs LOW-FA (white circles) normalized to FM (C) in HIGH-FA vs LOW-FA expressed as total FA Ra (D) in HIGH-FA vs LOW-FA normalized to FFM. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.
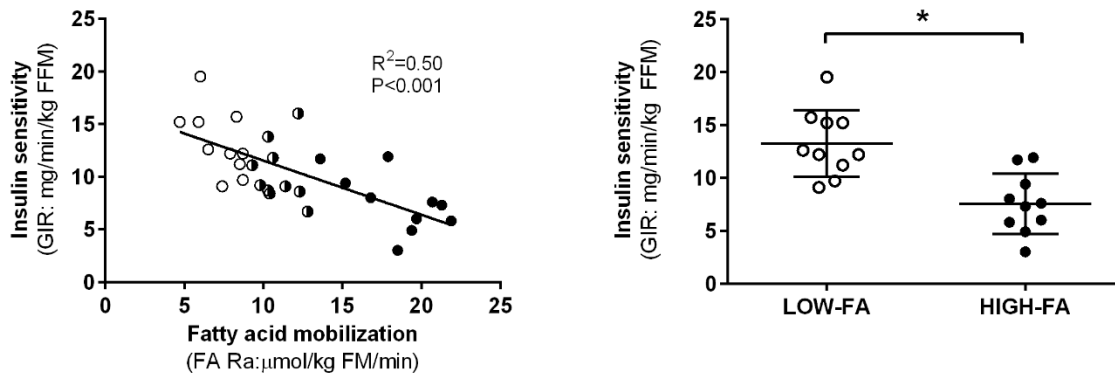
Figure 4-2: FA Ra and insulin sensitivity. (A) Correlation between insulin-mediated glucose uptake during the clamp (insulin sensitivity) and FA Ra (LOW-FA [n=10; white circles], MOD-FA [n=10; half black, half white circles], HIGH-FA [n=10; black circles]) (B) Insulin sensitivity in LOW-FA vs HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.
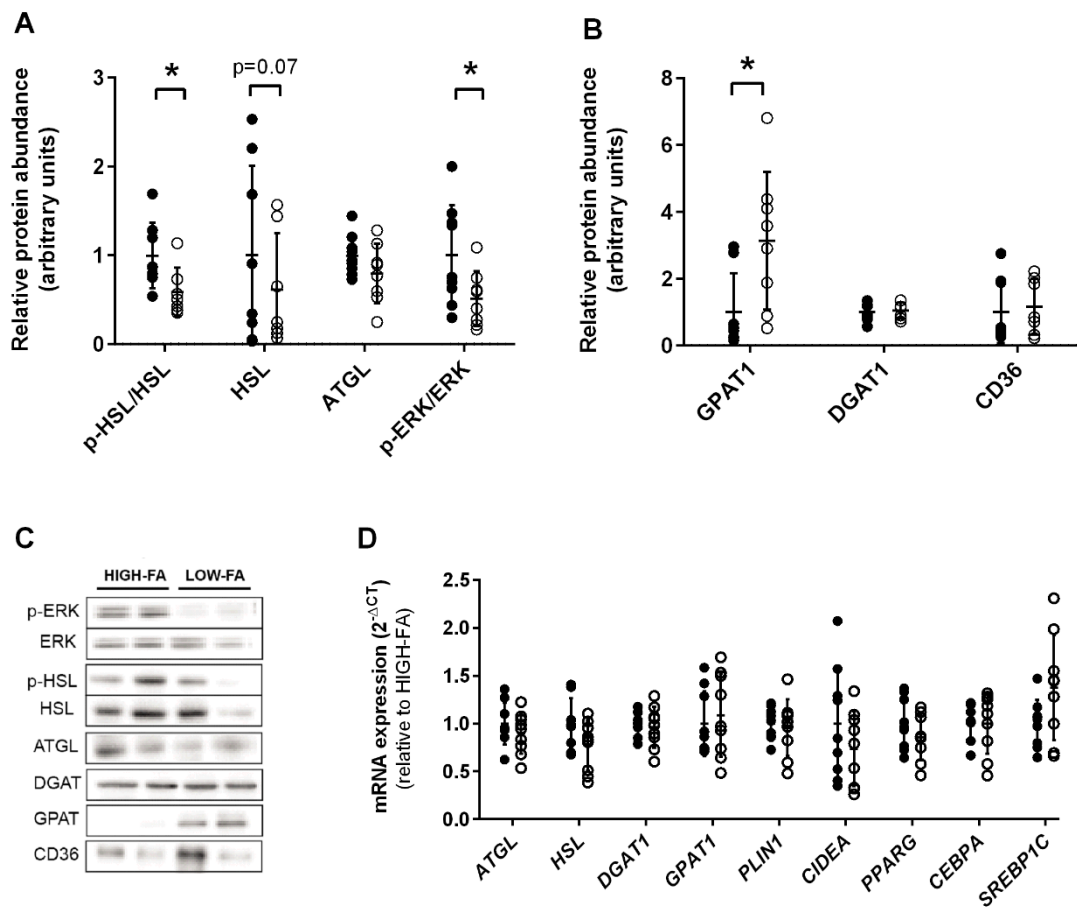
Figure 4-3: Markers of lipolysis, esterification, and fatty acid uptake. (A) Relative protein abundance of proteins related to lipolysis normalized to HIGH-FA. (B) Relative protein abundance of proteins related to esterification and fatty acid uptake expressed relative to HIGH-FA. (C) Representative images for western blotting analysis of proteins related to lipolysis, esterification, and fatty acid uptake. (D) mRNA expression of factors related to lipolysis, esterification, lipogenesis, and fatty acid storage in LOW-FA (n=9; white circles) compared with HIGH-FA (n=9; black circles). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M* and then expressed relative to HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.
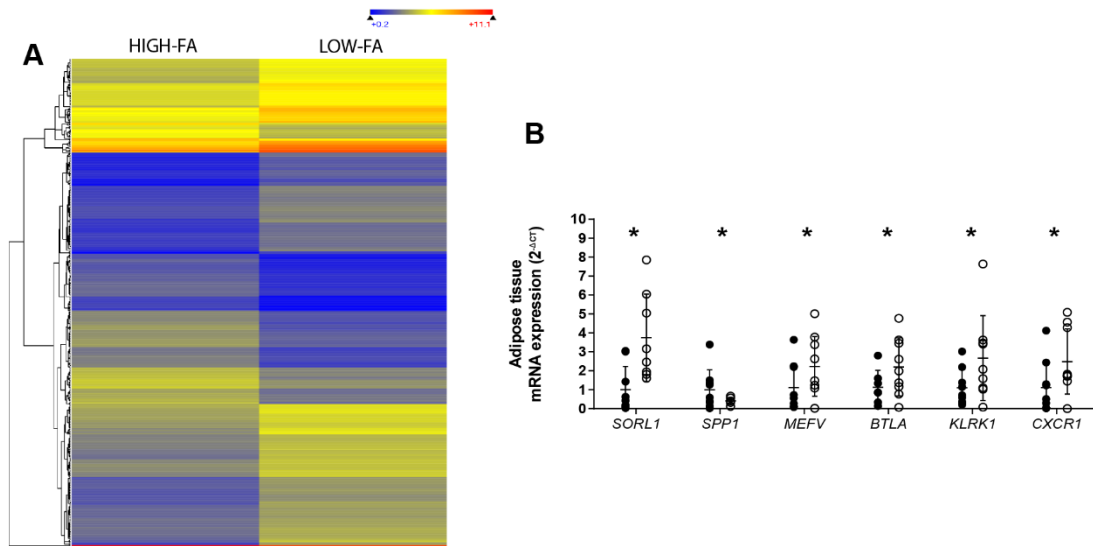
Figure 4-4: Microarray and gene expression analysis of adipose tissue. (A) Heatmap representing genes 2-fold differentially expressed in LOW-FA compared with HIGH-FA. (B) qPCR validation of mRNA expression of 6 genes found to be greater than 2-fold different in LOW-FA (n=9, white circles) compared with HIGH-FA (n=9, black circles). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M* and then expressed relative to HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.
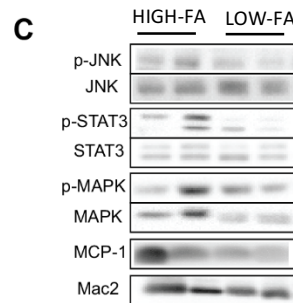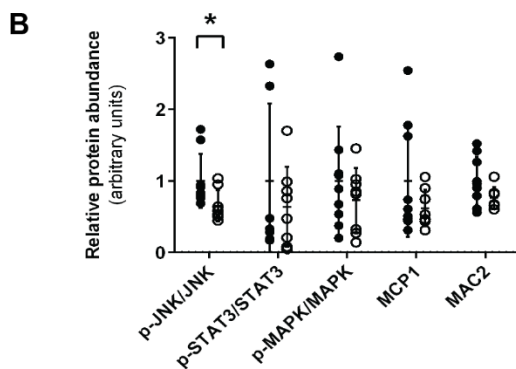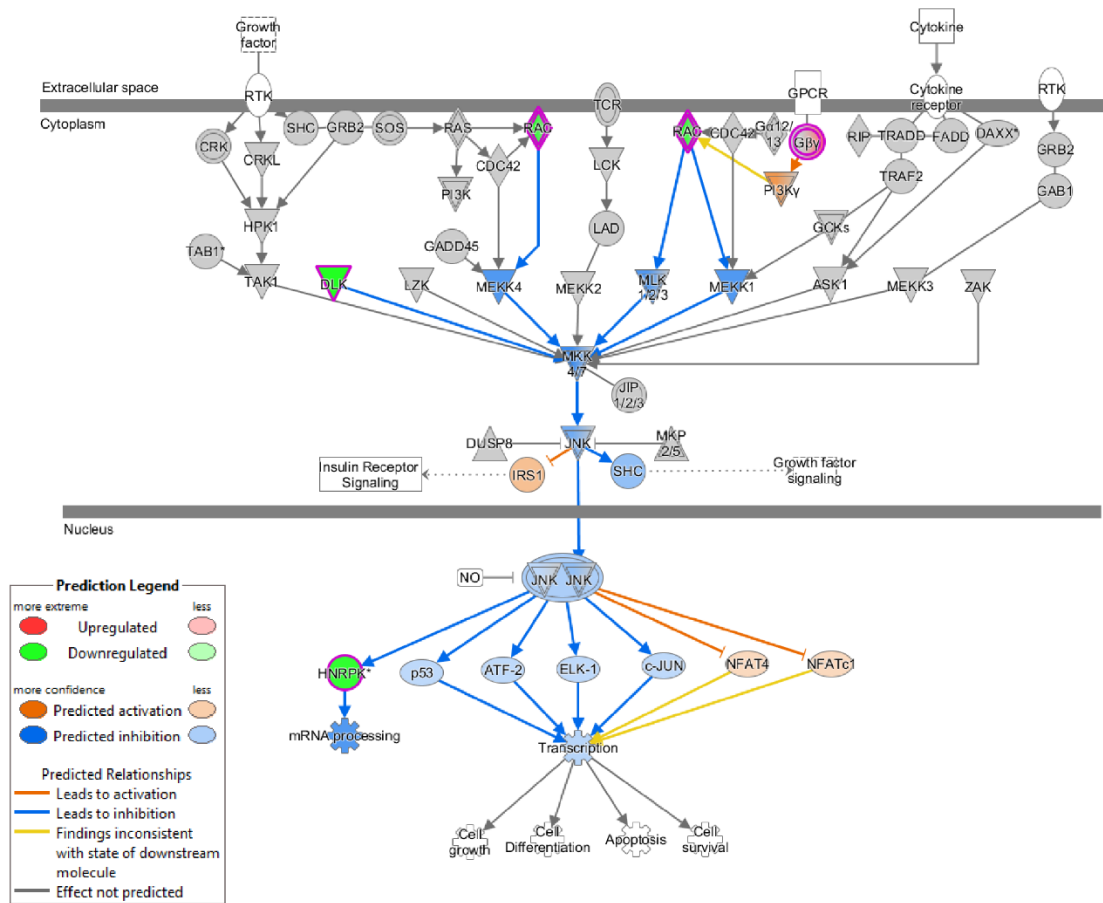
Figure 4-5: SAPK/JNK and stress/inflammatory pathway activation. (A) Ingenuity Pathway Anlaysis identified the SAPK/JNK pathway to be significantly downregulated in LOW-FA compared with HIGH-FA (p=0.04) (B) Relative protein abundance of proteins related to stress pathway activation and inflammation expressed relative to HIGH-FA (LOW-FA n=8, white circles; HIGH-FA n=9, black circles). (C) Representative blots. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.
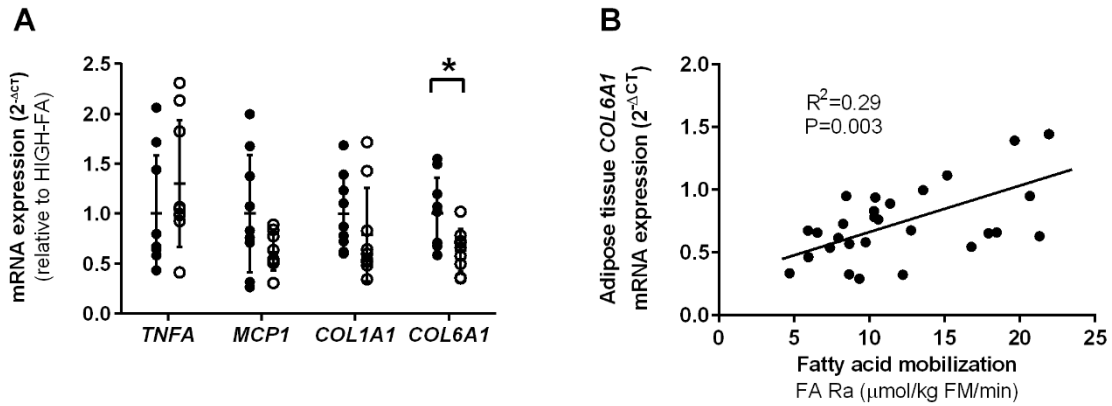
Figure 4-6: Markers of inflammation and fibrosis. (A) mRNA expression of factors related to inflammation and fibrosis in LOW-FA (n=7-9; white circles) compared with HIGH-FA (n=7-9; black circles). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M* and then expressed relative to HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA. (B) Correlation between *COL6A1* mRNA expression and FA Ra across 28 subjects.

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CHAPTER 5

Aerobic exercise elevates markers of angiogenesis and alternative macrophage activation in the subcutaneous adipose tissue of overweight to obese adults

Abstract

Alterations in the inflammatory state, metabolic function, and structure of subcutaneous adipose tissue (SAT) can greatly impact the development of insulin resistance in obesity. Exercise is often prescribed to improve metabolic health in obesity, but the effects of exercise on SAT are not well known. The primary aims of this study was to examine the effects of exercise on mRNA expression of markers of lipid metabolism (*ATGL, HSL, DGAT, GPAT, PPARG, SREBP1c, PLIN1*), inflammation (*TNFA, IL1B, NLRP3, MCP1, CD11C, CD206*), fibrosis (*COL6A1, COL1A1*), and hypoxia/angiogenesis (*HIF1A, VEGF, CD31, GLUT1*) in SAT, as well as adipocyte cell size. We recruited overweight-to-obese adults who exercise regularly (ACTIVE: n=8) or were sedentary (SED: n=12). The groups were well-matched for age (27±1 vs. 24±2 y), BMI (29±1 vs. 27±1 kg/m2), and body composition (30±1 vs 29±1 % body fat), but as expected, cardiorespiratory fitness was greater in ACTIVE vs. SED (VO$_2$peak: 51±3 vs. 42±1 ml/kg FFM/min; p=0.03). Abdominal SAT biopsy samples were obtained before and 1hr after 60min of aerobic exercise (~65% VO$_2$peak). The exercise session increased SAT mRNA expression of *VEGFA*, an important regulator if angiogenic processes, in both groups. In addition, SAT from ACTIVE subjects had a higher mRNA expression of the endothelial cell marker, *CD31* compared with SED. This elevation is suggestive an increase SAT capillarization in our habitual exercisers, which may be the cumulative effect of the transient increases in *VEGFA* with each of their regular exercise sessions. We also magnetically sorted CD14+ immune cells from SAT samples and found that expression of *IL6* was elevated in ACTIVE compared with SED. Exercise did not affect the expression of other inflammatory factors, markers if lipid metabolism, or extracellular matrix. In conclusion, exercise initiates increases in factors related to angiogenic processes and may promote alterations in macrophage inflammation in SAT.

Introduction

Regular exercise is known to have overall health benefits and is often prescribed to combat obesity-related metabolic complications that can lead to metabolic diseases, such as type 2 diabetes (T2D) and cardiovascular disease (CVD) (1). Elevated cardiorespiratory fitness is associated with reduced CVD risk factors, as well as reduced incidence of CVD and T2D (2, 3). While most of the focus on exercise adaptations that benefit metabolic health has centered on changes in skeletal muscle, exercise-induced changes in other tissues and organs may also contribute to the metabolic health effects of exercise (4). In particular, adaptations to exercise in adipose tissue may be important for mediating whole body metabolic outcomes since it is now well-recognized that adipose tissue health and function plays a prominent role in dictating obesity-related metabolic complications (5-7).

While the adipose tissue can actively secrete peptides (i.e. adipokines) that may have important systemic effects, complexities associated with the regulation of lipid storage within adipocytes can also have profound metabolic consequences (8-10). Limitations in the lipid storage capacity of adipose tissue can expose peripheral tissues to high levels of fatty acids, which can impair insulin signaling (11-13) and disrupt other cellular processes (14, 15). Several factors within adipose tissue are postulated to be important for determining its lipid storage capacity including lipase and esterification enzymes (16, 17), adipocyte cell size (18), extracellular matrix structure (19, 20), capillarization (21), and inflammation (22, 23). Several studies have reported beneficial effects of exercise on adipose tissue inflammation, fibrosis, and adipose tissue insulin sensitivity in humans, but these studies are confounded by a reduction in body fat mass that accompanied their exercise interventions(24-27), and even modest weight loss can profoundly impact adipose tissue and whole body metabolic outcomes (25, 27-29). However, recent animal studies show promising evidence for beneficial direct effects of exercise on adipose tissue structure and metabolic function (30-34).

It remains unclear whether exercise, acute or chronic, causes beneficial adaptations in adipose tissue that can improve obesity-related metabolic complications in humans. Therefore, the purpose of this study was to examine the effects of acute and habitual exercise training on the expression of factors related to adipose tissue fatty acid release and storage (e.g., lipolysis, esterification, lipogenesis, adipogenesis), the composition of the extracellular matrix, angiogenesis, adipose tissue cell size, and inflammation in adipose tissue. We hypothesized that

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acute exercise will stimulate factors that may lead to improved metabolic health and function of the adipose tissue, and that habitual exercisers will have markers of improved metabolic health in their adipose tissue compared to sedentary obese adults.

Methods

Subjects

A total of 20 overweight-to-mildly obese individuals, (body mass index [BMI]: 25-35 kg/m^2) between the ages of 18 and 40 were recruited for the study. Subjects completed a comprehensive behavior survey/questionnaire, and were categorized into the different cohorts based on their self-reported habitual physical activity behavior. Twelve of the subjects were non-exercisers (SED: n=12) while 8 subjects exercised regularly (ACTIVE: n=8) (i.e. at least 4 days/wk of aerobic/cardiovascular exercise for 30-60min/session at moderate and vigorous intensities Participants were not taking any medication known to effect metabolic outcomes. All participants were non-smokers and weight stable (within ± 2kg for ≥ 6 months). None of the subjects had any history of metabolic or cardiovascular disease that would impact study outcomes. Written, informed consent was obtained from all subjects before initiating participation and all study procedures were approved by the University of Michigan Institutional Review Board.

Preliminary testing

Maximal oxygen consumption (VO$_2$max)

Subjects completed a graded exercise test that involved a 4 minute warm-up, followed by an increase in exercise workload (increased speed and grade on treadmill or increased resistance on bike) every 1-2 minutes until the subject reached volitional fatigue. The participant had the choice to perform the test on either a stationary bike (Lode ergometer) or treadmill, and importantly, the modality of exercise chosen was the exercise modality used for the experimental procedures. Maximal oxygen consumption and maximal heart rate were recorded and used for calculating the exercise workload during the experimental trial.

Oral glucose tolerance test (OGTT)

Subjects arrived at the laboratory after an overnight fast to assess their glucose tolerance. Subjects who exercise regularly abstained from exercise for 3 days before the OGTT. Subjects drank a flavored solution containing 75 grams of glucose (Fisherbrand^{TM} GTT Beverage) and then we collected blood from an intravenous (IV) catheter inserted in to their arm vein every 15 minutes for 3 hours. Blood samples were centrifuged at 2000rpm at 4°C for 15 minutes, and plasma was aliquoted and stored at -80°C until analysis for blood glucose and insulin concentrations.

Body composition

Body composition was assessed using hydrostatic weighing (35). Briefly, subjects were weighed underwater after exhaling all air except residual lung volume using a scale attached to a chair in the water. Water temperature was maintained at 37°C. Residual lung volume was estimated using appropriate equations based on age, stature, and body mass. The appropriate Siri equation was then used to estimate percent body fat.

Experimental protocol

All subjects arrived to the laboratory in the morning of testing after an overnight fast (~0700h, ~10h fast). Furthermore, subjects who were regular exercisers refrained from exercise for 3 days prior to their experimental trial. After resting quietly for 30 minutes, we collected a baseline blood sample followed by collection of an abdominal subcutaneous adipose tissue sample using a needle aspiration method. This aspiration procedure involves injection of local anesthetic (2% lidocaine) in a region about 5cm lateral to the umbilicus. Adipose tissue is removed using a 16g needle with suction applied with a 10cc syringe. A portion of the adipose tissue biopsy was cleaned with saline, dried, and quickly frozen in liquid nitrogen, while a separate portion of cleaned sample was placed in phosphate buffered saline for separation of mature adipocytes and the stromal vascular fraction. Subjects then exercised on a treadmill or cycle ergometer for 1 hour at a moderate intensity (approximately 65%of their pre-determined VO$_2$max). Exactly 1h after exercise, we repeated the adipose tissue biopsy procedure along with a final blood draw. The timeline of the experimental trial is outlined in Figure 5-1.

Analytical procedures

Plasma substrate measurements

Plasma glucose (Thermo Scientific, Waltham, MA, USA) and fatty acid (Wako Chemicals USA, Richmond, VA, USA), concentrations were measured using commercially available colorimetric assay kits. Plasma insulin concentration was measured with a chemiluminescent immunoassay method (Siemens IMMULITE 1000, Flanders, NJ, USA).

Isolation of mature adipocytes and resident immune cells.

In order to differentiate changes in the various cell populations in the adipose tissue in response to exercise, we separated the mature adipocytes from the stromal vascular fraction. First, the adipose tissue was digested in Collagenase Type I (1mg/ml) in Hank's Balanced Salt Solution (HBSS) while gently rocking at 37°C for one hour. The digested tissue was strained through a 300µm nylon mesh filter to remove debris. The digested tissue was then be allowed to sit until all of the mature adipocytes floated to the top of the digested tissue. The SVF was then removed from underneath of the adipocytes. The mature adipocytes were used for RNA expression and cell sizing analysis (described below). The SVF was spun and pelleted at 500g for 7 minutes to allow for the removal of the supernatant and any residual mature adipocytes remaining. After 3 washes in PBS supplemented with 2% fetal bovine serum (to assist with maintaining cell viability), a commercially available immunomagnetic bead sorting kit (EasySep Human CD14 Positive Selection Kit, #18058, Stem Cell Technologies) was used to isolate the immune cell fraction from the SVF. The cell surface marker CD14 has been shown to be a marker specific to the macrophage population (36, 37), therefore, we used this marker as a means for identifying and isolating the adipose tissue macrophage population.

Adipose tissue mRNA expression

For isolation of RNA, sections of subcutaneous adipose tissue (~50mg), mature adipocytes, and CD14+ cells were homogenized in RNA Stat-60 (Tel-Test Inc., Friendswood, TX). RNA was isolated by chloroform extraction using a commercially available RNA isolation kit (Aurum total RNA fatty and fibrous tissue kit, Bio-Rad Laboratories, Inc.) and quantified spectrophotometrically. Reverse transcription was performed with 0.25µg of total RNA with the

High-Capacity cDNA RT kit (4368813, Life Technologies, Grand Island, NY). Real-time quantitative PCR was used to assess the mRNA expression levels of the genes listed in Table 5-1. Primer and probe sequences were designed for each gene's mRNA sequence using predesigned PrimeTime qPCR Assays (IDT). The qPCR data was normalized to expression of 2 housekeeping genes (peptidylprolyl isomerase A (PPIA) and beta-2-microglobulin (B2M)) using the -ΔCt method (38) and expressed as fold induction ($2^{-\Delta Ct}$) of mRNA expression.

Adipocyte size

We quantified the distribution of adipocyte cell size by imaging mature adipocytes using a brightfield microscope. A small portion of the isolated mature adipocytes (isolation described above) was added to an equal volume of formalin, gently mixed, and stored at 4°C. When ready to image the cells, 10µl of the suspension of fixed adipocytes were added to 20µl of mounting medium on a microscope slide and covered with a coverslip. Quantification of cell size was then performed using the protocol described by Parlee et al. (39) using ImageJ software.

Calculations

Index of whole body insulin sensitivity

Plasma glucose and insulin concentrations measured before and during the OGTT were used to assess insulin sensitivity using the Matsuda Composite Index (40). The Matsuda Composite Index has been found to be a reasonably accurate marker for insulin sensitivity when compared with the hyperinsulinemic-euglycemic clamp (40).

Statistical analysis

We used a two-way ANOVA with Tukey post-hoc analysis to assess significant differences in mRNA and adipocyte cell size between groups and in response to the single session of exercise. A Student's t-test was used to compare glycerol release in mature adipocytes and adipose explants before and after exercise. A P-value of <0.05 was considered statistically significant.

Results

Subject Characteristics

There were no differences age, body mass, body mass index (BMI), body fat percentage, fat mass (FM), or fat free mass (FFM) between SED and ACTIVE (Table 5-2). In addition, there were no differences in markers of insulin sensitivity or glucose tolerance between the groups (Table 5-2). As expected, however, ACTIVE had a higher VO_{2peak} compared with SED (52±3 vs. 42±2 ml/kg FFM/min, respectively; p=0.01) (Table 5-2).

Adipose tissue

Markers of angiogenesis, vascularization, and hypoxia

The acute exercise session significantly increased (p<0.05) mRNA expression of *VEGFA* (Figure 5-2A). Because VEGFA is a primary mediator of increased angiogenesis (41), we interpret this finding to suggest acute exercise may trigger an increase in angiogenesis in adipose tissue. In line with this finding, we found the expression of the endothelial cell marker, *CD31*, to be elevated (p<0.05) in our regular exercisers (i.e., ACTIVE > SED; Figure 5-2B). mRNA expression of *HIF1A* was also greater in ACTIVE compared with SED, but there was no effect of acute exercise on *HIF1A* expression in either group (Figure 5-2C). mRNA expression of *GLUT1*, a marker for elevated hypoxia, was not different between groups, nor was it altered after acute exercise (Figure 5-2D).

Markers of inflammation

mRNA expression of all genes measured related to inflammation (*TNFA, IL1B, NLRP3, MCP1, CD11C, CD206, CD163*) were not different between ACTIVE vs. SED before exercise or 1 hour after the exercise session in the whole tissue homogenate (Figure 5-3). In most whole tissue samples, the mRNA expression of *IL6* was undetectable or had ΔCt values higher than 35, therefore we did not use this data. The expression of *IL1B, NLRP3, MCP1, CD11C,* and *CD206* were also not different between groups in the CD14+ enriched fraction (Figure 5-4). However, *IL6* expression in the CD14+ enriched fraction was 4-fold greater in ACTIVE vs. SED (main effect for group; p<0.05) (Figure 5-4G). While the acute session of exercise did not significantly affect any of the inflammatory markers we measured in either the whole tissue homogenate (Figure 5-2) or the CD14+ enriched fraction (Figure 5-3), we did find trends for the exercise

session to lower the expression of *TNFA* (p=0.09) and *IL6* (p=0.055) and in the CD14+ fraction (Figures 5-4A and 5-4G).

Lipid metabolism

In vitro and *ex vivo* lipolytic rates (both unstimulated and isoproterenol-stimulated) measured in mature adipocytes and adipose tissue explants collected before exercise were largely similar in ACTIVE and SED (Figure 5-5). Importantly, the acute exercise session blunted isoproterenol-stimulated glycerol release in explants from ACTIVE subjects (Figure 5-5B), but this effect did not quite reach statistical significance in SED. Examining the acute effects of exercise in ACTIVE and SED combined, we found that acute exercise significantly lowered isoproterenol-stimulated glycerol release in both mature adipocytes and adipose tissue explants (p<0.05; Figures 5-5C and 5-5D). There were no effects of acute exercise on the expression of genes related to lipolysis (*ATGL, HSL*), esterification (*GPAT, DGAT*), lipid storage (*PLIN1, CIDEA*), lipogenesis, or adipogenesis (*SREBP1C, PPARG*) in mature adipocytes extracted from adipose tissue samples collected 1h after exercise compared with before exercise in either ACTIVE or SED, and no difference in the expression of any of these factors between groups (Figure 5-6).

Extracellular matrix and adipocyte cell size

mRNA expression of *COL6A1* and *COL1A1* were similar in ACTIVE and SED subjects before exercise or 1 hour after the exercise session (Figure 5-7). Additionally, we found no differences in the proportion of small adipocytes (<5000µm^2) or in the average adipocyte size between the groups. The distribution of adipocytes cell size was very similar between ACTIVE and SED (Figure 5-8).

Discussion

The main findings of this study suggest that exercise may trigger an increase in angiogenesis and capillarization in the subcutaneous adipose tissue of overweight and obese subjects. More specifically, a single session of aerobic exercise increased adipose tissue mRNA expression of *VEGFA* in both habitual exercisers and non-exercisers. An elevation in *VEGFA* after each exercise session may underlie an increased adipose capillarization in the habitual

exercisers, as suggested by higher expression of the endothelial cell marker, *CD31* in adipose tissue from this group. Habitual exercisers also had a higher expression of IL6, a cytokine important for alternative macrophage activation, in the CD14+ enriched fraction of their adipose tissue. We did not find any other effects of acute or habitual exercise on our measured markers of adipose tissue fibrosis, adipogenesis, adipocyte cell size, or other markers of inflammation. Overall, our data suggests that exercise may initiate angiogenic processes and cause alternative macrophage activation in the subcutaneous adipose tissue of obese adults.

Inadequate adipose tissue vascularization and hypoxia have been implicated as primary mediators of metabolic complications in obesity (42, 43). Angiogenic processes are stimulated by reductions in oxygen tension and nutrient availability, which signal for vessel formation primarily through the VEGF family of proteins (44). The VEGF family of proteins is considered to be one of, if not the most important regulators of endothelial cell growth (41). Binding of VEGF to VEGF receptors on endothelial cells initiates a signaling cascade that promotes proliferation, migration, and vessel formation (41). While we did not observe differences in baseline measures of *VEGFA* between ACTIVE and SED, a single session of aerobic exercise increased *VEGFA* mRNA in both groups. Repeated exercise bouts in the ACTIVE group that elevate *VEGFA* gene expression may lead to stable increases in capillarization as evidenced by the elevated mRNA expression of *CD31* in ACTIVE compared with SED. To our knowledge, only one study has examined whether exercise training without weight loss can alter capillarization in adipose tissue in obese humans (45). Walton et al. (45) reported that 12 weeks of aerobic exercise training in a group of insulin-sensitive obese adults was sufficient to increase CD31 mRNA expression and the density of capillaries in subcutaneous adipose tissue. Interestingly, Walton et al., (45) found no effect of exercise training on adipose tissue capillary density in their subjects who were "insulin resistant". The average BMI for their insulin resistant cohort was ~35kg/m^2, compared with ~26kg/m^2 in their insulin sensitive cohort, therefore it is possible that exercise has different effects on the angiogenic processes in the adipose tissue of obese adults who are more obese and/or insulin resistant. The reason why exercise may not augment angiogenesis and capillarization in obese adults with insulin resistance is not clear. Perhaps the higher fat mass in the insulin resistant cohort has higher inflammation, fibrosis, or other metabolic abnormalities that may inhibit angiogenic processes.

Hypoxia in adipose tissue can increase inflammation and disrupt normal glucose and lipid metabolism in obesity (42, 46). Our finding that adipose tissue *GLUT 1* expression (i.e., a surrogate marker of hypoxic conditions and changes in oxygen tension (47-49)was not different between our habitual exercisers and non-exercisers provides crude data to suggest that adipose hypoxia may not be different between these groups. We recognize that this evidence for no difference in hypoxia between our groups may appear to contradict our findings suggesting higher capillarization in our habitual exercisers, but because our subjects were only over weight-to-mildly obese (average BMI ~28kg/m^2) it is possible that the differences in capillarization may not manifest into measurable differences in markers of hypoxia in this population.

HIF1-α is a transcription factor that is also tightly linked with hypoxia. In response to reduced oxygen tension, HIF-1α targets VEGFA (44, 50), along with promoting inflammation and fibrosis (44, 51, 52). Thus, HIF1-α signaling can upregulate angiogenic processes, in part through VEGF signaling, but simultaneously increases expression of inflammatory factors that may cause insulin resistance and metabolic disturbances. Although we found adipose tissue HIF1A mRNA expression to be greater in our habitual exercisers compared with non-exercisers, it is the protein abundance and cellular localization of HIF1-α that are functionally linked to hypoxia, rather than its mRNA expression. In fact, in vitro studies demonstrate that induction of hypoxia in 3T3-L1 cells has no effect on *HIF1A* mRNA (53), while in human primary adipocytes hypoxia reduced *HIF1A* mRNA expression (49). In both of these studies (49, 53), the abundance and transcriptional activity of HIF-1α protein was increased during hypoxic conditions, further demonstrating the lack of connection between *HIF1A* mRNA, HIF-1α activity, and hypoxic conditions. Unfortunately, tissue limitations in our study prevented us from making measurements of HIF-1α protein content and localization, so we could not assess differences in the abundance or functional activity of HIF-1α. It remains inconclusive based whether the measured difference in *HIF1A* mRNA in our study had any indirect impact on our metabolic outcomes.

Although it may seem counter-intuitive, an enhanced ability to effectively expand subcutaneous adipose tissue during periods of nutrient excess may actually help attenuate the metabolic complications commonly associated with weight gain and obesity. This phenomenon has been referred to as the "Expandability hypothesis" (9, 10). A major underlying premise of the expandability hypothesis is that an enhanced ability to effectively store excess energy in

adipose tissue will help sequester excess fatty acids, reducing systemic fatty acid flux into the systemic circulation - and thereby help prevent excessive fatty acid uptake into insulin-responsive peripheral tissues (e.g. skeletal muscle and liver). Importantly, this hypothesis does not imply that gaining weight and increasing adiposity is healthful, but rather that when experiencing episodes of weight gain, subcutaneous adipose tissue that can more effectively store the excess energy may provide protection from metabolic disorders such as insulin resistance. The vast majority of adipose tissue expansion is due to adipocyte hypertrophy (i.e., increasing fat cell size), and the structure and composition of the extracellular matrix (ECM) may dictate the ability of adipocytes to adequately expand and store nutrients (54).

Collagen VI is a primary form of collagen found in adipose tissue (55), and the magnitude of collagen VI expression in adipose tissue is linked to insulin resistance (55). In agreement, we found *COL6A1* expression to be proportional to systemic fatty acid release from adipose tissue in STUDY #2 of my dissertation, reinforcing the notion that fibrosis in adipose tissue may contribute to the development of whole body insulin resistance. It has been suggested that high abundance of collagen VI can restrict adipocyte expansion – conversely, lower abundance of collagen VI allows the adipocyte to expand more freely, and thereby more effectively store fatty acids during episodes of nutrient excess (55). Interestingly, exercise may help modify adipose ECM. It has been reported that when compared with sedentary mice on a high fat diet, mice that exercised while eating the same high fat diet exhibited lower fibrosis (33). In a similar study by Kawanishi et al., exercising mice had larger adipocytes compared with their non-exercising counter-parts on a high-fat diet, suggesting their lower adipose fibrosis may allow adipocytes to expand more freely to accommodate the excess nutrients (32). We did not find any differences in *COL6A1* or *COL1A1* between our regular exercisers and non-exercisers suggesting that exercise training may not have the same effect in human subjects under weight stable conditions. However, because the mice in Kawanishi, et al. (32, 33) were exercising and gaining weight simultaneously it is possible that these adaptations may occur only with the stimuli of exercise together with nutrient excess.

Although adipocyte hypertrophy is the primary mechanism for storing excess energy in adipose tissue, adipose tissue may also expand through hyperplasia and create new adipocytes to accommodate the increased need for lipid storage. During exercise, there are several factors released into the circulation during and/or after exercise (e.g. fatty acids, IGF-1, cortisol) that are

known activators of adipogenic processes, so we hypothesized that exercise may upregulate expression of factors critical to adipogenesis (e.g. *PPARG* and *CEBPA*). Stanford et al. (34) demonstrated that mice exposed to a short-term endurance exercise training program without weight loss greatly increased their abundance of small adipocytes, suggesting enhanced adipogenesis. In contrast to our hypothesis, however, we did not find differences in expression of some critical regulators of adipogenesis or in the adipocyte cell size between ACTIVE and SED. Our current data set in overweight and obese adults that are metabolically healthy suggests exercise may not have a potent effect on adipogenic processes in subcutaneous adipose tissue in weight stable humans.

Although our findings suggest exercise may not impact adipose tissue fibrosis or adipogenesis in weight stable human subjects, prior exercise does appear to suppress subsequent lipolytic activity. Our findings that acute exercise lowered ex vivo lipolytic response is in agreement with previous work reporting a reduction in beta adrenergic stimulated lipolysis in subcutaneous adipose tissue one hour after a moderate session of aerobic exercise (56). The reduced lipolytic sensitivity to catecholamines after a single session of exercise that we observed may be metabolically beneficial by reducing systemic release of fatty acids into the circulation that are detrimental to whole-body insulin sensitivity at high levels for extended periods of time. Experiments performed in vitro and in situ (i.e., microdialysis) have demonstrated that a sustained exposure to catecholamines can desensitize the adrenergic lipolytic response (57, 58), so the lower lipolytic response measured 1h after acute exercise may be a consequence of elevated plasma catecholamines that occur during the exercise session. Prolonged stimulation of beta adrenergic receptors cause inhibition of the G-protein coupled receptor (GPCR) signaling cascade to a subsequent exposure of agonists (59) and is thought to be an important physiological feedback mechanism that protects cells from receptor overstimulation. The process of the desensitization is not fully elucidated, but involves a complex regulation of various phosphorylation sites on the receptors that control the internalization of the receptors to the cytoplasm (60). With extended exposure to catecholamines, the beta adrenergic receptors are retained in the cytoplasm and do not return to the cell membrane, thus reducing the beta adrenergic signaling response to a second agonist exposure. In contrast to the acute exercise effects, endurance exercise training without weight loss does not appear to change lipolytic sensitivity when measured at least a few days after the last exercise training session (61). Our

findings that in vitro lipolytic response to isoproterenol was similar between ACTIVE and SED supports this notion that adaptations to chronic exercise training, do not impact adrenergic lipolytic response. We also did not find any differences in basal lipolysis between regular exercisers and non-exercisers.

While some studies have reported higher or lower basal lipolytic rates after exercise training, differences in subject population (i.e. lean, obese, T2D), timing of sample collection, and methodologies may account for some of this disparity. For example, the effect of exercise training on lipolytic response to catecholamines is confounded by weight-loss that often accompanies endurance exercise programs (62, 63). Weight loss and a resultant loss of body fat mass (and smaller adipocyte diameter) is known to augment lipolytic sensitivity to catecholamines (64-66).

Several studies have reported that exercise training lowers adipose tissue inflammation in humans, which may lead to improved whole body metabolic outcomes (26, 27). Unfortunately, most of these exercise studies were confounded by weight-loss, which is known to have a robust impact on lowering adipose tissue inflammation (25, 27, 28). The independent effects of exercise on adipose tissue inflammation are far less clear. Recent evidence from some animal studies suggest a beneficial effect of both acute and chronic exercise on inflammation and metabolic function of adipose tissue independent of weight loss and adiposity (31, 34). Our finding that ACTIVE had a higher expression of IL6 in the CD14+ fraction of the adipose tissue compared with SED suggests that habitual exercise training may alter the inflammatory state of the adipose tissue resident immune cells. In agreement with our findings, IL6 expression and signaling within adipose tissue has previously been found to be upregulated after an exercise session in mice (31), with implications for altering the metabolic function of the adipose tissue. This finding is intriguing since IL6 has been implicated as being important for shifting the inflammatory status in macrophages from a more pro-inflammatory "M1" phenotype to a more alternatively activated "M2" state. However, since we did not find changes in the mRNA expression of IL6 or other markers of inflammation in adipose tissue after a single session of exercise, it remains unclear why ACTIVE would have a higher IL6 expression in their CD14+ fraction.

It is well-known that most of the improvements in insulin sensitivity associated with exercise are associated with the most recent exercise session, and removal of exercise for as little

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as 48 hours can return insulin sensitivity to levels similar to untrained individuals (67-69). Therefore, because our ACTIVE subjects performed the OGTT three days after their most recent exercise session, as expected glucose tolerance was indeed similar between ACTIVE and SED. Our data does not necessarily mean that exercise training will not have beneficial effects on adipose tissue that would translate into measurable improvements in metabolic health. It is important to note that other than being overweight and/or mildly obese, our SED subjects were still in general good health, and thus, the effect of exercise training in our ACTIVE group may be very hard to detect when compared with our relatively healthy SED cohort. It may also be possible that the metabolic benefits of exercise on adipose tissue are only measurable when combined with weight gain. Alterations in adipose tissue observed in studies from Kawanishi et al. (32, 33)occurred when exercise was combined with weight gain. Perhaps the exercise stimulus "primes" the adipose tissue for healthful adaptability to weight gain, but these adaptations only occur when nutrient excess also stimulates processes to store more lipids.

Findings from this study expand on the limited knowledge of the effects of exercise on adipose tissue structure and function in humans, however there are several limitations. The mRNA expression data in this study provide valuable insight, especially when examining the acute response one hour after a session of exercise, where adaptive responses in protein abundance would likely not be measurable. Notably, many adaptations to exercise result from the cumulative effect of transient increases in mRNA transcripts that occur with each exercise bout, eventually leading to an accumulation of adaptive proteins (4, 70). However, clearly many aspects of metabolic processes and cellular function are regulated at the level of protein translation and post-translational modifications, without a direct link to changes in the levels of mRNA (71). Unfortunately, limitations in tissue sample yield prevented us from making measurements at the protein level. We also acknowledge that the number of subjects in this study is small, and thus, small changes in metabolic outcomes in adipose tissue may have been missed, especially with the very large variability in adipose tissue outcomes in humans. However, we feel that this data set provides a solid foundation for further research focused on the aspects that have shown promise for responding to exercise (e.g., angiogenesis and macrophage polarization). Additionally, we used a "conventional" exercise stimulus of 1hr at ~65% VO$_2$peak, but there may be very different responses to different exercise modalities and intensities (i.e. high-intensity interval training, strength training).

In conclusion, our study suggests that a single session of aerobic exercise may lead to an increase in the mRNA expression of VEGFA, the primary mediator of angiogenesis, in adipose tissue. This response may lead to an increase in adipose tissue capillarization as evidenced by the elevated mRNA expression of CD31. We did not find an effect of exercise on markers of adipogenesis or fibrosis. Exercise training may also play a role in alternative activation of immune cells, as habitual exercisers had a higher mRNA expression of IL6 in the CD14+ enriched fraction of their adipose tissue compared with their sedentary counterparts. Our ex vivo experiments demonstrate that a single session of exercise can transiently reduce beta adrenergic stimulated lipolysis in the post-exercise period, however, there is no effect of exercise training. Further studies are needed to examine the effects of exercise on adipose tissue structure and function, especially to investigate whether any changes in the adipose tissue measurably improve metabolic health in obesity. Our data suggests that exercise may alter adipose tissue health and metabolic outcomes by elevating angiogenic processes and IL6 expression in adipose tissue.

Gene symbol	Protein encoded
PPARG	proliferator-activated receptor gamma (PPARγ)
SREBP1C	sterol regulatory element-binding protein 1 (SREBP1c)
CEBPA	CCAAT/enhancer-binding protein alpha (CEBPα)
GPAT1	glycerol-3-phosphate Acyltransferase-1 (GPAT1)
DGAT1	diacylglyceride acyltransferase 1 (DGAT1)
ATGL	adipose triglyceride lipase (ATGL)
HSL	hormone-sensitive lipase (HSL)
FABP4	fatty acid binding protein 4 (FABP4)
PLIN1	perilipin 1 (PLIN1)
COL6A1	collagen VI
COL1A1	collagen I
VEGFA	vaascular endothelial growth factor (VEGF)
HIF1A	hypoxia-inducible factor (HIF1α)
PECAM1	cluster of differentiation 31 (CD31)
MCP1	monocyte chemoattractant protein-1 (MCP-1)
TNFA	tumor necrosis factor alpha (TNFα)
NLRP3	NOD-like receptor family, pyrin domain containing 3 (NLRP3)
IL1B	interleukin-1 beta (IL1β)
ITGAX	"M1" macrophage marker CD11c
MRC1	"M2" macrophage marker CD206
CD163	"M2" macrophage marker CD163
SLC2A1	Glucose transporter 1 (GLUT1)

Table 5-1: qPCR targets in the subcutaneous adipose tissue

	SED	ACTIVE
Sex (M/F)	(5/7)	(3/5)
Age	27±2	27±1
Body mass (kg)	82±3	80±3
BMI (kg/m2)	27±1	29±1
Body fat (%)	31±2	29±2
FM (kg)	26±2	24±3
FFM (kg)	57±3	57±3
VO_{2peak} (ml/kg FFM/min)	42±2	52±3*
Matsuda ISI	8.2 ± 1.4	5.6 ± 0.3
HOMA-IR	1.3 ± 0.2	1.6 ± 0.1
Glucose AUC	868 ± 56	972 ± 60
Insulin AUC	8647 ± 1483	11323 ± 1101

* $p < 0.05$ compared with SED.
Data presented as Mean ± SD.

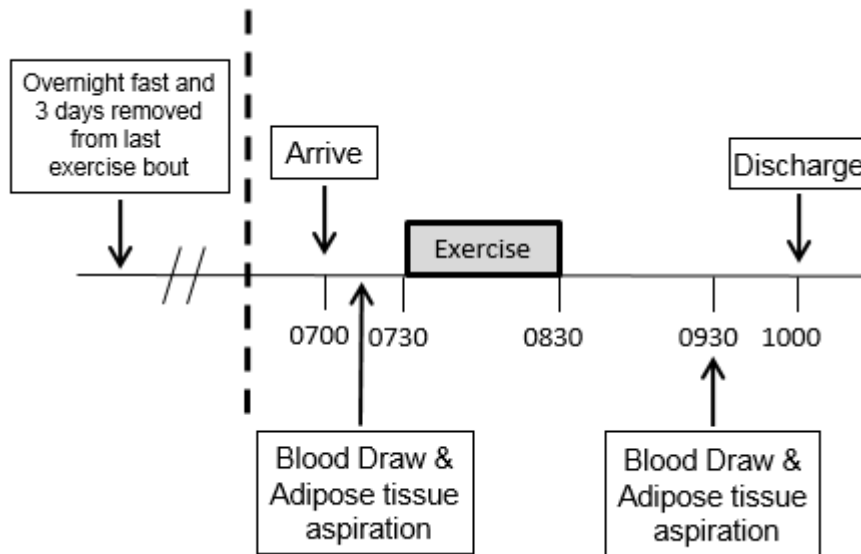**Table 5-2:** Physical characteristics of subjects

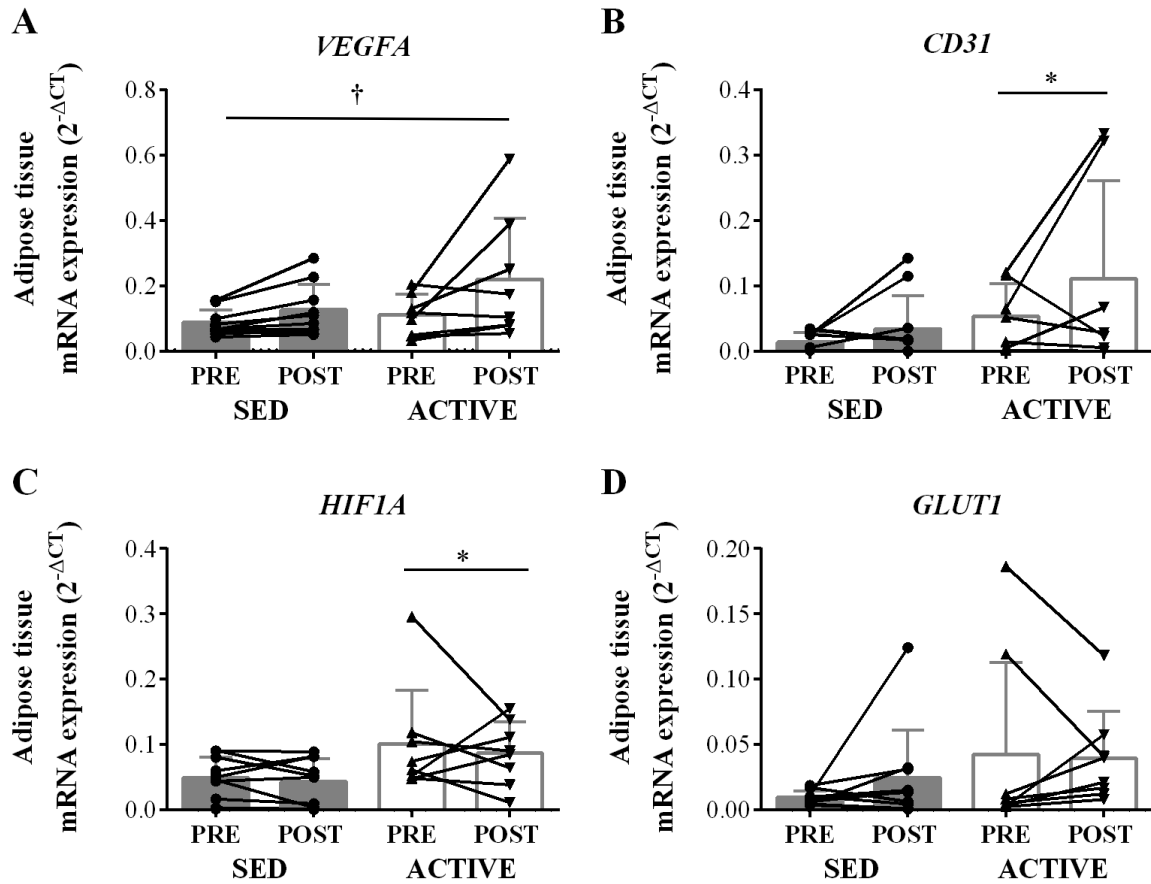**Figure 5-1:** Timeline of study trial day

Figure 5-2: mRNA expression of factors related to angiogenesis, capillarization, and hypoxia in the whole adipose tissue homogenate in SED compared with ACTIVE both before (PRE) and 1hr after exercise (POST). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M*. Data expressed as MEAN ± SD. *P<0.05 vs. SED, main effect of group. †P<0.05, main effect of exercise.

A *TNFA*

B *IL1B*

C *NLRP3*

D *MCP1*
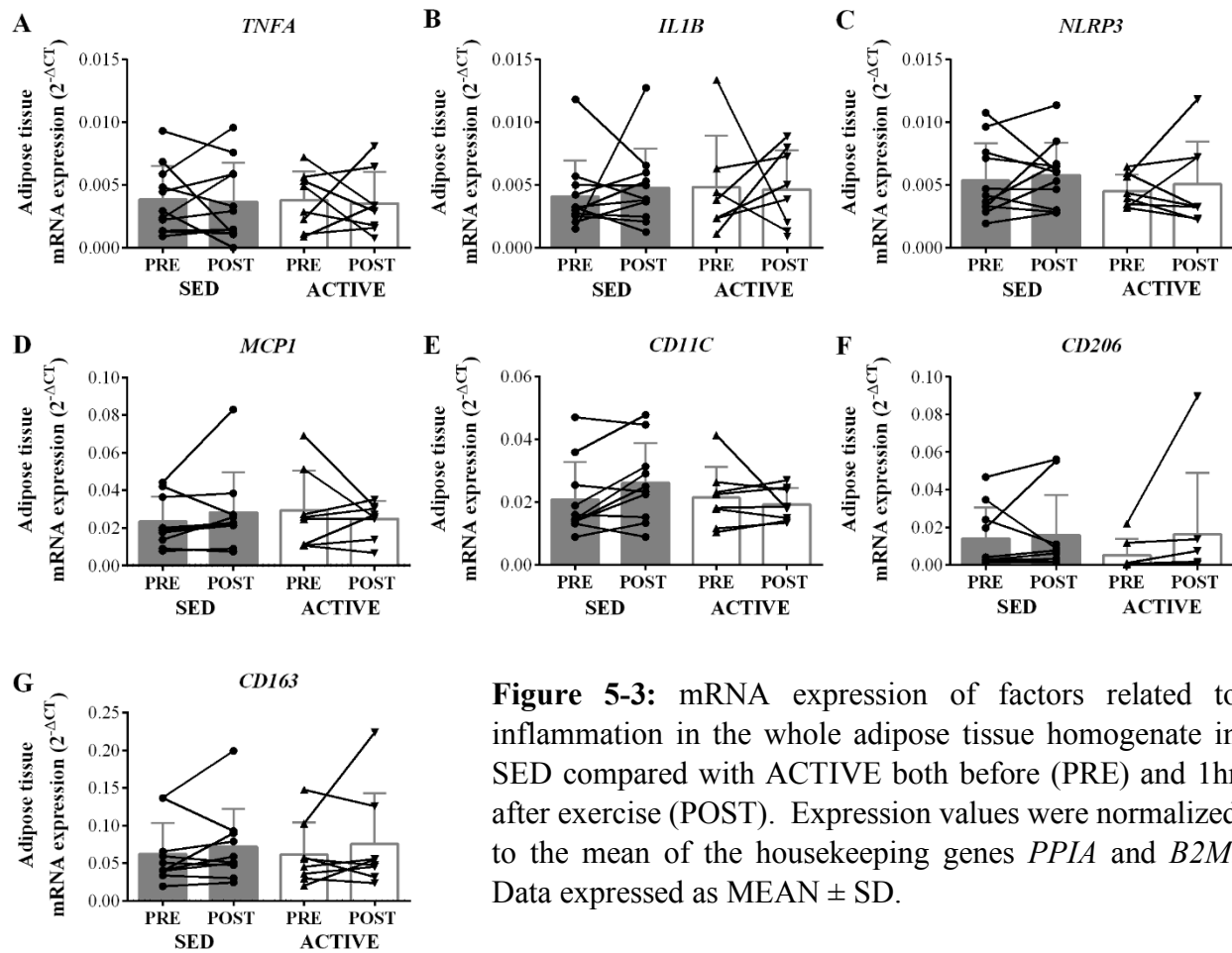
E *CD11C*

F *CD206*

G *CD163*

Figure 5-3: mRNA expression of factors related to inflammation in the whole adipose tissue homogenate in SED compared with ACTIVE both before (PRE) and 1hr after exercise (POST). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M*. Data expressed as MEAN ± SD.

A *TNFA*

B *IL1B*

C *NLRP3*

D *MCP1*

E *CD11C*

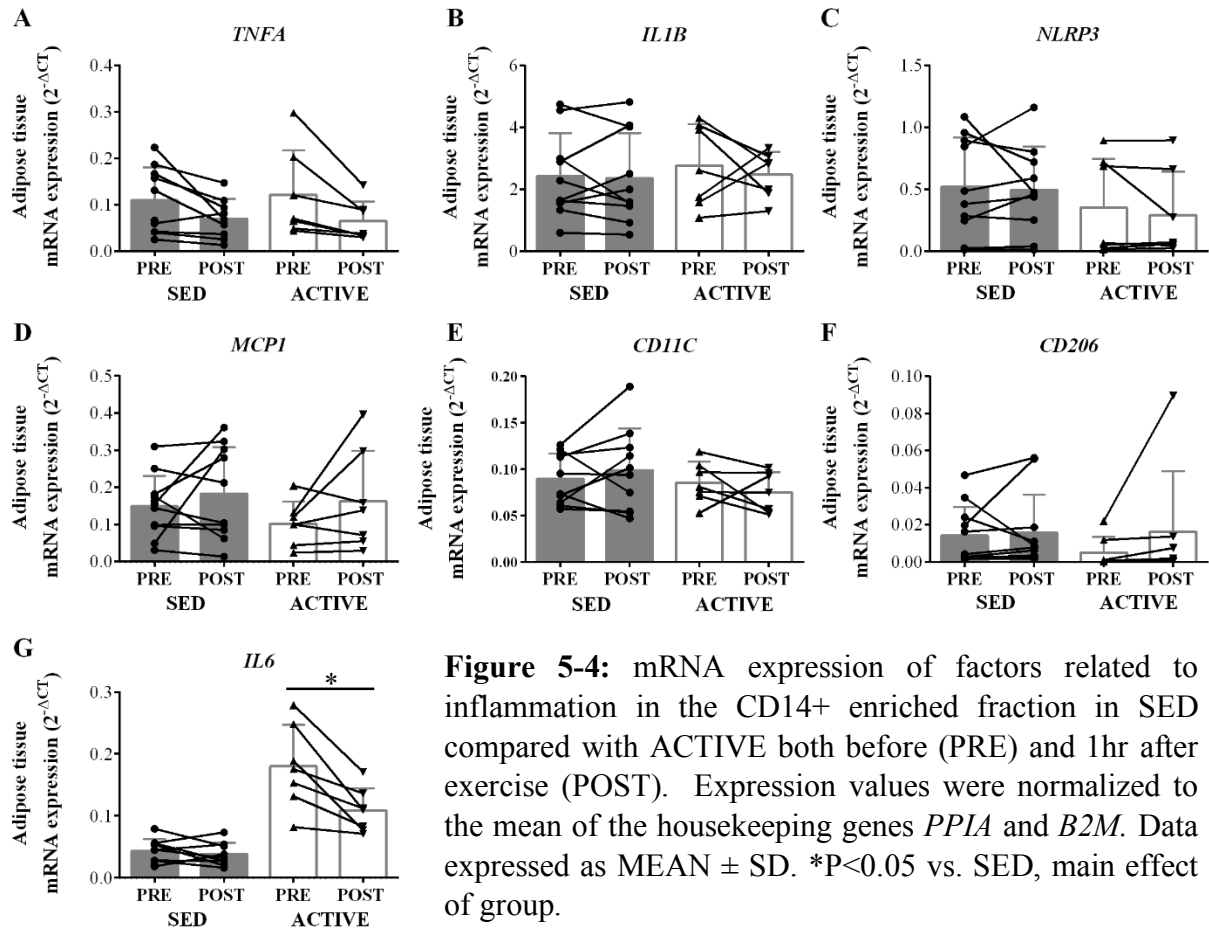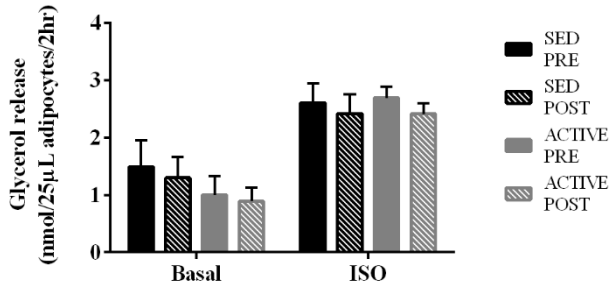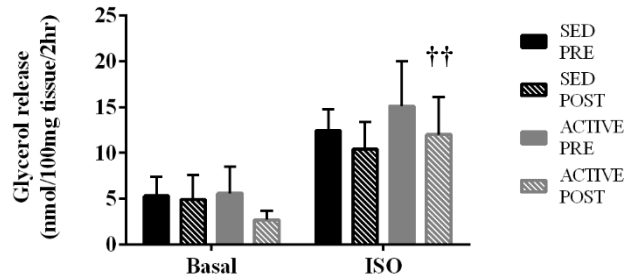
F *CD206*

G *IL6*

Figure 5-4: mRNA expression of factors related to inflammation in the CD14+ enriched fraction in SED compared with ACTIVE both before (PRE) and 1hr after exercise (POST). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M*. Data expressed as MEAN ± SD. *P<0.05 vs. SED, main effect of group.
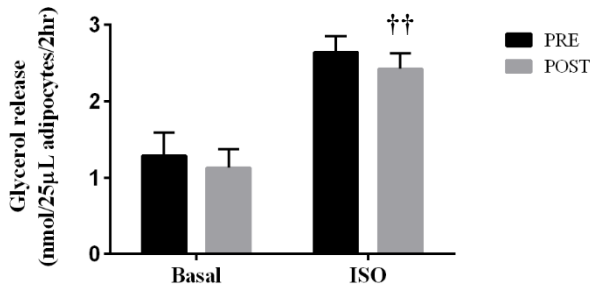
Figure 5-5: Glycerol release measured in mature adipocytes (A and C) and adipose tissue explants (B and D) taken before exercise (PRE) and 1hr after exercise (POST). Basal conditions (Basal) had no stimulation while ISO had incubation in isoproterenol. Data expressed as MEAN ± SD. †† $P<0.05$ vs. PRE.
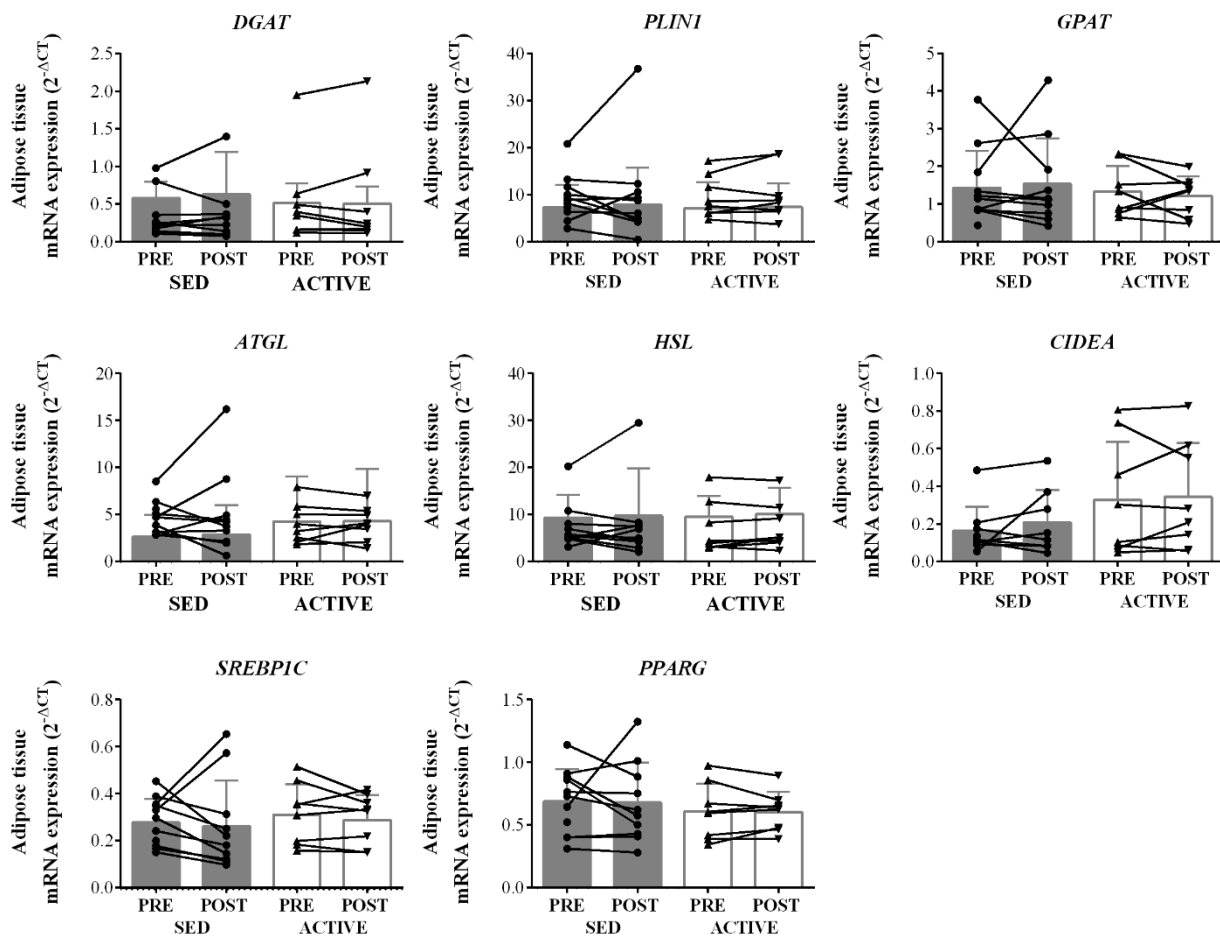
Figure 5-6: mRNA expression of factors related to related to lipolysis (*ATGL, HSL*), esterification (*GPAT, DGAT*), lipid storage (*PLIN1, CIDEA*), or lipogenesis/adipogenesis (*SREBP1C, PPARG*) in mature adipocytes extracted from adipose tissue samples collected before or after exercise in SED compared with ACTIVE both before (PRE) and 1hr after exercise (POST). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M.* Data expressed as MEAN ± SD.

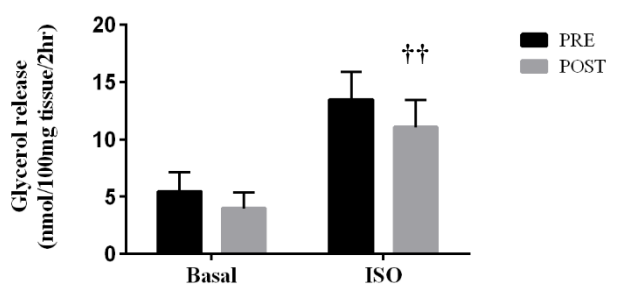**Figure 5-7:** mRNA expression of factors related to extracellular matrix in the whole adipose tissue homogenate in SED compared with ACTIVE both before (PRE) and 1hr after exercise (POST). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M*. Data expressed as MEAN ± SD.

Figure 5-8: Distribution of frequencies of adipocyte cell size

References

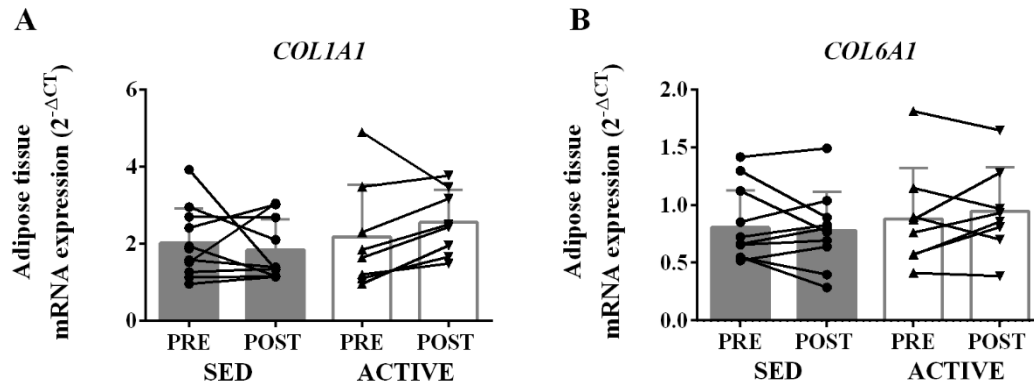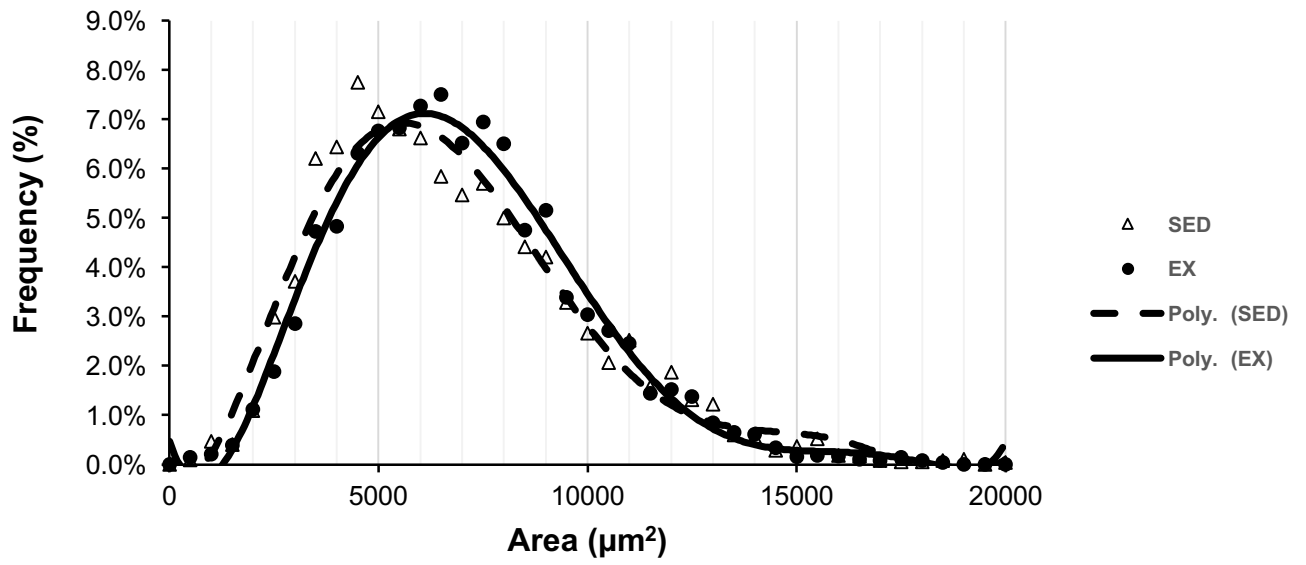1.   **Colberg, SR, Albright, AL, Blissmer, BJ, Braun, B, Chasan-Taber, L, Fernhall, B, et al.** Exercise and type 2 diabetes: American College of Sports Medicine and the American Diabetes Association: joint position statement. Exercise and type 2 diabetes. *Medicine and science in sports and exercise* 42: 2282-303, 2010. doi: 10.1249/MSS.0b013e3181eeb61c

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CHAPTER 6

Overall Discussion

Obesity has become a major health issue in the United States with an annual healthcare cost of ~$147 billion. The development of insulin resistance, which is central to metabolic diseases such as type 2 diabetes and cardiovascular disease, is common in obesity. With over one-third of the United States population considered obese, with no signs of declining, it is important to understand the relationship between obesity and the development of insulin resistance for the development of targeted treatments that may alleviate insulin resistance and thus, obesity-related disease. My dissertation work primarily focused on the role of subcutaneous adipose tissue in metabolic health since it is now known that adipose tissue "health" and metabolic function is associated with obesity-related insulin resistance. Alterations in various factors and processes within adipose tissue, such as lipid storage, inflammation, extracellular matrix composition and structure, adipogenesis, and angiogenesis, have all been implicated in determining the magnitude of many obesity-related metabolic health complications, including insulin resistance. Furthermore, although exercise is often prescribed to improve metabolic health in obesity, it remains unknown whether exercise causes adaptations in adipose tissue structure and function that may contribute to improvements in whole-body metabolic health. The overall objective of my dissertation studies was to assess how subcutaneous adipose tissue may contribute to whole-body metabolic outcomes in obesity, and whether exercise can modify adipose tissue in a manner that may lead to improved overall metabolic health.

Together, my dissertation studies have fulfilled this purpose and provided insight into important aspects of adipose tissue physiology and biology that may translate to whole-body metabolic health outcomes. More specifically, my dissertation studies have demonstrated: A) basal fatty acid flux from subcutaneous adipose tissue is an important determinant of insulin resistance and metabolic health (STUDY 1), B) adipose tissue from obese adults who maintain relatively low rates of fatty acid flux have alterations in factors related to triglyceride storage (higher) and release (lower), lower fibrosis, and lower markers of activation of some key inflammatory pathways (STUDY 2), and, C) endurance exercise may modify adipose tissue by triggering events related to an increase in angiogenesis and capillarization. (STUDY 3). Further details of the findings in each of these studies are described and discussed in detail in Chapters 3-5. In this overall summary, I will provide an integrative discussion on the collective significance

and importance of the findings of my dissertation and how they relate to existing knowledge in the field.

It has become very evident that the adipose tissue is a far more complex tissue than its classical description as simply a benign storage site for surplus energy. Adipose tissue constitutes adipocytes, extracellular matrix components, blood vessels, immune cells, and adipose stem cells, all of which can respond markedly to weight gain or loss. The integrative responses and interactions between adipocytes and other cells within adipose tissue can impact metabolic homeostasis, especially in the context of nutrient excess and obesity. Biological pathways related to inflammation, fibrosis, adipogenesis, angiogenesis, hypoxia, and lipid metabolism within adipose tissue are all implicated as important pathways impacting whole body metabolic outcomes. Alterations in one or more of these pathways may ultimately effect the lipid storage capacity of adipose tissue, leading to excessive systemic availability of fatty acids, which is known to cause metabolic abnormalities in other peripheral tissues such as skeletal muscle, liver, and pancreas. Because excessive mobilization of fatty acids into the systemic circulation is responsible for inducing profound systemic insulin resistance (1-6), one major theme of my dissertation studies targeted adipose tissue's role in storing and releasing fatty acids.

Findings from my dissertation support the "adipose tissue expandability hypothesis" which suggests that the ability to appropriately expand adipose tissue to accommodate excess nutrients is key to the maintenance of metabolic health. Although it has been well established that increasing systemic fatty acid mobilization induces systemic insulin resistance (1-6), STUDY #1 of my dissertation expands on these findings by demonstrating that the magnitude of basal fatty acid mobilization (FA Ra) from adipose tissue in obese adults was proportional to their insulin resistance. In other words, obese adults who maintain a lower FA Ra can preserve normal insulin sensitivity, despite having excess fat mass. This maintenance of a relatively low FA Ra in STUDY #1 was also accompanied by attenuated markers of inflammatory signaling in skeletal muscle. These findings from STUDY #1 provide valuable insight into why some obese adults may be protected from developing insulin resistance. However, without adipose tissue biopsies in STUDY #1, it was not possible to ascertain mechanisms that may underlie the relatively low fatty acid mobilization in the NORM-Si cohort. Therefore, to follow-up on the

important findings in STUDY #1, STUDY #2 was primarily designed to address what factors underlie the variability in fatty acid mobilization in otherwise similar obese adults.

A majority of systemic fatty acids are derived from subcutaneous adipose tissue, and fatty acid flux from adipose tissue is ultimately controlled by the coordination of triglyceride hydrolysis, esterification, and fatty acid trafficking. An imbalance in these processes could result in high rates of fatty acid flux and impaired metabolic health. In STUDY #2, adipose tissue from obese adults who had low FA Ra had lower basal phosphorylation of HSL (p-HSL^{ser660}) suggesting lower catecholamine induced lipolytic activity. In addition, findings from STUDY #2 were the first to our knowledge to demonstrate obese adults with low FA Ra have an elevated abundance of GPAT, an important enzyme for catalyzing the first step of committing a fatty acid to be stored as a triglyceride. Our human data coincides with *in vitro* work demonstrating increased triglyceride storage with GPAT overexpression. These data from STUDY #2 expand on our findings from STUDY #1 about the link between fatty acid flux and insulin sensitivity, and further advance our understanding about the association between adipose tissue metabolic function and metabolic health by demonstrating factors regulating lipolysis and esterification in adipose tissue may be important for protecting some obese adults from insulin resistance.

Components of adipose tissue structure can also contribute to the tissue's ability to store fatty acids. The extracellular matrix (ECM) provides a structural and signaling network for the adipose tissue while the capillary network within adipose tissue is necessary for nutrient and gas exchange in the tissue. Alterations in the adipose tissue ECM and/or vasculature may impact the capacity of the adipose tissue to control fatty acid flux in obesity, as well other important aspects related to metabolic health (e.g., hypoxia, inflammatory status, etc.). The gene ontology outcomes in STUDY #2 revealed that obese adults with low FA Ra had lower enrichment of pathways related to ECM structure, organization, and disassembly in their subcutaneous adipose tissue compared with obese adults with elevated FA Ra, supporting that ECM structure in adipose tissue is important for whole body outcomes. To our knowledge, these are the first data in human adipose tissue to link elevated markers of fibrosis in subcutaneous adipose tissue with elevations in FA Ra and adds an essential link in understanding the relationship between adipose tissue fibrosis and whole-body insulin sensitivity. Collagen VI is one of the most prominent collagens expressed in adipose tissue, and absence of collagen VI in adipose tissue of mice

results in improved whole body metabolic health on a high fat diet. Interestingly, in these mice, absence of collagen VI lead to an increased hypertrophy of the adipocytes, suggesting that elimination of this key component of the ECM may allow for adipocytes to expand with less restriction, leading to greater lipid storage, and thereby may help protect against ectopic lipid deposition in other tissues. We measured mRNA expression of collagen VI in STUDY #2 and found that it was proportional to the degree of FA Ra, supporting the notion that lower ECM deposition and assembly in obese adipose tissue can have a protective effect on whole body metabolic health. Unfortunately, without adipose tissue samples for histological analysis in STUDY #2, we cannot confirm that lower collagen VI expression was related to greater hypertrophy of adipocytes. We did not find any evidence that adipogenic or angiogenic processes were contributing to differences in metabolic health among our obese subjects in STUDY #2. Importantly, our subjects in STUDY #2 were weight stable and all measurements were made in the post-absorptive state, thus there was no stimulus for adipose tissue expansion. We postulate that differences in markers for these processes may only be apparent during a period of nutrient excess and weight gain when the adipose tissue is stimulated to expand, and not during basal/weight-stable conditions. Regardless, STUDY #2 of my dissertation helps advance our understanding about the potential impact of the ECM in human adipose tissue on whole body metabolic health in obesity.

In obesity, there is also an increase in inflammation within the adipose tissue that can disrupt metabolic function locally as well as systemically. Inflammation is tightly linked to poor metabolic outcomes, but it is also highly proportional to the degree of fibrosis and hypoxia in adipose tissue. Elevated inflammation occurs via proliferation and altered activation of resident immune cells as well as through recruitment and infiltration of more immune cells. The reduced markers of fibrosis in subcutaneous adipose tissue of our insulin sensitive obese adults in STUDY #2 led us to hypothesize that we would also observe lower markers in inflammation in their adipose tissue. However, GO analysis of the microarray data in STUDY #2 indicated that obese adults with low FA Ra had enrichment of pathways related to antigen receptor-mediated signaling, defense response, and lymphocyte differentiation, suggesting greater immune activity in their adipose tissue compared with obese adults with elevated FA Ra. The immune response is highly complex, and while it is tempting for us to speculate whether the inflammatory environment in the adipose tissue is simply "pro-inflammatory" or "anti-inflammatory", the

immune activity in the adipose tissue can be far more nuanced. Most studies primarily focus on the role of macrophages in regulating inflammation in the adipose tissue but there are certainly other infiltrating immune cells dictating the inflammatory response. We found many of the most highly upregulated genes to be associated with T cells and antigen-receptor signaling, and it is now appreciated that changes in CD4+ and CD8+ T cells can dictate macrophage recruitment and inflammatory response to obesity. The enrichment of the lymphocyte differentiation pathways may be from propagation of the more "protective" CD4+ T cells that may dampen metabolic disturbances with increased fat mass. However, without specific cell sorting it is impossible for us to conclusively determine this. Obese adults with low FA Ra also had lower activation of the SAPK/JNK pathway that is important for the activation and inflammatory status of myeloid cells. Findings from STUDY #2 suggests that the relationship between overall inflammatory status of the adipose tissue and whole body metabolic health is complex and may not be a simple linear relationship between classic markers of inflammatory status and metabolic health. The enhanced insulin sensitivity observed in some obese adults in STUDY #1 and STUDY #2 may be partially dictated by "protective" immune responses in adipose tissue that preserve adipose tissue function in the face of obesity.

With STUDY #2 identifying factors in adipose tissue that may contribute to metabolic outcomes in obesity, the next goal of my dissertation was to determine if an intervention can modify adipose tissue to develop a more "healthful" phenotype. It is well-known that weight loss interventions can improve adipose tissue and whole body metabolic health, however, little is known about the direct effects of exercise, independent of weight loss. STUDY #3, was designed in part to test whether endurance exercise can impact adipose tissue structure and function in a manner that would resemble the phenotype from metabolically healthy obese adults in STUDY #2. However, in general we did not find an effect of acute or chronic exercise on the mRNA expression of factors important for metabolic health that we identified in STUDY #2, such as lipid handling factors (i.e. HSL, GPAT) or ECM (i.e. COL6A1). Importantly, however, our main finding from STUDY #3 demonstrating that a single session of exercise stimulated an increase in mRNA expression of VEGFA, which is considered a primary regulator of angiogenesis, suggests that exercise may trigger an upregulation of angiogenic pathways in adipose tissue. Accompanying this increase in VEGFA expression, we also found an elevated gene expression of CD31 (a marker of endothelial cells) in adipose tissue from habitual exercisers. We interpret

this to suggest that the repeated transient elevation in VEGFA signaling that regularly occurs in the habitual exercisers induced structural adaptation to the adipose tissue vasculature (i.e., increased adipose capillarization). We also found a higher mRNA expression of IL6 in the CD14+ enriched fraction of the adipose tissue and we are intrigued by this finding since IL6 signaling in myeloid cells can be an important mechanism for promoting alternative activation or "anti-inflammatory" properties in immune cells. However, there were no significant changes in the expression with an acute bout of exercise so it remains unclear exactly what may be causing a sustained elevation in IL6 mRNA in the immune cells. While it is tempting to speculate that increased capillarization and possible changes in immune cell activation may improve metabolic outcomes in obesity, it remains possible that these changes in adipose tissue will not have a measurable impact on overall metabolic health without a change in fat mass. Regardless, the data from STUDY #3 demonstrates that exercise can initiate angiogenic processes and potentially induce alternative activation in immune cells in subcutaneous adipose tissue. By increasing angiogenesis with regular exercise, the adipose tissue may exhibit improved lipid storage capacity through reductions in hypoxic stress. Further, exercise may initiate alternative activation of immune cells and alter the inflammatory state of the adipose tissue. STUDY #3 demonstrated that exercise may improve mechanisms of adipose tissue metabolic health, but these adaptations do not mimic the same mechanisms and pathways we observed in STUDY #2 that protected adipose tissue and metabolic health in some obese adults.

Together, my dissertation studies demonstrate that the health and function of adipose tissue is an important mediator of overall metabolic health in obesity and that exercise may be a useful intervention for improving adipose tissue structure and function. The capacity of the adipose tissue to sequester and store excess nutrients and protect other tissue depots from ectopic lipid deposition is key for protection against insulin resistance in the face of obesity. Various pathways including reduced lipolysis, increased esterification, reduced ECM enrichment, and altered immune activity were related to maintenance of low fatty acid flux in insulin sensitive obese adults. In addition, exercise may be a useful intervention to improve adipose tissue metabolic health by increasing capillarization and reducing metabolic dysfunction related to hypoxic stress. My dissertation has provided exciting new information regarding adipose tissue biology and the control of metabolic outcomes in obese adults.

CONCLUSIONS

Obesity is often accompanied by the development of insulin resistance, which is central to many chronic cardio-metabolic diseases, such as type 2 diabetes and cardiovascular disease. These diseases are associated with lower quality of life and increased mortality. In addition, the economic burden and national healthcare costs of obesity-related disease is enormous, with more than 20% of national health care expenditures directed toward treating obesity-related illness and disease. It is important to find therapeutic targets and treatments that can prevent or reverse insulin resistance, which would not only reduce the economic burden associated with obesity, but even more importantly, would mitigate the tremendous personal challenges to many of those who suffer from obesity-related diseases. My dissertation provides new information regarding adipose tissue biology and the control of metabolic outcomes in obese adults. In particular, STUDY #1 and #2, demonstrated that the rate of fatty acid mobilization from adipose tissue was directly associated with insulin resistance, and obese adults with high rates of fatty acid mobilization exhibit alterations in factors related to lipid handling, inflammation, and fibrosis in adipose tissue. To follow-up on the findings, future work could focus on clarifying the role of fibrosis in the control of lipid handling in adipose tissue. Examining the relationship of adipose tissue fibrosis and fatty acid mobilization in lean adults could help determine if this relationship occurs before the onset of obesity, and whether the levels of fibrosis are predictive of future metabolic outcomes with controlled weight gain studies. These types of studies may provide a timeline of adaptations in the adipose tissue to help understand of the excessive fibrosis is a cause or a consequence of the elevated fatty acid mobilization. Further measurements to verify our findings are needed as well, such as histological quantification of ECM in the adipose tissue. In addition, in STUDY #3, it was demonstrated that exercise training may increase angiogenic processes and lead to an increase in capillarization in adipose tissue. These adaptations may "prime" adipose tissue for improved metabolic responses during periods of weight gain by mitigating hypoxic conditions associated with expanding fat mass. Future studies could be designed to examine if exercise can mitigate poor metabolic responses during periods of weight gain. We hypothesize that adipose tissue adaptations and the regulation of fat storage in response to overeating in people who exercise regularly may differ considerably when compared with non-exercisers, resulting in more favorable health outcomes in the exercisers despite the same degree of weight gain. Furthermore, histological examination of the adipose tissue should be used to verify that exercise training does indeed increase capillary density in the adipose tissue. The data from my dissertation projects, combined with follow up mechanistic analysis, could allow for targeted treatments to prevent excessive fatty acid mobilization from adipose tissue in obesity and reduce poor metabolic outcomes.

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