DISSERTATION

TESTING THE METABOLIC SINK POSTULATE: SUBCUTANEOUS ADIPOSE TISSUE THE PROTECTIVE DEPOT

Submitted by

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ABSTRACT

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Adipose tissue distribution and not body mass index is the major predictor of risk for obesity-related chronic disease. Specifically, central adiposity, intra-abdominal/visceral adipose tissue accumulation, is associated with adverse metabolic outcomes such as, but not limited to, insulin resistance syndrome, cardiovascular disease, and hypertension [1, 2]. Conversely, peripheral adiposity, subcutaneous/gluteofemoral adipose tissue accumulation, is considered protective against metabolic disease [3, 4]. It is proposed that the subcutaneous adipose depot functions as a "metabolic sink" to sequester and store lipid from circulation, preventing ectopic deposition. Therefore, an individual with high overall fat mass, primarily located in the lower body subcutaneous adipose depots could be metabolically healthy while obese.

While subcutaneous adipose tissue (SAT) has been associated with greater insulin sensitivity and lower risk of adverse metabolic outcomes, it has not been fully examined for exact mechanisms or causality. The broad goal of this proposal was to identify and understand how adipose tissue contributes to the development, progression, and possibly resistance to metabolic disease. The specific goal of this dissertation was to examine how SAT protects against metabolic dysregulation.

One of the protective properties of lower body subcutaneous adipose tissue (LBSAT) is its ability to expand and proliferate with new/healthy, lipid-filling adipocytes. We examined

adipose tissue compensation following intra-abdominal fat removal and glucose homeostasis. Peroxisome proliferator-activated receptor-γ (PPARγ; an activator of adipogenesis) knockout mice and control mice received either Sham surgery or intra-abdominal lipectomy. The inability of cell proliferation following lipectomy in PPARγ knockout mice induced glucose intolerance. Control mice with intra-abdominal lipectomy had increases in peripheral adipose mass, cell size redistribution, and improved glucose tolerance.

The Foster lab previously demonstrated that removal of LBSAT caused skeletal muscle, but not liver, lipid accumulation in standard CHOW and high fat diet (HFD) mice. Additionally, LBSAT removal resulted in deterioration of systemic glucose tolerance and muscle insulin sensitivity in HFD animals only. Hence, we proposed that muscle triglyceride deposition per se was not sufficient to explain systemic glucose intolerance. One purpose of this dissertation was to further examine the protective properties of SAT and to investigate the fundamental mechanisms that contribute to impairment of glucose tolerance.

We sought to extend our previous research with a systematic approach. We hypothesized that SAT has a dose-dependent association with systemic glucose regulation and maintenance of insulin sensitivity in nearby muscle. Our focus here was to examine the relation between peripheral adipose tissue and glucose homeostasis. This was accomplished with progressive removal of adipose tissue: ~20%, 40%, or 80% of the total SAT. Mice fed HFD for 13 weeks exhibit a dose-dependent decline in systemic glucose tolerance. This was accompanied by a decline in femoral muscle insulin response in the basal state but not the insulin-stimulated state. Muscle triglycerides were significantly higher in all surgery groups. Other contributing factors were eliminated, including circulating factors, adipocyte distribution and compensation, or liver

triglycerides. Therefore, we have demonstrated a dose-response effect of progressive SAT removal on glucose intolerance and basal muscle insulin insensitivity.

In addition to metabolic outcomes, we seek to identify a lipid signature that is linked to diet-induced impairments in glucose tolerance. Liquid chromatography and mass spectrometry (LCMS) were used to identify differential diet patterns of lipid species between CHOW and HFD. Mice that did not have fat removed and were fed a healthy chow diet have intramuscular triglycerides that are consistent with longer chain fatty acids (more carbons) and a higher degree of unsaturation (less hydrogens). They also have high abundance of phosphatidylserine and phosphatidylinositol. Diet-induced obesity is associated with femoral muscle lipids that include diacylglycerides and sphingomyelin. Overall, when examining the total lipid profile in muscle, healthy fats were more influential than unhealthy ones.

In summary, inhibition of adipocyte proliferation results in glucose intolerance following intra-abdominal fat removal. Progressive subcutaneous fat removal results in a dose-dependent deterioration of systemic and muscle glucose homeostasis. Thus, peripheral fat does indeed serve as a “metabolic sink” that sequesters excess energy and preserves metabolic regulation. Our data suggest that muscle lipid accumulation per se is not detrimental to health, but the types of lipids that are stored should be considered.

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CHAPTER 1: DETRIMENTAL AND PROTECTIVE FAT: BODY FAT DISTRIBUTION AND ITS RELATION TO METABOLIC DISEASE[1,2]

Summary

Obesity is linked to numerous co-morbidities that include, but are not limited to, glucose intolerance, insulin resistance, dyslipidemia and cardiovascular disease. Current evidence suggests, however, obesity itself is not an exclusive predictor of metabolic dysregulation but rather adipose tissue distribution. Obesity-related adverse health consequences occur predominately in individuals with upper body fat accumulation, the detrimental distribution, commonly associated with visceral obesity. Increased lower body subcutaneous adipose tissue, however, is associated with a reduced risk of obesity-induced metabolic dysregulation and even enhanced insulin sensitivity, thus storage in this region is considered protective. The proposed mechanisms that causally relate the differential outcomes of adipose tissue distribution are often attributed to location and/or adipocyte regulation. Visceral adipose tissue effluent to the portal vein drains into the liver where hepatocytes are directly exposed to its metabolites and secretory products whereas the subcutaneous adipose tissue drains systemically. Adipose depots are also inherently different in numerous ways such as adipokine release, immunity response and

[1] A modified version of this chapter is published as Booth A, Magnuson A, Foster M. Detrimental and protective fat: body fat distribution and its relation to metabolic disease. Horm Mol Biol Clin Investig. 2014 Jan;17(1):13-27.

[2] The aim of this work was to conduct a literature review on the role of body fat distribution with respect to complications in metabolic regulation. This discussion outlines the implications of adipose tissue location and provides background for the anatomical differences in fat depot storage capacity.

This chapter includes the complete published manuscript for this literature review titled *Detrimental and protective fat: body fat distribution and its relation to metabolic disease* (Andrea Booth, Aaron Magnuson, Michelle Foster, Horm Mol Biol Clin Investig., 2014). My contributions to this publication included writing multiple sections of the body of the text, review for accuracy and cohesiveness of the entire manuscript, editing and corrections prior to submission.

regulation, lipid turnover, rate of cell growth and death and response to stress and sex hormones. Proximal extrinsic factors also play a role in the differential drive between adipose tissue depots. This review focuses on the deleterious mechanisms postulated to drive the differential metabolic response between central and lower body adipose tissue distribution.

Introduction

High body mass index (BMI), obesity, is a risk factor for type-2-diabetes [5], dyslipidemia, hypertension [6], non-alcoholic fatty liver disease (NAFLD) [7], atherosclerosis, cardiovascular disease (CVD) [8] and is a strong predictor of mortality [9]. However, neither BMI nor positive energy balance is an exclusive predictor of this metabolic dysregulation. Current evidence suggests adipose tissue distribution to have a substantial impact on systemic metabolism and thus is an important determinant of disease risk. Compartments of body mass distribution are generally split into two, visceral and subcutaneous adipose tissue depots. Visceral adipose tissue is located in the intra-abdominal cavity where it surrounds and connects various organs, while the subcutaneous lays between muscle and hypodermis. Visceral adiposity is highly associated with several pathological conditions including, but not limited to, metabolic syndrome. Indeed, a number of studies demonstrate a higher risk of type-2-diabetes is associated with greater intra-abdominal adiposity [10-14]. Visceral obesity also correlates to atherogenic abnormalities that include dyslipidemia [15-17] (increased circulating triglycerides and free fatty acids and increased LDL to HDL ratio), inflammation and increased risk of thrombosis in the heart (CVD), arties, veins or capillaries [18-20]. Correspondingly, dyslipidemia is associated with NAFLD; hence, visceral obesity is demonstrated to be a predictor of NAFLD [21]. Unlike visceral adiposity, which is associated to be detrimental to health, lower body subcutaneous

adiposity, adipose tissue accumulation in the thigh, buttocks and lower stomach, is proposed to be protective. It has been established that visceral adiposity has a greater association with metabolic syndrome than subcutaneous [22, 23]. Others demonstrate subcutaneous adipose tissue accumulation to be associated with cardioprotection [24]. Specifically, this study linked subcutaneous adiposity with lower circulating triglyceride concentration and lower prevalence of decreased HDL cholesterol levels when compared with visceral adiposity [24]. However, not all regions of subcutaneous adipose tissue are commonly connected to metabolic protection. For example, some implicate subcutaneous adiposity, specifically truncal/abdominal, in the pathogenesis of insulin resistance [25-27], atherosclerosis and cardiometabolic risk [28, 29]. Instead, gluteofemoral subcutaneous adiposity is associated with metabolic protection. High thigh adiposity is associated with lower glucose and triglycerides, higher HDL, insulin sensitivity and decreased risk for type-2-diabetes and metabolic syndrome [3, 30-34]. Although the mechanisms that causally relate the different metabolic outcomes of adipose tissue distribution are not fully understood, they are often attributed to location and/or adipocyte specific physiology.

Location

Although visceral adipose tissue, omental and mesenteric depots, has a greater association to the pathogenesis of obesity-related disease, it only constitutes ~10% of total body adiposity whereas subcutaneous is approximately 85% [35]. This relatively low amount of visceral adipose tissue, however, is proposed to play a role in obesity-induced health consequence due to its anatomical location. Visceral adipose depots surround, cover, attach and share vasculature with abdominal organs. This relationship can become detrimental

predominantly with venous drainage to the liver where insulin-sensitive hepatocytes are directly exposed to the metabolites and secretory products released by visceral adipocytes into the portal vein [36-38]. Indeed, excessive adipose tissue accumulation and subsequent release of numerous adipokines, pro-inflammatory cytokines, glycerol, lactate and fatty acids into the hepatic portal vein have major influence on hepatic processes such as glycogenesis, gluconeogenesis and very low-density lipoproteins (VLDL) synthesis [39-44].

Of the many factors known to play a role in hepatic metabolic processes free fatty acid induced alterations are best defined. The portal hypothesis proposed in 1990 by Bjorntorp postulates that excessive visceral adipose tissue free fatty acid effluent is an inducer of hepatic insulin resistance [45]. In support of this, studies demonstrate chronic exposure of the liver to free fatty acids modifies several key metabolic processes such as stimulating hepatocyte gluconeogenesis [38, 46], hepatic lipid synthesis and storage, as well as reducing enzymes involved in fatty acid oxidation [47-49]. Excessive fatty acid effluent also decreases hepatic insulin binding and degradation [50] and consequently results in systemic hyperinsulinemia [51] and additional attenuation of insulin suppression of hepatic glucose production (*i.e.* hepatic insulin resistance) [52]. Taken together dysregulation of the lipid and insulin signaling pathways perpetuate reciprocal causation in which the ectopic lipid accumulation further exacerbates hepatic insulin resistance [51, 53] which in turn increases dyslipidemia [54]. However, the adipose depot/region responsible for obesity-induced increases in portal vein free-fatty acid release remains controversial. Some estimate the subcutaneous adipose depots in obese humans to supply the majority of free fatty acid flux to the portal vein [55] and systemic circulation [55, 56]. Visceral adipose tissue is estimated to contribute minimally to portal vein fatty acid flux [55, 56], thus some postulate obesity-induced pathophysiology of the liver may primarily be due to

the limited ability of subcutaneous adipose tissue to store excess energy and visceral obesity is a secondary consequence of this action. Others demonstrate, however, that visceral adiposity is associated with an increase in post-prandial [57] and post-absorptive [58] systemic fatty acid concentration and similarly portal vein fatty acid concentration appears to increase proportionally with visceral fat accumulation [55].

The deleterious consequences of visceral adiposity are, in part, due to free fatty acid flux to the liver, but portal effluent also includes potentially deleterious factors such as adipokines and cytokines, also known as adipocytokines, which are adipose derived cytokines or hormones. This list currently includes, but is not limited to, adiponectin, apelin, chemerin, interleukin-6 (IL-6), leptin, monocyte chemoattractant protein-1 (MCP-1), omentin, plasminogen activator inhibitor-1 (PAI-1), resistin, retinol binding protein 4 (RBP4), tumor necrosis factor-alpha (TNFα) and visfatin. Although the proposed roles of these adipokines and cytokines in obesity-induced pathophysiology will be discussed later in more detail, it is important to note that some of these factors have been identified to de differentially released between portal and systemic circulation. Studies in obese humans demonstrate IL-6 and visfatin concentrations to be higher in portal circulation than systemic, but adiponectin, chemerin, leptin, MCP-1, resistin and TNFα [59-63] are similar between the two sample areas. To our knowledge, the differences between portal and systemic release of apelin, PAI-1, omentin, RBP4 has yet to be investigated.

Inherent Differences in Visceral and Subcutaneous Depot Regulation

Adipokines

Adipose tissue plays an integral role in regulating systemic physiologic and pathologic processes via cell signaling molecules, adipokines, or cytokines. In obesity, the release of

adipokines becomes dysregulated leading to a proinflammatory environment that subsequently changes the composition of residing adipose tissue immune cells, such as M1-macrophages and cytotoxic $CD8^+$ T-cells [64]. As previously discussed, the association between visceral adiposity, insulin resistance, inflammatory disorders and cardiovascular disease may, in part, be a consequence of effluent to the liver [65]. Although the expression and release of the majority of adipokines are increased in both visceral and subcutaneous adipose depots with obesity [66], the pathogenic differences of adipose distribution to some extent likely result from the disproportion of these increases between these two regions.

Of the adipokines, the physiological roles of leptin, resistin and adiponectin are among those that are best characterized in both lean and obese individuals. Leptin is a lipostatic signal, with expression directly proportional to increases in adiposity, that plays a fundamental role in regulating energy intake (anorexigenic/satiety indicator) and expenditure. Leptin signaling has also been demonstrated to play a role in immune homeostasis, thus in obesity this adipokine can also lead to immune dysfunction. Specifically, increases in circulating levels of leptin as occurs in obesity contribute to enhanced production of Th1-type proinflammatory cytokines, a reduction of Th2-type anti-inflammatory cytokines [67] and increases in activity of adipose-derived macrophages [68]. The role of leptin in inflammation occurs through its binding on immune cells which initiates the JAK/STAT signaling cascade and subsequently induces growth and function of that target immune cell [69]. Particularly, leptin receptors are expressed on T lymphocytes where it acts to modulate/regulate maturity of this cell population [70]. Leptin also produces damaging cytokines and impairs insulin action through its participation in the activation of signaling pathways related to glucose homeostasis such as MAPK/ERK and PI3K/Akt [69]. Its direct secretion from mature large adipocytes [71] is differential between subcutaneous and

visceral adipose tissue depots. Studies in obese men and women demonstrate leptin mRNA expression was ~2.8-fold higher in the subcutaneous adipose depot than omental and determined a direct relationship between cell size and location [72]. Consistent with this, another study in lean and obese women determined leptin secretion was also increased ~ 2-fold in subcutaneous adipose tissue compared with omental [71]. Overall, increased leptin expression and secretion from the subcutaneous adipose tissue depot is due to the larger cell size in this region [71]. Some postulate subcutaneous lipid deposition plays a greater role in communicating whole body energy status.

Studies in rodents demonstrate a role of adipocyte secreted resistin in glucose homeostasis [73, 74]. Resistin is associated with impaired glucose tolerance and insulin action and is demonstrated to increase in blood circulation without corresponding increases in adipose tissue mRNA expression in obese rodents [75]. Unlike rodents, resistin in humans is not expressed by adipocytes, but rather is primarily secreted by macrophages located within adipose tissue depots [76]. Therefore, in humans circulating resistin is not directly related to the adiposity levels, but rather degree of inflammation within the adipose tissue depots. Therefore evidence demonstrates adipose tissue macrophage infiltration associated with obesity causes an increase in resistin expression and release [77]. Although the role of resistin in inflammation is well defined in humans, its relation to insulin resistance and type-2-diabetes is controversial [78]. Several proinflammatory stimuli increased in obesity such as lipopolysaccharide, interleukin 6 (IL-6), IL-1β and TNF-α have been demonstrated to increase resistin production [79]. In addition, resistin has been demonstrated to upregulate the expression of IL-6, IL-12 TNF-α and monocyte chemoattractant protein 1 (MCP-1) [80, 81]. Despite the controversial association of resistin and glucose homeostasis in humans, there remains a strong association between increases in resistin

and inflammatory dysregulation that, in part, likely contributes to the chronic low-grade inflammation linked to metabolic syndrome. In rodents, gene transcripts for resistin are significantly lower in the visceral adipose depot than that observed from all other fat depots including subcutaneous [65]. Gene expression of resistin is also differentially regulated in rodent adipocytes with obesity-induced decreases occurring in visceral adipose tissue depots, but not subcutaneous [82]. In a recent study of women undergoing abdominoplasty, extracted omental adipocytes secreted 250% more resistin than cells extracted from subcutaneous [83]. Studies of resistin gene expression and secretion among humans and rodents are inconsistent, thus more research is needed to determine the role of this hormone in metabolic complications.

Unlike leptin and resistin, adiponectin release is associated with enhanced glucose uptake and lipid oxidation through activation of AMP-activated protein kinases, thus adiponectin increases are linked to improved insulin sensitivity and decreased fatty acid toxicity [84]. Adiponection also regulates anti-inflammatory pathways by numerous mechanisms including inhibition of TNFα production, modulation of T-cell activity, and promotion of M2 macrophage differentiation [85]. In humans excessive adipose tissue accumulation decreases adiponectin mRNA levels thus the protective insulin sensitive effect of this hormone is decreased which consequently contributes to the detrimental effects of obesity. Gene expression of adiponectin is differentially regulated between adipose tissue depots, but specificity of this depends on the multimeric isoforms. In general total adiponectin mRNA and protein concentrations are inversely correlated with subcutaneous, but not visceral, adiposity [86]. Visceral adipose tissue has lower gene expression of the total adiponectin compared with subcutaneous depots but high-molecular weight adiponectin is higher in visceral adipose tissue [87]. The primary active form of adiponectin is the high-molecular weight isoform, which is predominantly released from visceral

adipose tissue, indicating that the potency of adiponectin depends not only on adiposity, but also on adipose depot origination.

The list of adipose tissue adipokines continues to grow with recently described vaspin, omentin, chemerin, and galectin. Vaspin is a serine protease inhibitor that is highly expressed in visceral adipose tissue and positively correlated with obesity [88]. Though vaspin is primarily derived from visceral adipose tissue, it is expressed in multiple organs like stomach, liver, pancreas, and skin in humans. In rodents, vaspin effects on the liver are beneficial by way of relieving ER stress and attenuating metabolic dysregulation [89]. While vaspin gene expression and protein content is undetectable in lean rodents, it is positively correlated with body weight and its expression in obese rodents is higher in visceral adipose tissue than subcutaneous [88]. In humans vaspin mRNA expression is increased in obesity, but is not differentially expressed among adipose depots or correlated with metabolic parameters [90]. The role of vaspin in obesity-induced co-morbidites remain to be elucidated. Omentin is a visceral adipose tissue specific adipokine inversely associated with obesity that is secreted from stromal vascular cells within the depot rather than adipocytes [91]. Much like adiponectin, omentin stimulates glucose uptake via activation of Akt, which stimulates insulin-mediated pathways [91]. In obese humans omentin gene expression is decreased in the visceral, but not subcutaneous, adipose tissue depot compared with lean individuals [90]. Chemerin is characterized as a chemoattractant protein as it stimulates dendritic cells and macrophages to a site of inflammation by promoting adipocyte differentiation. The role of chemerin in adipogenesis is supported in cell culture studies that demonstrate mRNA expression of chemerin protein and its receptor increase rapidly as adipocytes mature [92]. Serum chemerin concentration is positively correlated to body mass index in men and women and its gene expression is higher in subcutaneous adipose tissue

compared with visceral [93]. These data propose that the primary source of chemerin is from subcutaneous fat depots; however, more research is needed to understand how chemerin may contribute directly or indirectly to obesity and its metabolic diseases. A group of adipokines related to inflammation, immune function, and adipocyte differentiation is the family of galectins, specifically galectin-12. The presence of galactin-12 on the outer shell of lipid droplets within adipocytes is associated with a suppression of lipolysis via degradation of cAMP levels which in turn down regulates protein kinase A activity, ultimately reducing the release of free fatty acids [94]. Studies in mice demonstrate that expression of this protein is inversely correlated with adiposity and is lower in visceral adipose tissue compared with subcutaneous [95].

Overall expression and release of most adipokines involved in metabolic dysregulation are higher in subcutaneous adipose tissue than visceral despite the associated link between central adiposity and metabolic dysfunction. Perhaps differential regulation is a product of location, hence visceral effluent directly to the liver is potentially more damaging than systemic effluent from the subcutaneous depot, which ultimately accounts for lower secretion from visceral adipocytes. Further investigations are needed to compare portal and systemic concentrations of adipokines in obese and lean individuals. Of the multiple metabolites and products released from adipose tissue, adipokines may play a inconsequential role relative to free fatty acids and cytokines, however as previously indicated adipokines when dysregulated subsequently induce pro-inflammatory pathways.

Inflammation

Obesity is often described as a state of chronic low-grade systemic inflammation that occurs as a consequence of intrinsic adipose tissue immune system activation [96]. Although the inciting etiology of obesity-induced adipose tissue production and release of proinflammatory cytokines remains to be elucidated, adipose tissue hypoxia (insufficient oxygen supply to expanding and/or proliferating adipocytes) is often proposed to play a fundamental role [97]. Emerging evidence suggest that adipose tissue compartmental distinctions in immunity regulation, specifically differential regulation of immune response within the visceral cavity, may contribute to the pathophysiology of central adipose accumulation. Studies have begun to identify and characterize the consequences of differential regulation of inflammatory adipokines, adipose derived cytokines/mediators of inflammation, within distinct adipose depot regions. The interleukin ((IL): IL-1) cytokine family is identified to play a fundamental role in the relationship between immunity and the risk for developing metabolic syndrome [98-100], thus cytokines within this family are suggested propagators of visceral adiposity-induced inflammation [99]. Thus far two cytokines in the IL-1 family are demonstrated to play a fundamental role especially in visceral adipose tissue immune response, IL-1β and IL-18 [101, 102]. The protein expression of these two cytokines are observed at a level 10-fold higher in visceral than subcutaneous adipose tissues depots [99]. Consistent with this IL-1β and IL-18 production is induced by cellular components of pathogenic bacteria such as lipopolysaccharides (LPS) [102, 103]. In obesity, especially that induced by high saturated fat/western diets, it has been demonstrated that the gut becomes increasingly permeable, and in turn creates more opportunity for the penetration of such substrates as LPS. Dysregulation of the pathways involved with IL-1β and IL-18 can lead to the exacerbation of chronic inflammation in relation to visceral adiposity and subsequently

induce metabolic disease. The exact source of IL-1β is unknown, but it is a proposed product of macrophages originating in adipose tissue, subsequently promoting cytokine production during the development and progression of obesity. However, adipocytes also secrete IL-1β and express caspase-1, a component of the inflammasome required for the activation of IL-1β, but at lower levels than macrophages [99]. In this manner, inflammation is not only increased by macrophages and the pro-inflammatory cytokines that they produce, but also the expanding visceral adipose depot as well by way of adipocyte secretion.

Cytokines outside of the IL-1 family that have also been identified to be active in the inflammatory and immune response and are found to be higher in visceral adipose tissues are IL-12, Interferon gamma, monocyte chemoattractant protein 1 (MCP-1) , IL-6, IL-8, and plasminogen activator inhibitor 1 (PAI-1) [104-106]. IL-18 in combination with IL-12 induces production of interferon gamma (IFN-gamma), a macrophage-activating factor, by TH-1 cells [107]. IFN gamma has been demonstrated to be elevated in visceral adipose tissues of obese individuals. Therefore the pathogenesis of obesity induced systemic inflammation and metabolic disease may be the contribution of the innate immune response consisting of macrophages and other innate immune molecules as well as adaptive immunity that is mediated by T-helper cells, specifically type 1 [107]. MCP-1 has been demonstrated to play an important role in the infiltration of macrophages in adipose tissue, specifically visceral adipose tissue [108] that consequently leads to the release of pro-inflammatory cytokines. Studies demonstrate that increased levels of MCP-1 are highly correlated with obesity-linked visceral adiposity [108]. This is central to the induction of systemic inflammation that results from increased visceral adiposity as these cytokines begin to interact with tissues outside of the visceral depot [109, 110]. IL-6 and IL-8, also elevated in visceral adipose tissue compared with subcutaneous, are

both linked to the increased recruitment of macrophages and are active in the immune response. The main function of IL-8 is the recruitment of neutrophils to a site of injury or infection [111] and IL-6 is predominately involved in the regulation of immune function [112]. IL-6, like IL-8, is involved with neutrophils in the initial inflammatory response but through several other chemokine signals (CSC, and CC) facilitates the immune transition to a more sustained response, if necessary, involving mononuclear cells [112]. PAI-1 regulates thrombus formation via inhibition of tissue-type plasminogen activator which is an anticlotting factor [113]. Consistent with this increased release of PAI-1 is a risk factor for cardiovascular disease and is strongly associated with insulin resistance [114]. PAI-1 concentration is directly associated with visceral adiposity but further investigation is needed to determine its role as the driving factor or consequence of obesity-induced metabolic dysregulation [106].

Studies demonstrate that the obesity-induced macrophage infiltration that results from adipocyte death during rapid depot expansion is greater in the visceral adipose tissue depot than subcutaneous [115]. The enzyme ALOX15b is proposed to play a fundamental role in regulation of macrophage induced pro-inflammatory cytokines in adipose tissues especially those demonstrated to be produced at higher levels in visceral adipose tissues (e.g. IL-12, IL-6 and IFN gamma) [105]. Specifically, the proposed inciter of obesity-induced inflammation, adipose tissue hypoxia (insufficient oxygen supply to expanding and/or proliferating adipocytes), is demonstrated to trigger ALOX15b expression in macrophages in human omental adipose tissue which subsequently induces chemokine production and T-cell migration [105].

The visceral adipose depot, specifically the omentum in humans, is a potent source of immune molecules [116]. The omentum differs from subcutaneous adipose tissue in its innate immune ability which is likely due to higher number of lymph nodes associated with the need to

respond to pathogens in the peritoneal cavity, such as gut release of lipopolysaccharide (LPS) [117]. Indeed, this immune depot in the intra-abdominal cavity is referred to as the peritoneal defense mechanism [118]. In addition to lymph nodes, the omentum contains milky spots that house large numbers of macrophages and other immunocompetent molecules [116, 117]. As previously discussed, macrophages and adipocyte dysregulation are indicated in the pathogenesis of metabolic syndrome related to obesity. Once the immune response is stimulated, the lymphatic system can also induce lipolysis in adipocytes surrounding lymph nodes for an energy source [119]. Conversely low grade chronic inflammation can also initiate adipogenesis and adipose tissue hypertrophy [120] which further exacerbates visceral obesity metabolic dysreglation.

Unlike adipokine release, which is predominately higher in subcutaneous adipose tissue than visceral, the proinflammatory immune response initiated from adipose derived cytokines or resident macrophages is consistently higher is the visceral depot. Because obesity is defined as a state of chronic low-grade inflammation, differential immune regulation among visceral and subcutaneous adipose depots likely plays a key role in their contrasting contributions to the pathogenesis of obesity-related dysregulation.

Lipid Regulation

The acquisition and release of fatty acids from adipocytes is fundamental for the regulation of energy homeostasis. Visceral and subcutaneous adipocytes, however, have been demonstrated to have differential response to lipid storage and lipolytic stimuli. Studies in non-obese humans demonstrate that adipocytes from the omental depot have greater rates of free fatty acid storage than those located subcutaneously. The difference in storage flux is related to the

adipocyte rate limiting steps involved in factors regulating fatty acid incorporation into triglyceride. Proteins involved in fatty acid transport across the plasma membrane (plasma membrance-associated fatty acid binding protein; FABP), intracellular activation/trapping (Acyl-CoA; ACS) and final conversion to triacylglycerol (diaclyglycerol acyltransferase; DGAT) are higher in adipocytes from the omental depot compared with those from subcutaneous [121]. Lipolysis regulation is also differentially regulated in the adipocytes of the visceral and subcutaneous depots. Major regulators of lipolysis include catecholamines and insulin. The action of catecholaimes are regulated through adrenoceptor located on the cell-surface of adipocytes. In comparison with subcutaneous adipocytes, those isolated from the visceral depot have an increased expression of beta-1, -2 and -3 adrenergic receptors [122, 123]. Consistent with this, other studies demonstrate higher rates of catecholamine-induced lipolysis in visceral adipocytes. In lean humans, visceral adipocyte lipolytic response to beta-agonist such as epinephrine, norepinephrine and isoprenaline is up to 10 times higher than subcutaneous adipocytes [122]. This lipolysis sensitivity is also demonstrated to be higher in the visceral adipocytes from obese humans [124].

Total adipose tissue deposition is also determined by the rate of antilipolytic signaling. Insulin a prominent inhibitor of adipocyte lipolysis blocks liberation of free fatty acids by inducing a cascade of signals to reduced signaling pathways fundamental for lipolysis (protein kinase A: PKA) and consequently enzymes responsible for hydrolyzing stored triglycerides to free fatty acids (i.e. hormone sensitive lipase: HSL) [125]. As with lipolysis, antilipolytic regulation is also different between visceral and subcutaneous depots. Studies in obese humans demonstrate an inverse correlation between the antilipolytic effect of insulin and visceral adiposity, but not subcutaneous adiposity [126]. Indeed, adipocytes from the visceral depot are

less responsive to the antilipolytic effect of insulin than those from the subcutaneous depot [127, 128]. In the visceral depot a higher concentration of insulin is needed to produce an equivalent suppression of lipolysis as occurs in non-visceral adipocytes [129]. This is likely an outcome of lower binding affinity of insulin to visceral adipocytes [130], which is consistent with a reduction in insulin receptor substrate (IRS)-1 protein expression in visceral adipose tissue [128]. Other key regulators of lipid storage balance that have higher expression in visceral adipocytes than subcutaneous include hormone-sensitive lipase, lipoprotein lipase and fatty acid synthase [131-133]. Overall, these studies indicate that intra-abdominal adipose tissue has a higher turnover of free fatty acids than adipose tissue in the lower body, which is a consequence of higher incorporation, increased lipolysis and decreased sensitivity to the antilipolytic effects of insulin.

Adipogenesis and Apoptosis

Growth of pre-adipocytes to adipocytes, as well as death of mature adipocytes, also vary by location. Adipose depot growth is characterized by the number of new cells that develop compared to apoptosis of existing cells. Factors that influence growth of new adipocytes include hormones, transcription factors, and cell signaling molecules, all of which affect activity of the master regulator, peroxisome proliferator-activated receptor-γ (PPAR- γ). Specifically, adipose-derived stem cells show enhanced adipogenesis properties in the subcutaneous and pericardial regions when compared to the visceral region [134]. It has been shown in rats that adipocytes in subcutaneous depots differentiate more rapidly and extensively than those in the visceral depot, presumably due to differential gene expression and responsiveness to growth factors [135]. This finding may contribute to the protective effect of subcutaneous versus visceral fat, owing to the

fact that size and age of adipocyte are key factors in their response to insulin and lipid storage capacity. Cells send out signals to initiate apoptosis in response to stress, thus programmed cell death of mature adipocytes is in part dependent on their perceived threat from circulating molecules. In vitro, visceral adipose tissue is more susceptible to TNFα, which acts as an apoptotic stimulus, than subcutaneous fat [136]. As discussed, the pro-inflammatory cytokine TNFα is upregulated in obesity, suggesting that visceral adipocytes are more vulnerable to death from release of these damaging signals.

Stress, Glucocorticoids and Visceral Adiposity

Growing evidence suggest that repeated stress, recurrent activation of the stress response, may play a role in visceral adipose tissue deposition, thus may also be a contributing factor toward the development of low-grade systemic inflammation that is linked to the pathogenesis of metabolic dysregulation. Clinical studies demonstrate that people repeatedly subjected to psychosocial stress have associated disturbances in hypothalamic-pituitary-adrenal (HPA) axis and increased visceral adiposity, thus suggesting an increase in HPA axis activity may drive lipid distribution to the visceral depot [137]. Despite these correlative clinical studies, the pathogenic mechanisms linking stress with visceral adipose depot redistribution remain unclear. Recent studies demonstrate that stress-induced alterations in adipose tissue metabolism are linked to crosstalk with the immune system. The cytokine IL-1β is one of the proposed regulators of stress-induced visceral adipose tissue accumulation. IL-1β mediated adipose depot redistribution is proposed to be a consequence of changes that occur in subcutaneous adipose tissue. After a stressor, IL-1β release from subcutaneous adipose tissue, but not visceral, increases 5-fold [138]. Although IL-1β is best characterized as an immune modulator, this cytokine has also been

demonstrated to decrease genes involved in adipogenesis [139] and increase lipolysis by decreasing expression of genes involved in lipid metabolism [140] such as lipid droplet-associated fat specific protein 27 (FSP27) [141]. Taken together these studies demonstrate repeated stressors that induced frequent IL-1β signaling in subcutaneous adipose tissue reduces the ability of this depot to absorb or retain lipids, thus altering adipose distribution by shunting lipid deposition to the visceral cavity [138]. IL-1β is also proposed to play a role in glucocorticoid activation by stimulating the enzyme 11-beta hydroxylsteroid dehydrogenase type 1 (11β-HSD1) which converts inactive cortisol/corticosterone to active forms [142].

Evidence suggests glucocorticoids play a prominent role in the development of visceral adiposity, e.g. Cushing's syndrome hypercortisolemia is associated with visceral adipose accumulation. It is well established that glucocorticoid production and secretion, cortisol in humans, is associated with obesity, specifically visceral obesity [143, 144]. Studies demonstrate that urinary cortisol excretion rates are 1.6 fold higher is women with central/abdominal adipose tissue distribution than those with peripheral adipose accumulation [145, 146]. However, many studies suggest that obesity-induced increases in cortisol excretion is not due to over activity of the hypothalamic-pituitary-adrenal axis but rather increased glucocorticoid activation within adipose depots (for review see: [147]). Growing evidence substantiates alterations in adipose tissue specific intracellular cortisol metabolism, specifically the fundamental role of 11β-HSD1 in stressed-induced obesity pathophysiology. Local alteration of glucocorticoids have been demonstrated to be regulated in a tissue-specific manner pre-receptor via the enzyme 11β-HSD1 that, as mentioned previously, converts the inactive glucocorticoid cortisone to its active form cortisol. In mice, selective overexpression of 11β-HSD1 in adipose tissue results in visceral obesity and comorbidities associated with it such as insulin resistance, glucose intolerance and

hyperlipidaemia [148]. Consistent with this human studies confirm that 11β-HSD1 expression is increased in adipose tissue during obesity [149, 150]. Although evidence discriminating differences in subcutaneous and visceral gene expression is inconsistent (for review see: [151, 152]) data does demonstrate visceral adipocytes are more responsive to the effects of glucocorticoid actions. Glucocorticoid mRNA expression is 2 to 4-fold greater in visceral adipose tissue compared with subcutaneous, hence there are more binding sites and greater binding of glucocorticoids in the visceral cavity [153-155]. The increased response of glucocorticoids in the visceral cavity ultimately alters adipogenesis in such manner that in combination with high insulin release, as occurs in obesity, numerous genes involved in lipid deposition are increased [156].

Endocannabinoids and Visceral Obesity

The endocannabinoid (EC) system is also implicated in redistribution of lipids to the visceral cavity. This system plays a role in glucose and lipid regulation thus when dysregulated leads to site-specific lipid accumulation. The EC system modulates adipose tissue metabolism through type 1 cannabinoid receptors (CB_1) where increases in ligand and receptor are demonstrated to increase pre-adipocyte differentiation to mature adipocytes as indicated by upregulation of peroxisome proliferator-activated receptor γ (PPAR-γ), increased triacylglycerol accumulation and subsequently increased cell size [157]. CB_1 stimulation enhances triacylglycerol by various mechanism such as, but no limited to, increased activation of lipoprotein lipase [158], inhibition of lipolysis [157], decreased fatty acid oxidation [159], increased fatty acid synthase and increased glucose uptake [160]. Increased blood and adipose tissue levels of ECs have been reported in obese/overweight individuals [161]. In humans,

increased EC release is positively associated with visceral, but not subcutaneous, obesity [157, 162, 163], however, the role of CB_1 alterations is controversial and needs further investigation. Overall, it is postulated that in obesity enhanced EC system activity within the visceral depot is responsible for the excessive accumulation of fat within this depot compared with the subcutaneous.

Extrinsic Factors

Extrinsic factors play an important role in regulation of adipose tissue depot metabolism, yet they are generally underemphasized. Studies demonstrate that obesity is associated with sympathetic nervous system over activity to the visceral adipose depot [164-168]. Although it has been postulated that sympathetic nervous system differentially innervates adipose depots its characterization have yet to be elucidated. Another important extrinsic factor related to adipose tissue expansion is development and homeostasis is adipose depot vasculature. Studies demonstrate that adipose depot size is directly related to blood flow capacity [169] and blood flow in visceral adipose tissue is higher on a per gram weight basis than subcutaneous [170]. In addition, others show that differential adipose tissue angiogenesis may contribute to metabolic disease pathogenesis. Visceral adipose tissue is has a lower capillary density and decreased potential to expand its capillary network during depot expansion compared with subcutaneous [171]. Hence, increased impairment of adipose tissue angiogenesis could lead to hypoxia-induced inflammation, which is the proposed initiator of adipose tissue dysfunction.

Sex Hormones (Estrogen)

Numerous studies demonstrate that women in their mid-life tend to have a shift in adipose tissue distribution from lower body subcutaneous deposition to visceral adipose accumulation [172]. Consistent with diet-induced visceral obesity, consequences of mid-life adipose tissue redistribution include insulin resistance [173] and dyslipidemia [174], which may partially explain the greater risk for cardiovascular disease in aging women. This shift in adipose tissue distribution in women is called "perimenopausal obesity" because the adipose shift tends to occur concomitantly with events surrounding cessation of reproduction. Although the etiology of this shift is not completely understood, it is proposed that progressive declines in hormones such as estrogens play a role. Evidence suggest that estrogens, specifically 17β-estradiol, plays a role in the metabolism and regional distribution of adipose tissue. Estrogen has been demonstrated to regulate lipid storage via lipoprotein lipase, promote leptin expression and increase adipocyte proliferation [175, 176]. Consistent with decreased estrogen release in humans, studies in rodents demonstrate lower estrogen levels following removal of the ovaries results in intra-abdominal/visceral adiposity gain [177]. Estrogen replacement in these rodents, however, reversed these effects. Antilipolytic alpha2A-adrenergic receptors are a fundamental factor in the maintenance of the typical female lower body adipose tissue distribution. Accumulation of lipids to lower body adiposity by estrogen is due to its ability to up-regulate alpha2A-adrenergic receptors, which subsequently attenuates lipolysis of adipocytes specifically in this region [178]. Therefore, decrease in estrogen would enhance lower body subcutaneous adipose tissue lipolysis and favor intra-abdominal adipose deposition.

Thiazolidinedione (TZD)

Thiazolidinediones (TZDs) are ligands for the peroxisome proliferator-activated receptors (PPARs) that have implications for the treatment of diabetes. Long term TZD treatment in diabetic patients increase insulin sensitivity, reduces C-reactive protein (a marker of inflammation) and decreases the cardiovascular risk associated with visceral adiposity [179]. However, research suggests that the insulin-sensitizing action of TZD is a consequence of depot specific promotion of preadipocyte differentiation [180]. TZDs have adipose depot-specific responses that favor accumulation in lower body subcutaneous adipose tissue and current data supports that adipocyte proliferation in this region is responsible for TZD-induced improvements in insulin sensitivity [180, 181]. TZD treatment, however, does not alter adipocyte number or size of adipocytes in the visceral adipose tissue depot. The insulin sensitizing effect of TZD treatment is paradoxical given its fat promoting properties, specifically in subcutaneous adipose tissue. However, this result is reasonable based on adipocyte re-structuring towards smaller fat cells with greater capacity for lipid storage and insulin action.

Conclusions

Adipose tissue distribution, but not total adiposity, has a substantial impact on systemic metabolism and thus is an important determinant of disease risk. Numerous potential mechanisms may causally relate the different metabolic outcomes of adipose tissue distribution. These factors likely interact synergistically with one another to exacerbate the metabolic perturbations of obesity. Detrimental visceral adiposity and its association with the pathological conditions of obesity could be a consequence of limited expansion of the protective subcutaneous adipose tissue. Therefore, obesity-induced pathophysiology of the liver may

primarily be due to lipid spillover from subcutaneous adipose depots, which would eventually alter portal effluent. The obesity pathophysiology connection, however, cannot exclusively be attributed to location and free fatty efflux providing visceral adipose depot adipocytes structure, physiologic and metabolic characteristics are different from subcutaneous adipose depot. This review demonstrates there is a disproportionate increase of pro-inflammatory events that occur in visceral adipose tissue during obesity. It is likely that the induction of inflammation in the visceral adipose tissue not only links obesity with metabolic disorders but is also the origin of obesity-induced dysregulation.

CHAPTER 2: ADIPOSE TISSUE: AN ENDOCRINE ORGAN PLAYING A ROLE IN METABOLIC REGULATION[3,4]

Summary

Adipose tissue is a complex endocrine organ with an intricate role in whole body homeostasis. Beyond storing energy, adipose tissue is fundamental in numerous processes including, but not limited to, metabolism, food intake and immune cell function. Adipokines and cytokines are the signaling factors from adipose tissue. These factors play a role in maintaining health and are candidates for pathologies associated with obesity. Indeed excessive adiposity causes dysregulation of these factors that negatively affect health and contribute to numerous obesity-induced co-morbidities. In particular, adipokines are fundamental in regulation of glucose homeostasis and insulin signaling, thus aberrant production of these adipose derived hormones correlates with the development and progression of type 2 diabetes. Therefore, elucidation of adipose regulation is crucial for understanding the pathophysiological basis of obesity and metabolic diseases such as type 2 diabetes. In the present review, we summarize current data on the relation between adipokines and adipose depot derived cytokines in the

[3] A modified version of this chapter is published as Booth A, Magnuson A, Fouts J, Foster MT. Adipose tissue: an endocrine organ playing a role in metabolic regulation. Horm Mol Biol Clin Investig. 2016 Apr 1;26(1):25-42.

[4] The aim of this work was to conduct a literature review on intrinsic characteristics of adipose tissue and its role as an endocrine organ. We investigated the rate and contribution of adipocytokine secretion on metabolic regulation. This discussion outlines the implications of the unique components of adipose tissue biology and highlights depot differences that effect metabolic outcomes.

This chapter includes the complete published manuscript for this literature review titled *Adipose tissue: an endocrine organ playing a role in metabolic regulation* (Andrea Booth, Aaron Magnuson, Josie Fouts, and Michelle Foster, Horm Mol Biol Clin Investig., 2016). My contributions to this publication included writing multiple sections of the body of the text, review for accuracy and cohesiveness of the entire manuscript, editing and corrections prior to submission.

Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 2.1. This article is reproduced with permission, and only minimal modifications were made to meet formatting requirements. No other modifications were made, as per the licensing agreement.

maintenance of glucose homeostasis. Specifically, physiological and molecular functions of several adipokines are defined with particular focus on interactions within the insulin-signaling pathway and subsequent regulation of glucose uptake in both standard and obesity-induced dysregulated conditions. This same relation will be discussed for cytokines and inflammation as well.

Introduction

Excessive weight gain and subsequent obesity are becoming a worldwide problem [182, 183]. Weight increases following excess energy intake are primarily due to increases in adipose tissue deposition. Previously thought to function exclusively as insulation and an energy reservoir, adipose tissue is now characterized to be a complex organ with central nervous system (CNS) innervation and fundamental endocrine and immune roles [For review see [184]]. The adipose depot is a multifaceted structure that is comprised of adipocytes as well as pre-adipocytes, endothelial cells, fibroblast and immune cell types that include, but are not limited to, macrophages, dendritic cells, and T cells. All these cells together contribute to the tissues effluent release of metabolites, lipids, cytokines and adipokines. Under standard conditions, adipose tissue plays a role in whole body homeostasis by storing lipids that are used as an energy source during fasting, thus preserving protein, regulating metabolism and food intake (balances energy homeostasis), reproduction, and fueling immune response to pathogen invasion. Excessive adiposity, which occurs with obesity causes balance of the adipose organ to become dysregulated and subsequently produces a negative effect on health. Hence, obesity is causally linked to a cluster of chronic and complex diseases such as cardiovascular disease [1], metabolic

syndrome [185] and type 2 diabetes [2]. The complicated association between excessive adiposity, insulin resistance and metabolic diseases remains a subject of prevalent investigation.

The metabolic and endocrine functions of adipose tissue have been extensively investigated over the last two decades. It is well established that adipose tissue secreted factors play a role in whole body glucose homeostasis, but the specifics of how this regulation occur are still being elucidated. Current proposed regulators of this link include both adipose depot derived hormones and cytokines. Adipocyte secreted factors, "adipokines", are signaling molecules that regulate numerous biological processes by autocrine, paracrine and endocrine mechanisms. Adipokines are essential in the balance between appetite and satiety, regulation of body fat stores and energy expenditure, glucose tolerance, insulin release and sensitivity, cell growth, inflammation, angiogenesis and reproduction. Target organs/systems of adipose tissue include, but are not limited to, the brain, liver, muscle, heart, pancreas, thymus, spleen and lymph nodes. Cytokines released from the adipose depot also influence both local and systemic metabolism, but are slightly different from adipokines. First, cytokines can be secreted directly from adipose tissue, but are also released from other cell populations in the stromal vascular cells of the adipose depot such as preadipocytes, fibroblast and immune cells (macrophages, dendritic cells, and T cells). Second, cytokines are involved in cell signaling but primarily play a role in immune regulation and are generally not growth factors for non-immune cell populations. Overall, in both standard and dysregulated conditions it is valuable to understand specifically what manner adipocyte released factors can affect within depot cellular events as well as those systemic events regulated through endocrine exchanges.

Diet-induced obesity alters adipose tissue beyond just increases in depot size. This rapid and persisting adipose growth alters cell composition and leads to depot dysregulation and

subsequently altered adipocyte biology and function. Inflammation within adipose tissue is proposed to link obesity with the cluster of metabolic associated diseases [186]. A proposed trigger of this inflammation is hypoxia, which occurs in response to adipocytes that do not have access to depot vasculature [187]. Indeed, increases in adipocyte number increase circulating adipokine concentration while hypoxia independently exacerbates this adipokine release from individual adipocytes [188, 189]. Hypoxia is proposed to initiate obesity-induced inflammation and this together with enhanced adipokine release is fundamental in the loss of insulin sensitivity leading to elevated increases in glucose concentration. Taken together, excessive adipose deposition increases adipokine release and causes hypoxia among adipocytes, leading to increases in cytokines and recruitment of immune cells. This process is associated with insulin resistance and glucose intolerance. Here we will highlight the role of adipose depot released factors in glucose homeostasis and dysregulation events that occur with obesity. Figure 2.1 is a simplified representation of the insulin-signaling pathway. This figure summarizes where the below discussed adipokines are characterized to alter the insulin-signaling pathway.

Leptin

In a series of experiments, Zhang *et al.* [190] cloned and subsequently demonstrated an obesity gene, *ob,* fundamental to adipose tissue autocrine and paracrine signaling. The *ob* gene, which is highly conserved among all vertebrates including humans, is recognized to play a role in adipose tissue regulation since deficiency of the *ob* gene produces obesity. This gene was identified as an adiposity indicator because increases in *ob* gene expression are positively associated with increased adiposity. It was further demonstrated by Halaas *et al*. [191] that the proposed secretory protein encoded by the *ob* gene, identified as leptin, regulates adiposity by

modulating food intake and energy expenditure. Hence, peritoneal leptin injections reduce body weight in both lean and leptin deficient *ob/ob* mice [191]. Leptin injections in the central nervous system (CNS), *e.g.* arcuate nucleus, also dose dependently reduces food intake and body weight, hence the CNS is a fundamental site for leptin regulation [192]. Leptin can therefore be defined as a lipostatic signal that functions in regulating energy balance through control of food intake.

Obesity is a factor well characterized to increase risk for type 2 diabetes and increased leptin levels are a direct indicator of obesity. Hence, numerous epidemiologic studies demonstrate a positive association between leptin and type 2 diabetes in adults. In a 6-year clinical investigation, individuals with high plasma leptin concentration at study initiation had a greater risk for the development of type 2 diabetes over the duration of the study [193]. In support of this a meta-analysis reported higher plasma leptin to be associated with the occurrence of type 2 diabetes in men [194]. Another study, in both men and women, associating beta cell function, insulin levels, C-reactive peptide, intact pro-insulin, and Des-31,32 pro-insulin levels to leptin concentration reported a positive relation among leptin concentration with insulin and Beta cell function [195]. Specifically, insulin assessment via tertiles (low insulin levels (less than 72 pmol/L), normal insulin levels (72-108 pmol/L) and hyperinsulinemia (greater than 108 pmol/L)) while adjusting for age and fat mass revealed that higher insulin levels were associated with higher leptin levels. Beta cell function measured by homeostatic model assessment (HOMA) is also positively associated with leptin levels but is dependent upon fat mass. With groups separated according to beta cell function, as more or less than 100%, there is a positive association with increased leptin in those categorized as hyperinsulinemic even when adjusted for gender and fat mass. This led the authors to postulate that insulin resistance as measured by HOMA was positively correlated with increased leptin levels in both males and females [195].

Similarly, in moderately overweight men (mean body mass index (BMI) of 26.8) with type 2 diabetes, higher fasting leptin concentration is positively correlated with BMI and fasting insulin while negatively correlated with glucose clearance rates [196]. These correlations remained significant when adjusted for fat mass or BMI. With separation by insulin sensitivity tertile, the highest fasting leptin levels are in patients with the greatest insulin resistance. This indicates a functional relationship between leptin and insulin resistance in patients with type 2 diabetes, with increased insulin resistance being associated with increased leptin levels [196]. In opposition of data collected from adults, a study in adolescents demonstrates that the development of type 2 diabetes is associated with hypoleptinemia [197]. The authors suggest contradictory findings are likely due to subjects being juveniles, BMI matched control group, and/or varying ethnic backgrounds among leptin studies.

There is evidence that links leptin to the development of type 2 diabetes through molecular mechanisms; however, they are not completely understood. Here we will highlight findings by first discussing exogenous leptin administration in an obese model and then extrapolate what occurs during leptin dysregulation. Under standard conditions, leptin enhances insulin sensitivity via alterations in muscle metabolism. In rats, three mechanisms involved in muscle insulin regulation, including phosphorylation of AS160, ceramide and diacylglycerol (DAG) content, and distribution of fatty acid translocase/Cluster of Differentiation 36 (FAT/CD36) to the subsarcolemma and intramyofibrillar mitochondria, were investigated with regard to leptin-induced alterations [198]. Briefly, leptin administration to diet-induced obese rats reverses impaired glucose uptake, via enhanced insulin signaling. In the muscle of obese rats, leptin treatment increases AS160 and AKT phosphorylation and concomitantly decreases concentration of harmful lipid intermediates, DAG and ceramide. Decreases in lipid deposition,

however, are not due to alterations in the fatty acid transport proteins (FAT/CD36) but rather an increase in the oxidative capacity of the mitochondria. This increased oxidative capacity is due to an increase in phosphorylation of the alpha2 subunit of 5'AMP-activated protein kinase (AMPK) and inhibition of acetyl-CoA carboxylase (ACC) which allows transport of fatty acids (FAs) into the mitochondria by carnitine palmitoyltransferase 1 (CPT-1) [199]. AMPK is also a known activator of AS160 in the insulin-signaling cascade, thus is also linked to insulin sensitivity [200]. Overall, this study indicates leptin improves glucose uptake and insulin sensitivity in high fat diet (HFD) fed rats by several concurrent mechanisms; increased AS160 and AMPK phosphorylation, decreased amounts of dangerous lipid intermediates in the muscle, and increased mitochondrial oxidative capacity [198]. Leptin also alters insulin regulation through insulin-like growth factor binding protein-2 (IGFBP-2) mechanisms. Leptin regulation of muscle metabolism occurs by central and peripheral nervous system regulation and direct muscle stimulation. This was investigated through a series of experiments in sheep and human myotubes that addressed leptin mediated central nervous system control of metabolism, leptin mediated release of IGFBP-2 via the sympathetic nervous system (SNS), and direct action of leptin on muscle [201]. Intracerebroventricular (icv) administration of leptin lowers plasma glucose levels; hence, leptin mediates glucose levels via the central nervous system. Central administration of leptin also dose-dependently increases concentration of IGFBP-2 in muscle, but not circulating plasma, demonstrating SNS stimulation of muscle. This was associated with an increase in phosphorylated AKT, a key protein in phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-induced glucose uptake pathway, and a decrease in the levels of phosphatase and tension homologue (PTEN), inhibitor of PI3K/AKT. Leptin also dose dependently increases IGFBP-2 mRNA and protein directly in muscle. Leptin-induced increases in muscle IGFBP-2 production

are dependent upon STAT-3 and PI3K signaling. Taken together, leptin has a direct and indirect effect on increasing IGFBP-2 mRNA expression and protein concentration that subsequently mediates enhanced glucose uptake via increased phosphorylated AKT and decreased levels of PTEN in the insulin-signaling pathway. Leptin exerts insulin sensitizing effects centrally through the SNS, as well as directly through STAT-3 and PI3K/AKT [201]. Overall, these studies suggest that leptin enhances insulin sensitivity in muscle and high circulating levels of leptin may help spare muscle tissue from the negative aspects of harmful lipid deposition while protecting insulin sensitivity.

At the onset of adiposity, increases in leptin directly enhance muscle glucose regulation while decreasing food intake and body weight through CNS alterations; however, this signaling cascade can become dysregulated if obesity is not alleviated. As a lipostatic indicator, leptin serves as an afferent signal in a negative feedback loop that functions to maintain suitable fat storage. However, obesity is associated with exceedingly high leptin levels without counteractive feedback; hence, resistance to adipose reducing effects of leptin develops [202]. Knight *et al.* [203] demonstrated that leptin resistance only developed when preceded by hyperleptinemia. This was accomplished by clamping leptin levels in *ob/ob* mice to that of normal weight mice. In comparison to obese wild-type controls the clamped *ob/ob* mice remained sensitive to exogenous leptin even after extended exposure to a HFD and obesity [203]. Hence, in chronic obesity, the ability of leptin to exert insulin-sensitizing effects may be decreased due to the development of leptin resistance.

Contradictory to the positive insulin sensitizing effects in muscle, leptin is involved in inflammatory processes that are associated with obesity and the development of type 2 diabetes. Leptin has been linked to inflammation but the mechanisms are not completely clear. This is of

interest as inflammation is associated with and demonstrated to induce insulin resistance [204-206]. Leptin is involved in cell-mediated immunity. First indications of immune regulation by leptin was observed in *ob/ob* mice which had decreased levels of immune cell types needed for pathogen clearance, hence leptin deficiency impaired immune response [207]. Indeed, leptin not only regulates the proliferation of naïve T-cells but also influences cell polarization towards Th1 (pro-inflammatory) while suppressing Th2 (anti-inflammatory) cytokine response [67]. Leptin also stimulates pro-inflammatory cytokine release from B cells. Specifically, in this cell group leptin activates janus activator kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), p38 mitogen activated protein kinase (p38MAPK), and extracellular signal-regulated kinase (ERK1/2), resulting in release of pro-inflammatory cytokines IL-6 and TNF-alpha. This was confirmed by showing that blocking any one of the previously mentioned pathways inhibited the release of IL-6 and TNFα [208]. Together, these studies demonstrate functional interactions between leptin, immunity and pro-inflammation, and thus provide mechanisms by which high leptin levels could exacerbate chronic inflammation and disturb insulin regulation.

Leptin is best characterized as being pro-inflammatory in nature, but emerging studies suggest anti-inflammatory roles as well. Leptin treatment in *ob/ob* mice increases production of cAMP in macrophages located within the adipose depot, this subsequently inhibits NF-κβ mediated expression of pro-inflammatory cytokines by activation of class IIa histone deacetlyases (HDAC) while increasing expression and production of IL-10, an anti-inflammatory cytokine [209]. The previous changes, however, are inversely associated with leptin resistance. This pathway also interacts with insulin signaling. Specifically, diet-induced obese mice with macrophage specific HDAC4 knock-out have higher plasma glucose, free fatty acids, insulin and macrophage infiltration in white adipose tissue compared with wild-type obese [209]. Consistent

with this, administration of rolipram, an insulin sensitizing drug, improved glucose tolerance and insulin sensitivity in wild type but not HDAC4 macrophage knockout mice on HFD [209]. In total, these experiments demonstrated that induction of the cAMP-HDAC4 pathway in macrophages might promote insulin sensitivity in obesity by inhibiting the translocation of NF-κβ and subsequent production of pro-inflammatory cytokines. In humans, single nucleotide polymorphisms (SNPs) in the HDAC4 gene are positively associated with high BMI and/or waist circumference, thus the previous discussed pathway may be conversed across species [209].

Overall, leptin is a hormone that modulates glucose homeostasis through both central and peripheral mechanisms. Centrally leptin plays a role in integrating metabolic signals that balance energy intake and expenditure. Peripherally this adipokine regulates lipid and glucose homeostasis in numerous tissues. Although leptin is demonstrated to have a positive effect on the previous factors, deleteriously high levels cause desensitization and resistance which leads to overall dysregulation of the leptin axis and worsening of obesity-related co-morbidities including diabetes.

Adiponectin

Adiponectin, encoded by the gene *AdipoQ,* is the highest circulating adipokine with plasma concentrations ranging from 3-10 μg/ml [210, 211]; this is ~40-fold higher than levels of circulating leptin [212]. Blood levels are commonly higher in women than in men and have an inverse relationship with weight status for both genders, hence unlike most adipokines, higher BMI is associated with lower adiponectin concentration [210]. Adiponectin is associated with many metabolic processes, including lipid trafficking and glucose homeostasis [213-215], as such it is proposed to play a role in the pathogenesis of insulin resistance and diabetes.

Adiponectin exerts its effects through two receptors, AdipoR1 and AdipoR2, which have similar form and function but differential expression among various tissues. Both receptors consist of 7 transmembrane domains similar to that of G-protein coupled receptors with the exception of orientation within the membrane which is opposite in terminus direction. Both adiponectin receptors associate only with isoforms of the adiponectin protein [216]. AdipoR1 is found primarily on the outer membrane of skeletal muscle myocytes whereas AdipoR2 resides mostly on the outer membrane of hepatocytes within the liver [217]. Intracellular signaling cascades that occur after ligand binding involve increased AMPK and peroxisome proliferator-activated receptor α (PPARα) activity [217], with subsequent increases in mitochondrial biogenesis and fatty acid oxidation. Adiponectin receptor stimulation also increases the activity of the enzyme ceramidase that cleaves and lowers cellular ceramide molecules and simultaneously increases the concentration of sphingosine 1-phosphate [218]. High ceramide levels are detrimental to the cell and have been implicated in apoptosis and cell growth arrest [219].

The hormone adiponectin is unique in that it is positively correlates with lean body types and is linked to insulin sensitivity and high density lipoprotein (HDL-C) levels [213, 215]. Accordingly, adiponectin and its receptors are decreased in individuals who are either obese or non-obese but pre-diabetic [220]. Through its AMPK stimulatory activities, adiponectin has similar effects of exercise with respect to increased glucose uptake and suppression of hepatic glucose output. While adiponectin has a variable effect on glucose uptake, it down-regulates endogenous glucose production through reduced expression of key gluconeogenic enzymes [221]. Overall, this hormone is considered an insulin-sensitizing agent by means of increasing phosphorylation events in the insulin-signaling cascade and inhibition of muscle and liver

triglyceride deposition by enhancement of beta-oxidation and fatty acid combustion pathways [222, 223].

Studies demonstrate an inverse relation between circulating adiponectin concentration and fasting glucose concentrations. Insulin-sensitizing effects of adiponectin are predominantly a product of altered liver metabolism. In wild-type, *ob/ob*, and non-obese diabetic (NOD) mice, a single physiologic injection of purified adiponectin decreases circulating fasting glucose levels by approximately 30% and is associated with decreases in hepatic glucose output [224]. The greatest suppression of circulating glucose was four hours post-injection and was associated with enhanced insulin sensitivity [224]. Another study demonstrates a 65% decrease in hepatic glucose production following adiponectin infusion and suggests it is due to a reduction in glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression, key enzymes in the gluconeogenic pathway [221]. Furthermore, adiponectin-induced glucose alterations are primarily mediated in the liver because alterations to peripheral glucose uptake are minimal, hence endogenous glucose production is reduced without affecting glucose utilization in peripheral tissues. Consistent with rodent studies, there is a negative correlation between plasma adiponectin levels and endogenous glucose production in humans [225].

Adiponectin is also proposed to alter glucose metabolism and insulin sensitivity via phosphorylation and activation of AMPK in both liver and skeletal muscle. Overall, this pathway begins with adiponectin receptor binding and initiation of a series of phosphorylation steps that result in increased glucose uptake by myocytes or decreased glucose production by hepatocytes [84]. Furthermore, this pathway increases phosphorylation of ACC, which subsequently increases fatty acid oxidation [84]. In support of this, the lack of adiponectin in HFD fed mice causes rapid development of glucose intolerance and inhibits response to PPARγ agonist, a

robust stimulator of AMPK activity [226]. This suggests that adiponectin is required for improving insulin sensitivity through the AMPK pathway.

Others demonstrate via adiponectin knockout models that this hormone is associated with the insulin-signaling pathway, with its removal impairing phosphorylation of insulin receptor substrate 1 (IRS-1) and AKT [227]. These impairments decreased insulin response resulting in diminished glucose uptake and hepatic insulin resistance [227]. In humans, fasting plasma adiponectin is positively correlated with insulin receptor tyrosine phosphorylation, a key step in the insulin-signaling cascade and vital for maintaining insulin sensitivity [228]. These data indicate a separate but related connection of adiponectin to phosphorylation events in the insulin-signaling cascade.

Visceral adiposity, a highly associated risk factor for metabolic disease, is linked with low adiponectin levels [229]. Chronic low-grade inflammation, driven by enhanced visceral adiposity, is exacerbated by decreasing adiponectin. It is unclear, however, whether it is casual or consequence, hence it is yet to be determined if pro-inflammatory factors suppress adiponectin release or if decreased adiponectin permits increased release of inflammatory cytokines. Regardless, low adiponectin concentration is associated with increased circulation levels of inflammatory markers such as TNFα, C-reactive protein, IL-6, and reactive oxygen species [230].

Overall, adiponectin is an adipocyte secretory molecule that defends against obesity-related diseases such as type 2 diabetes. Its inverse relationship with insulin resistance, visceral adiposity, and inflammatory markers support its role as a protective adipokine. Adiponectin concentrations remain a significant indicator of glucose tolerance and metabolic homeostasis.

Resistin

Named for resistance to insulin, resistin is an adipocyte produced and released hormone that was discovered in a mouse model [231]. Resistin was originally discovered as a gene that is upregulated during adipocyte differentiation, but later was identified to be secreted [74]. Circulating resistin increases with increasing adiposity in both genetic and diet-induced obese mouse models [74, 75], though some suggest *resistin* mRNA is reduced in obesity [232, 233]. Differences among studies were later confirmed to be assay methods [75], hence it is now well established that resistin alters insulin sensitivity. Mouse models demonstrate resistin is associated with insulin resistance. First, exogenous injections of resistin in lean mice reduce insulin sensitivity and induce glucose intolerance [74]. Second, *ob/ob* obese mice that are resistin deficient have improved fasting glucose levels compared with *ob/ob* mice with resistin [234]. Humans also produce and secrete resistin from adipose tissue depots, but production is not from adipocytes. Adipocyte *resistin* mRNA expression has no correlation with obesity in humans for it is undetectable in adipocytes in obese individuals [77]. In humans, *RETN* is responsible for the production of resistin [74] which is produced and secreted from monocytes and macrophages [76] rather than adipocytes. This implies that resistin in humans plays a role in immune/inflammatory responses that is reminiscent of those prompted by TNFα [77]. Despite the species differences, humanized-resistin mice are more susceptible to insulin resistance [235]. Overall, elevated resistin is associated with glucose intolerance, reduced insulin sensitivity, hyperglycemia, and increased levels of circulating free fatty acids in rodents [74, 75, 234, 235]; whereas in humans it is associated with atherosclerosis and cardiometabolic disease [236, 237].

Receptors with the affinity for resistin have been identified in mice. These thus far include an isoform of decorin on adipose progenitor cells [238] and tyrosine kinase-like orphan

receptor (ROR) 1 on adipocytes [239]. Resistin through these receptors is proposed to regulate adipogenesis, white adipose tissue expansion, and glucose homeostasis. Though resistin-specific receptors have not been confirmed in humans, it is suggested that resistin may bind to toll-like receptor 4 (TLR4) [240]. Consistent with its release from immune cells within adipose tissue in humans, resistin further plays a role in pro-inflammation through TLR4-mediated events. In support of this, recent studies demonstrate human resistin to be a cytokine that induces low-grade inflammation via monocytes [241]. In particular, adenylyl cyclase-asssociated protein 1 (CAP1) is the resistin receptor responsible for mediating the inflammatory action of monocytes [241]. CAP1 is detected on the cellular membrane of promonocytic THP-1 cells (human monocytic cell line) but primarily resides in the cytosol until induced by resistin to translocate to the membrane, the mechanisms of these events, however, are currently unknown [241]. Binding of resistin to CAP1 upregulates cAMP concentration, protein kinase activity and NF-κβ related transcription of inflammatory cytokines [241]. Specifically, in humans resistin binds directly to the proline-rich Src homology 3 (SH3) domain on CAP1. This leads to adenylyl cyclase activation, which converts ATP into cAMP, which then promotes activity of cAMP-dependent protein kinase A (PKA) and results in NF-κβ activity in monocytes. Likewise, the activation of NF-κβ upregulates mRNA expression and protein levels of pro-inflammatory cytokines such as IL-6, TNFα, and IL1β. The previous effects are amplified when CAP1 is overexpressed, but lessened when PKA is inhibited because of subsequent decreases in NF-κβ activity [241]. Knockout of CAP1 blocks resistin-induced increases in intracellular cAMP concentrations and subsequent inflammatory reactions [241]. Because human resistin is secreted from monocytes and its receptor translocates to the cellular membrane of monocytes, it is proposed to be an autocrine and paracrine signal that is enhanced during obesity [241]. Overall, in humans resistin via CAP1 induces inflammation,

which if unresolved, can result in exacerbating chronic low-grade inflammation associated with metabolic disease and type 2 diabetes.

Apelin

Apelin has two predominate isoforms and is the primary endogenous ligand of the APJ G-protein coupled receptor that is expressed in a variety of tissues, most notably the lung, heart, adipose tissue, kidney, spleen and brain [242]. Functions of apelin are dependent on the site of receptor binding, some examples include stimulation of angiogenesis and hypotensive vessel outcomes [243], cardiac contractions in the heart [244], fluid homeostasis [245], and inhibiting glucose-stimulated insulin secretion by the pancreas [246]. As a ubiquitous peptide, apelin is secreted by neurons, cardiomyocytes, and endothelial cells, however it is considered an adipokine because it is produced and secreted by adipocytes [247].

Apelin is proposed to be anti-obesigenic and anti-diabetic. It is highly expressed in the central nervous system (CNS), specifically the hypothalamus, regulating food intake and drinking behavior [248]. In support of this, icv injection of apelin-13 in rats causes a decrease in food intake in both fed and fasted conditions [249, 250]. Icv injection of apelin-13 is also demonstrated to increase water intake, hence apelin also plays a role in fluid homeostasis [251]. In addition to food and fluid balance, apelin is proposed to reverse insulin resistance and development of diabetes by diminishing insulin response to increases in blood glucose concentrations. Specifically, apelin inhibits insulin production from INS-1 cells (insulin secreting cell line) by activating a PI3K-dependent protein, which suppresses cAMP levels, an amplifier of glucose-induced insulin secretion [252]. As apelin has been found in pancreatic juices secreted from rats, it also appears to be involved in exocrine processes as well as endocrine [253]. Last,

apelin increases glucose uptake in isolated myocytes by activation of AMPK, which phosphorylates AKT and facilitates glucose transport [254].

Overall, the positive effects of apelin on food and fluid regulation, control of insulin secretion, and glucose utilization, make the peptide a viable target for obesity and pre-diabetic therapies. However, apelin concentrations are elevated in individuals who are obese, have type 2 diabetes with and without obesity, and/or hyperinsulinemic [255], whereas those who are lean, dieting or received bariatric surgery have diminished levels [255]. This indicates that high circulating levels of apelin are not protective and, at best, high levels of apelin at the onset of obesity may initially protect against the development of insulin resistance. However, after prolonged obesity the hormone signaling becomes deficient or ineffective. This may occur by multiple mechanisms such as enzymatic degradation of apelin to an inactive form Pyr1 apelin [256], which has a lower binding affinity to the respective receptor [257] or apelin resistance via decreased AJP receptors [258]. Despite these two circumstances, exogenous apelin in obesity is still demonstrated to be an effective treatment [254]. Mechanisms that regulate endogenous dysregulation of the apelin-signaling pathway remain to be elucidated.

Visfatin

Previously known as both nicotinamide phosphoribosyltransferase (Nampt) [259] and Pre-B-cell colony-enhancing factor (*PBEF*) [260], visfatin is an adipokine with several physiological roles involved in the regulation of immune cells, insulin mimicking regulation and cellular energetics via NAD-dependent enzymes and nicotinamide adenine dinucleotide (NAD) biosynthesis [261-263]. First recognized in large amounts mainly in bone marrow, liver, muscle and brown adipose tissue [260], visfatin was further described to be released from white adipose

tissue [264]. Although originally characterized to be produced and released from adipose tissue depots, visfatin is produced and secreted by immune cells (leukocytes, in particular macrophages, monocytes and neutrophils) located among the adipocytes [265]. Visfatin is further characterized to be more abundant in the visceral adipose depot than subcutaneous depot [264], likely because of the higher prevalence of macrophages within the visceral depot [265].

Visfatin is involved in immune cell signaling. In vitro, visfatin promotes expansion of B-cell colonies in bone marrow when in the presence of both IL-7 and stem cell factor (SCF) [260]. Visfatin actions are also cytokine-like with promotion of pro-inflammatory molecule transcription and translation from monocytes, which include TNFα, interleukin-1β (IL-1β), and IL-6 [263, 266]. Via a MAPK inhibitor method, visfatin is determined to promote innate immune response through the MEK1 pathway, further activating NF-κβ [267].

In adipocytes, visfatin treatment upregulates expression of *PPAR-γ, CCAAT-enhancer binding protein-α (C/EBP-α), fatty acid synthase (FAS), DGAT-1, adipose P2 (aP2)* and *adiponectin*, hence increases adipocyte differentiation and maturation markers [266]. Therefore, a paracrine function of visfatin is adipocyte growth and expansion, but this adipokine also plays an endocrine role. Visfatin is proposed to activate the insulin receptor inducing insulin-like effects. Indeed, compared with wild type, transgenic mice with lower circulating visfatin have increases in fasting and postprandial glucose concentrations without altered insulin sensitivity [266]. Insulin-like functions of visfatin include increases in preadipocyte triglyceride accumulation and adipocyte glucose uptake, suppression of hepatocyte glucose uptake, increases in triglyceride accumulation in preadipocytes and triglyceride synthesis from glucose [266].

Despite these effects on insulin action, Jacques *et al.* determined in chondrocytes that visfatin does not directly interact with either the insulin receptor or insulin growth-like factor 1

receptor (IGF-1R) [268]. The authors speculate that visfatin may interact with the growth hormone receptor and/or induce elevated insulin growth-like factor 1 (IGF-1) [268]. Subsequently it is proposed that activation of IGF-1R induces the AKT or Erk1/2 pathway via MAPK signaling [268]. Another study, however, determined that IGF-1 signaling in chondroytes was inhibited by administration of visfatin [269]. The authors here conclude that visfatin signals the activation of ERK pathway via an unknown receptor which then inhibits the activation of AKT by IGF-1 [269]. These specific mechanisms of visfatin are yet to be determined on other insulin sensitive tissues that greatly contribute to homeostasis of glucose concentration.

Two meta analyses support an association between visfatin and metabolic diseases specifically demonstrating increased visfatin concentration is positively related to obesity, adiposity, cardiovascular disease, insulin resistance and type 2 diabetes [270, 271]. In clinical studies, some demonstrate visfatin concentration increases are positively associated with obesity [272] and diabetes [273], whereas others demonstrate only a positive correlation with circulating inflammatory markers such as C reactive protein [274]. Therefore, general opinion indicates that visfatin increases with increasing adiposity and markers of inflammation. Overall, despite the insulin mimicking effect of visfatin, it is postulated that during obesity the functions on immunity as an inflammatory cytokine dominates and subsequently exacerbates inflammation.

Omentin

Omentin is an adipocyte hormone primarily released from visceral adipose tissue; hence, it is nearly undetectable in subcutaneous adipose tissue. The amino acid sequence was originally identified as a protein called intelectin, which plays a protective role against bacterial translocation in the gut and proposed to be a defense mechanism for intestinal inflammation

[275]. Within the abdomen, omentin is released from stromal vascular cells of the omental adipose depot and not adipocytes, stimulates glucose uptake via enhancement of insulin signaling [276].

Omentin plays a protective role in glucose homeostasis and insulin signaling by modulating systemic metabolism in an autocrine and paracrine fashion. In human embryonic kidney (HEK-293T) cells, omentin treatment does not affect basal glucose uptake, but does increase insulin-stimulated glucose uptake by 50% [276]. Similar to adiponectin and apelin, omentin is proposed to enhance glucose utilization via phosphorylation of AMPK and AKT proteins [277]. Nonetheless, plasma omentin levels and mRNA are significantly higher in lean, but not overweight or obese subjects and plasma levels are negatively correlated with BMI [91]. Omentin concentrations are increased following decreases in BMI and associated with improved insulin sensitivity [278]. Omentin levels are higher in women than in men and low in morbid obesity, hence low circulating omentin is strongly associated with metabolic syndrome [90, 91].

Omentin is inversely correlated with inflammatory factors like IL-6 and TNFα, and has been shown to suppress the inflammatory response in cultured endothelial cells [279]. Inflammatory suppression is accomplished through the AMPK/eNOS signaling pathway by blocking Jun amino-terminal kinase (JNK) activation [280]. These favorable inflammatory factors may play a role in hampering systemic insulin resistance.

Omentin is yet another beneficial adipokine identified to enhance the effects of insulin on glucose metabolism in the body. As such, the release of omentin from visceral adipose depot, but not subcutaneous, is advantageous because of the depots proximal location with direct effluent to the insulin-sensitive liver. Omentin is predictive of metabolic consequences and co-morbidities

of obesity because lower circulating amounts are predictive of insulin resistance and decreased glucose utilization.

Vaspin

Visceral adipose tissue-derived serpin, shortened to vaspin, is an adipokine characterized to play a role in insulin regulation and glucose homeostasis [281]. First recognized as a member of the serpin protease inhibitor family, vapsin is a gene associated with visceral adiposity and type 2 diabetes as determined by representational difference analysis between Otsuka Long-Evans Tokushima fatty (OLETF) type-2 diabetes rat and Long-Evans Tokushima Otsuka (LETO), the diabetes-resistant counterpart [282]. Transcribed by the gene *Serpina12,* vaspin in humans is not detectable in lean subjects (BMI<25), but is detected in obese individuals as well as those with type 2 diabetes [281]. This adipokine in humans is regulated in a depot specific manner with higher detection in the visceral depot compared with the subcutaneous [281]. Vaspin mRNA expression and serum concentrations are increased in both human and rodent obesity [88, 281, 283, 284], although in humans it tends to be higher in women [285]. It is proposed that vaspin may be a beneficial adipokine that protects or attenuates co-morbidities associated with metabolic disease as a compensatory factor against insulin-resistance.

The role of vaspin as an insulin-sensitizing adipokine was demonstrated in two rodent models; a genetic obesity model (OLETF rats) and a high-fat and high-sucrose (HFHS) obese model (CRL:CD-1 mice) (ICR) [286]. In the OLETF rats, treatment with insulin or pioglitazone, an insulin-like molecule, upregulates adipocyte vaspin mRNA and circulating protein concentrations, suggesting vaspin increases may be a feedback response to alleviate insulin resistance [286]. In support of this, vaspin treatment reversed HFHS-induced hyperglycemia,

while also decreasing circulating leptin, resistin and TNFα concentration, but did not change hyperinsulinemia [286].

Vaspin is also demonstrated to regulate insulin sensitivity through adipose depot growth. Specifically, vaspin in 3T3-L1 cultured cells is proposed to promote the differentiation of preadipocytes [287]. This occurs by an increase in differentiation factors including PPARY, C/EBP, and free fatty acid-binding protein 4 (FABP4) [287]. The downstream effects are smaller differentiated adipocytes expressing decreased IL-6 mRNA and increased glucoe transporter type 4 (GLUT4) mRNA, contributing to reduced inflammation and increased insulin sensitivity, respectively. From these results, the authors suggest that vaspin induces phosphorylation of AKT and AMPK, improving glucose regulation while reducing inflammation. Overall, vaspin is a beneficial adipokine that currently is characterized to ameliorate metabolic dysregulation in type 2 diabetes and metabolic disease via enhancement of insulin sensitivity.

Retinol Binding Protein

A family of proteins, known as retinol binding proteins (RBP), function to bind and carry retinol (vitamin A) through cells, plasma, or interstitial fluid to its target, where it acts to modulate gene expression. The gene *RBP4* encodes for a specific isoform secreted by adipocytes that functions as a plasma indicator for decreases in blood glucose levels [288]. A study performed in children show a relation between RBP4, obesity, and insulin resistance, suggesting that RBP4 is related to adiposity and weight status [289]. Furthermore, high plasma RBP4 is associated with impaired glucose tolerance and type 2 diabetes in adults and in general is higher in women and seniors [290]. Protein levels of this adipokine are elevated in abdominal adipose tissue, the omentum, of obese subjects with and without type 2 diabetes [291]. Thus, visceral

adiposity is a better predictor of both RBP4 levels and insulin resistance than general measures of obesity like BMI [292, 293].

GLUT4, the primary transporter for glucose uptake in myocytes and adipocytes, is decreased in insulin resistant states and is inversely correlated with RBP4 levels [294]. Serum RBP4 levels are 2.5-fold higher in mice with lower adipose-GLUT4 compared with control mice, suggesting a possible contributing role of RBP4 in systemic insulin resistance [294]. Consistent with this, RBP4 interferes with insulin signaling in human adipocytes by enabling serine phosphylation of IRS-1, which attenuates availability of the GLUT4 transporter [295]. In addition, RBP4 increases expression of the gluconeogenic enzyme PEPCK, which leads to higher levels of blood glucose [294]. In obese humans, GLUT4 is also decreased in visceral adipose tissue [291].

Although the link between RBP4 and insulin resistance has been demonstrated in vivo, it is not clear if dietary intake of vitamin A has an effect on plasma RBP4 levels in humans. Vitamin A deficiency in a rodent model increases adiposity [296] and vitamin A supplementation prompts weight loss and increases in insulin sensitivity [297]. Thus, the relation between RBP4 and vitamin A with insulin resistance are incongruent. Overall, the exact mechanism linking RBP4 with insulin resistance is still unknown.

WISP-1

Research continues to identify a number of novel adipokines that play a potential role in the pathogenesis of type 2 diabetes. Recently characterized is adipokine Wnt 1 inducible signaling pathway protein 1 (WISP-1). WISP-1 is an extracellular matrix associated protein of the CCN gene family. It is a cysteine rich protein within connective tissue and is involved in the

WNT signaling pathway [298]. The WNT signaling pathway is classically associated with mechanisms involving cell proliferation, cell fate determination and cell polarity during both embryonic development and tissue homeostasis [299].

Human adipocytes, but not monocytes or monocyte-derived macrophages, express *WISP-1,* which plays a role in adipose depot expansion via adipocyte differentiation [298]. WISP-1, dose dependently increases mRNA expression of *IL-6, TNF-α, IL-1β and IL-10* in macrophages but not in adipocytes [298]. This is paralleled by increases in the expression of M1 (pro-inflammatory) macrophage markers (CCR7 and COX2) and a reduction of M2 (anti-inflammatory) macrophage markers (CD36 and CD136). These increases in *WISP-1* mRNA in adipose tissue samples from humans are also positively associated with high fasting insulin levels and macrophage infiltration and negatively with insulin sensitivity [298]. Although much remains to be elucidated this study generally demonstrates WISP-1, released from fully differentiated adipocytes stimulates pro-inflammatory cytokines release from macrophages and plays an unidentified role in increasing insulin concentration.

Adipolin

Enomoto *et al.* [300] identified the adipokine CTRP12, C1q/TNF-related Protein-12, also known as adipolin, in a mouse model of obesity, where adipolin mRNA and circulating protein were decreased. Systemic administration of adipolin in diet-induced obese mice reduces adipose tissue macrophage (ATM) infiltration and pro-inflammatory gene expression in the adipose depot [300]. These alterations are associated with an attenuation of obesity-induced insulin resistance and glucose intolerance. In addition, macrophage pro-inflammatory cytokine production stimulated by lipopolysaccharide (LPS) or TNF-α is decreased when immune cells

are pretreated with media from kidney cells (COS-7) that are transfected with adenovirus vectors for adipolin [300]. Together these data suggest that adipolin functions as an anti-inflammatory adipokine and exerts beneficial effects on glucose tolerance and insulin sensitivity [300]. In support of this, others demonstrate that administration of recombinant protein adipolin lowers blood glucose levels in wild type lean and *ob/ob* and diet-induced obese mice [301]. In hepatocyte and adipocyte cultures adipolin treatment directly (independent of insulin) activates the PI3K/AKT signaling pathway improving insulin sensitivity by promoting glucose uptake and reducing gluconeogenesis in the liver [301].

Bell-Anderson *et al.* [302] further demonstrated adipolin as a potential target for the treatment of type 2 diabetes. The beneficial actions of adipolin are also demonstrated in Kruppel like factor 3 (KLF3) null mice. These mice are characterized as resistant to diet-induced obesity with lower adiposity in the abdominal and subcutaneous adipose depots [302]. When compared to wild type controls KLF3-null mice were more insulin sensitive. Epididymal white adipose tissue, red skeletal muscle and liver microarray analysis identified that fam132a, the gene that codes for adipolin, was significantly upregulated in KLF3-null mice [302]. This was further confirmed with RT-PCR in multiple tissues from the KLF3-null mice including heart, lung, bone marrow and epididymal white adipose tissue. Consistent with this plasma adipolin concentration is increased in the KLF3-null mice. Overall, KLF3 binds directly to the promoter for fam132a and represses promoter activity and production of adipolin. Others demonstrate that adipolin expression in adipocytes is also regulated by Kruppel-like factor 15 (KLF15). Unlike KLF3, KLF15 is positively associated with adipolin [303]. As such, diet-induced obese mice have decreased expression of KLF15 and adipolin. Targeted ablation of KLF15 reduces expression of *adipolin*, hence adipolon expression is dependent upon KLF15 [303]. In 3T3L1 adipocytes

obesity-associated inflammatory factors such as TNFα reduce mRNA expression of *KLF15* and *adipolin* [303]. This, however, does not occur if cells are KLF15 adenovirus transfected or if JNK signaling is inhibited blocking subsequent TNFα production. Taken together obesity-induced inflammation increases JNK signaling which decreases expression of KLF15 and subsequently decreases promoter activity of adipolin, in the adipocyte [303]. Combined, the two previous studies identified both the inhibitor (KLF3) and the promoter (KLF15) for the adipolin gene (fam132a).

Overall adipolin influences glucose homeostasis by two paths, as an insulin sensitizing adipokine that can act directly on insulin signaling or by inflammatory mechanisms that may enhance the insulin-signaling cascade.

Subfatin

Subfatin, also known as Metrnl, was discovered in a gene array analysis seeking identification of novel adipokines stimulated by caloric restriction in diet-induced obese rats, hence the exploration of a metabolically beneficial adipokine [304]. Subfatin mRNA and protein is highly expressed in white adipose tissue and annotated as "Meteorin-like" [304]. Subfatin was first recognized for its similar transcription to Meteorin, a glial cell differentiation regulator expressed exclusively in the brain [305], however expression of subfatin has not been detected in the brain [304]. Additionally, its expression is higher in white adipose than brown adipose tissue of both mice and humans[304], and is higher in subcutaneous adipose tissue than abdominal [306]. Subfatin expression increases during the differentiation of preadipocytes and in obese mice fed a high-fat diet [304, 307].

Another study in mice reported increases of subfatin in muscle after exercise and in adipose tissue upon cold exposure to increase whole-body energy expenditure [307]. Rao *et al.* [307] determined that the presence of subfatin stimulates the infiltration of immune cytokines, specifically IL4 and IL13, into adipose tissue where they trigger pro-thermogenic actions. These immune cytokines, following recruitment by subfatin, also shift the monocyte population towards M2 macrophages, thus inducing a repair and wound healing response [307]. Subfatin is also characterized to regulate insulin sensitivity. Specifically, diet-induced obese mice, but not chow, deficient in subfatin are insulin resistant [306]. When overexpressed, however, subfatin enhanced insulin sensitivity in diet-induced obese mice, but again not chow fed [306]. Hence, subfatin restores insulin sensitivity in obese mice, but does not enhance insulin regulation in lean mice. Overexpression of subfatin also restores insulin sensitivity in genetic models of obesity such as leptin knockouts [306].

Subfatin enhances insulin sensitivity by increases in AKT phosphorylation [306], hence this factor is enhanced in subfatin overexpressing mice. In addition, this adipokine likely regulates insulin sensitivity via regulation of inflammation. As such, subfatin deficiency is also linked to an increase in TNFα expression, hence overexpression of subfatin decreases this inflammatory factor [306]. Lastly, subfatin promotes adipocyte growth with upregulation of PPARγ and subsequent increases adipocyte lipid accumulation and maturation [306].

Cytokines

Obesity is now defined as a state of chronic low-grade inflammation. This inflammation theory is gaining support as a fundamental link between obesity and the development of type 2 diabetes. As such, type 2 diabetes in lean or obese children is positively associated with an

increase in plasma pro-inflammatory cytokines, specifically TNFα and MCP-1 [204]. This relation is consistent in adults where insulin sensitivity is inversely associated with adipose tissue TNFα protein and plasma IL-6 concentration [205]. Another study demonstrates that elevated plasma IL-6 and TNFα concentrations are indicative of the development of type 2 diabetes over a 2 year period [206]. After adjusting for confounding factors, BMI, age and sex, IL-6 remained a predictive factor [206]. Overall, this supports a relation between inflammation and the subsequent development of type 2 diabetes.

Mechanistic evidence, thus far, supports the inflammation-diabetes link. *In vitro* adipocyte studies demonstrate that TNFα greatly suppresses the transcription and mRNA stability of C/EBPα and subsequently transcribed GLUT4 [308]. As a result, cellular protein content of C/EBPα and GLUT4 are also decreased. In addition, continuous exposure of adipocytes to TNFα decreases insulin receptor mRNA by ~50% [308]. Overall loss of TNFα in a mouse model, both genetic and diet induced is associated with lower levels of fasting glucose and insulin and increased insulin sensitivity as well as lower levels of circulating free fatty acids [309]. Lack of TNFα function via targeted mutation of the gene or its receptors increases insulin mediated autophosphorylation of the insulin receptor [310]. In total, these data in mice demonstrate that lack of TNFα in an obese state serves to protect from insulin resistance [309, 310]. Immune cells that infiltrate adipose tissue also play a role in glucose homeostasis. Adipose tissue from obese humans contains CD4 T cells that produce 3 to 7.5 times more IL-17 and IL-22 than non-obese subjects [311]. These cytokines, IL-17 and IL-22, inhibit glucose uptake in skeletal muscle isolated from rats, as measured by 2-deoxyglucose uptake, and induced insulin resistance in cultured human hepatocytes as measured by total phosphorylated AKT protein [311].

Clinical investigations demonstrate that calorie restriction, increased physical activity and subsequent weight loss are effective at reducing inflammation that likely contributes to insulin resistance and type 2 diabetes. In particular, weight loss reduces expression of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (*NLRP3)* inflammasome in adipose tissue, which reduces caspase-1 activation and subsequent secretion of the pro-inflammatory factors IL-1β and of IL-18 [312]. The relation between this inflammasome and insulin resistance was further explored in the mouse. Specifically, ablation of NLRP3 in mice increases insulin sensitivity in liver and adipose tissue and is associated with decreases in IL-18 and interferon gamma (INFγ) expression [312]. In addition, naïve CD4 T-cells are increased while CD4-effector T cells were decreased, suggesting a decrease in pro-inflammatory signaling. In NLRP3 knock out mice there is an increase in PI3K/AKT activity, hence improved insulin signaling, and a reduction of serine phosphorylation of the IRS-1 substrate that is commonly associated with insulin resistance [312]. Excess lipids in obesity are proposed to activate this NLRP3 inflammasome in macrophages via the production and build-up of harmful lipid intermediates, such as ceramides, leading to a proinflammatory cell that releases IL-1β [312]. This progression potentiates insulin resistance, however is inhibited in NLRP3 knock out mice with attenuation of macrophage IL-1β activation and secretion.

Overall, these studies suggest that adipose tissue is the nexus of immunity and metabolism. Adipose tissue disorders encountered in obesity cause alterations in cytokine release and composition of adipose-resident immune cell populations. The resulting changes appear to induce profound consequences for basal systemic inflammation. As such insulin resistance and type 2 diabetes are regulated by inflammation, hence are driven by the metabolic consequences

of excess adiposity. Figure 2.2 illustrates associations between inflammation and insulin regulation with the contributions of adipokines known to play a role in immunity.

Concluding Remarks

The understanding of the pathogenesis of obesity and associated co-morbidities has advanced rapidly over the past two decades. New adipokines are continuously being identified and categorized relative to their ability to promote or reduce obesity associated disease risk. Adipokines discussed in this review are thought to change glucose regulation by way of directly altering the insulin-signaling pathway (Figure 2.1) or indirectly by altering immune response and subsequent inflammation, which then alters components of the insulin-signaling pathway (Figure 2.2). Overall, most adipokines are demonstrated to play a beneficial role towards the enhancement of insulin sensitivity and glucose uptake and utilization. Others, however, are primarily detrimental and drive inflammation and insulin resistance. Leptin is extensively characterized and demonstrated to have multiple functions including the enhancement of insulin sensitivity, and exacerbation of inflammation and insulin resistance (Figure 2.3). These functions however are specific to cell types involved and environmental cues.

Glucose homeostasis is dependent upon the appropriate balance of circulating adipokines. One can postulate these differential adipokines create a coordinated response to fluctuating energy storage. As such, decreases in adiposity would augment this response to increase food intake and energy storage, whereas increases in adiposity would decrease food intake while increasing oxidation of lipid stores. Leptin, adiponectin, apelin, omentin, vaspin, adipolin subfatin and visfatin are all characterized to improve insulin sensitivity in rodent models, however epidemiological studies suggests obesity is associated with insulin resistance. Perhaps

these insulin-sensitizing factors are only effective during acute periods of rapid adipose tissue growth in an effort to maintain glucose homeostasis despite the intake of excessive calories. The beneficial effects, however, are lost with chronic obesity because helpful adipokines either decrease, *e.g*. adiponectin and omentin, or increase, *e.g*. leptin and apelin, but become less efficient via resistance, lower binding affinity or increased degradation.

Figures

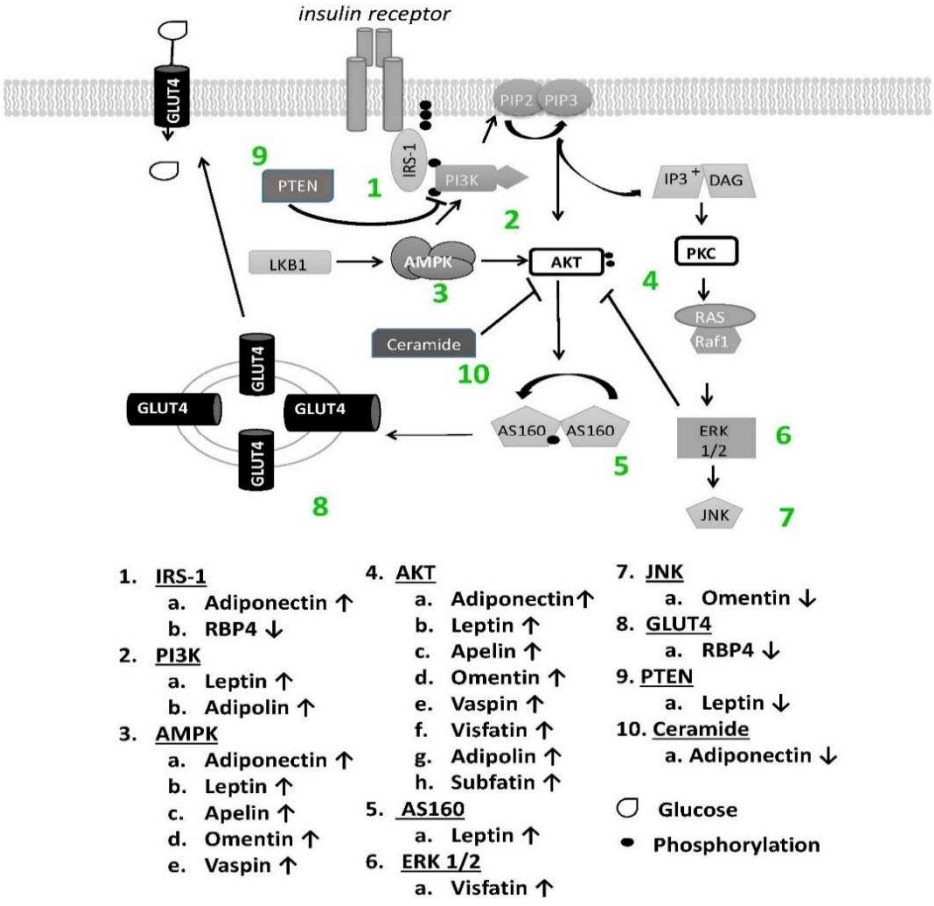


Figure 2.1: Adipokines and the Insulin-Signaling Pathway. Adipokines that are demonstrated to directly alter the insulin-signaling pathway. This is a simplified depiction of the insulin-signaling pathway that includes specific steps modulated by adipokines. The depiction is limited to what occurs under standard conditions. Certain steps, such as increases in AMPK or enhanced AKT phosphorylation, are increased by numerous adipokines. Arrows next to adipokine name indicates if process is increased or decreased by that particular factor. During obesity insulin resistance occurs because beneficial adipokines are either decreased or become inefficient by way of resistance, degradation or conversion to an inactive form.

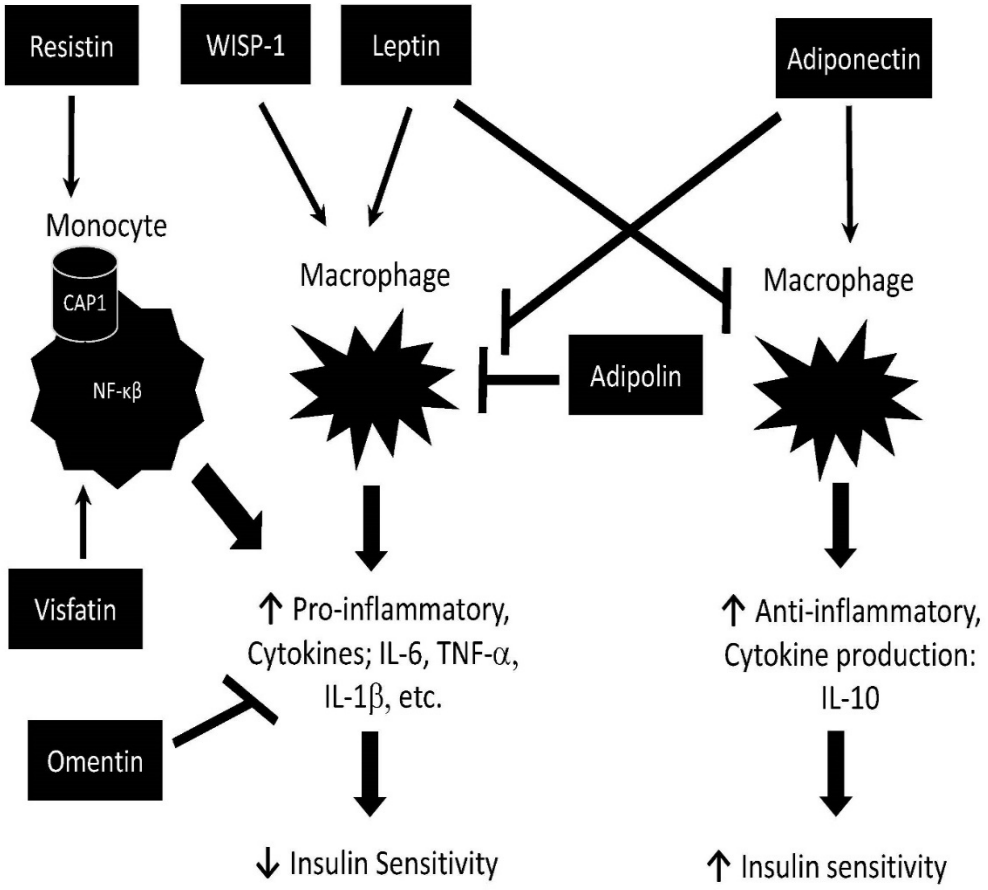


Figure 2.2: Adipokines, Inflammation and Insulin Sensitivity. Inflammation is described to be the fundamental link between obesity and the development of type 2 diabetes. Insulin sensitivity is influenced by the balance between pro- and anti-inflammatory cytokines. Certain adipokines alter insulin sensitivity indirectly by interactions with adipose depot immune cells such as monocytes and macrophages. Leptin, resistin, WISP-1 and visfatin induce pro-inflammatory immune cell types, whereas adiponectin prompts immune cells to secrete anti-inflammatory cytokines. Omentin and adipolin are demonstrated to inhibit production of harmful cytokines from pro-inflammatory cells.

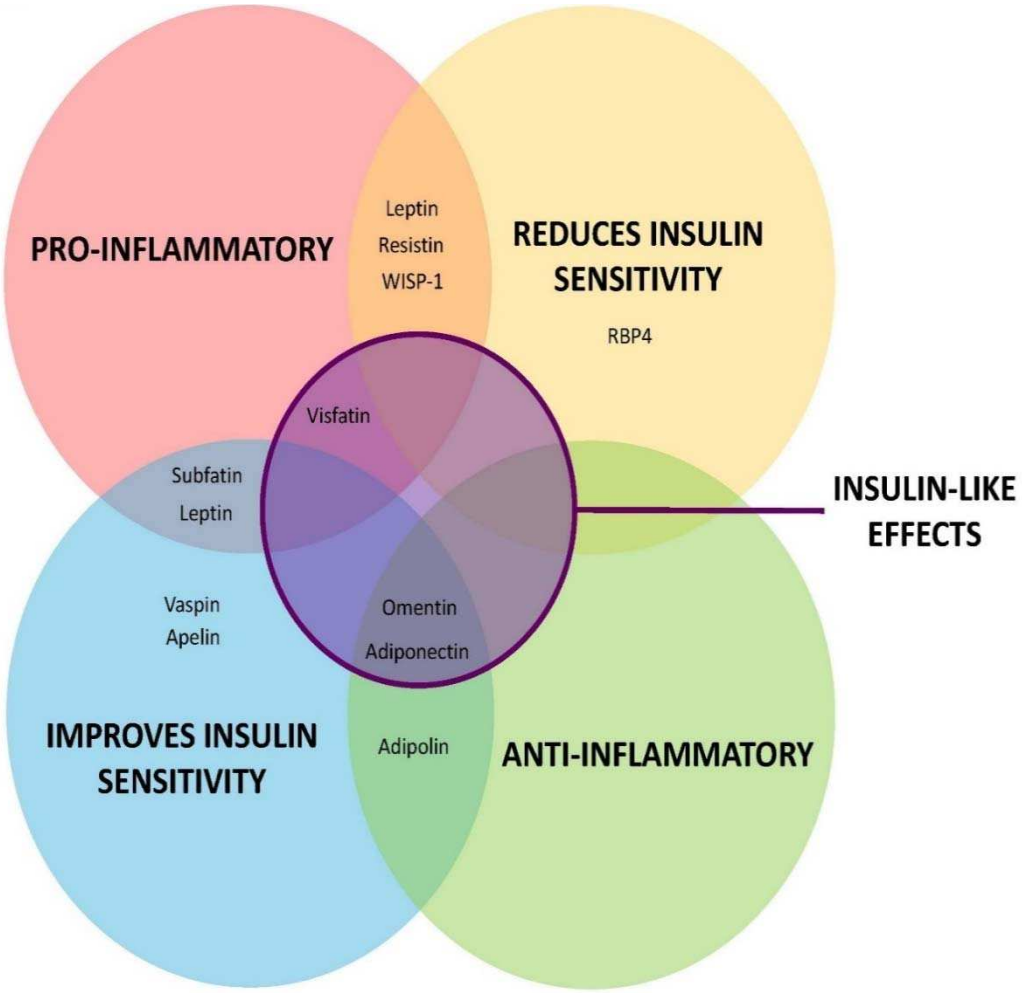


Figure 2.3: Adipokine Classification and Relation to Insulin Regulation. General postulated role of adipokines concerning insulin regulation as traditionally classified by inflammation, insulin regulation and insulin mimicking effects.

CHAPTER 3: INHIBITION OF ADIPOSE TISSUE PPARΓ PREVENTS INCREASED ADIPOCYTE EXPANSION AFTER LIPECTOMY AND EXACERBATES A GLUCOSE-INTOLERANT PHENOTYPE[5,6,7]

Summary

Adipose tissue plays a fundamental role in glucose homeostasis. For example, fat removal (lipectomy, LipX) in lean mice, which resulted in a compensatory 50% increase in total fat mass, is associated with a significant improvement of glucose tolerance. This study was designed to examine further the link between fat removal, adipose tissue compensation and glucose homeostasis using a peroxisome proliferator-activated receptor γ (PPAR γ; activator of adipogenesis) knockout mouse. The study involved PPARγ knockout mice (FKOγ) or control mice (CON) subdivided into groups that received LipX or Sham surgery. We reasoned that since the ability of adipose tissue to expand in response to LipX would be compromised in FKOγ

[5] A modified version of this chapter is published as Booth AD, Magnuson AM, Cox-York KA, Wei Y, Wang D, Pagliassotti MJ, Foster MT. Inhibition of adipose tissue PPARγ prevents increased adipocyte expansion after lipectomy and exacerbates a glucose-intolerant phenotype. Cell Prolif. 2017 Apr;50(2).

[6] The aim of this work was to conduct an experimental study on inhibition of adipose tissue proliferation via deletion of PPARγ following intra-abdominal lipectomy. Our lab carried out a controlled experiment using cre-lox technology to examine variances in glucose tolerance using a PPARγ knockout mouse model. We investigated the rate of peripheral adipose tissue hyperplasia with and without the presence of PPARγ, a master regulator of adipogenesis. This article outlines the importance of adipose tissue proliferation in the maintenance of glucose tolerance.

This chapter includes the complete published manuscript for this original research titled *Inhibition of adipose tissue PPARγ prevents increased adipocyte expansion after lipectomy and exacerbates a glucose-intolerant phenotype* (Andrea D. Booth, Aaron M. Magnuson, Kim A. Cox-York, Y. Wei, D. Wang, Michael J. Pagliassotti, and Michelle T. Foster, Cell Prolif., 2017). My contributions to this experiment included breading, housing, food and weight measurements, genotyping, GTT, lab assays, tissue collection, and general care of experimental mice. My contribution to this manuscript included data calculations, table and figure preparation, statistics, as well as input and review of content for accuracy and cohesiveness, including editing and corrections prior to submission.

Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 3.1. This article is reproduced with permission, and only minimal modifications were made to meet formatting requirements. No other modifications were made, as per the licensing agreement.

[7] Thanks to Hamlin Barnes who completed RNA extraction for qPCR procedure. Funding, NIH grant K01DK087816.

mice, so would improvements in glucose homeostasis. In CON mice LipX increased total adipose depot mass (~60%), adipocyte number (~45%), and shifted adipocyte distribution to smaller cells. Glucose tolerance was improved (~30%) in LipX CON mice compared to Sham. In FKOγ mice, LipX did not result in any significant changes in adipose depot mass, adipocyte number or distribution. LipX FKOγ mice were also characterized by a reduction of glucose tolerance (~30%) compared with sham. Inhibition of adipose tissue PPARγ prevented LipX-induced increases in adipocyte expansion and produced a glucose intolerant phenotype. These data support the notion that adipose tissue expansion is critical to maintenance and/or improvements in glucose homeostasis.

Introduction

The metabolic consequences of obesity are closely aligned with how lipids are distributed among adipose tissue depots. Dyslipidemia, type 2 diabetes and insulin resistance are more likely to occur in obese individuals characterized by central/abdominal and upper body lipid storage [1, 2, 45, 313, 314]. In contrast, these comorbidities are less likely in individuals with [26, 315, 316] lower body subcutaneous (SAT) adipose tissue expansion [31]. Indeed, lower body SAT expansion is a primary characteristic of the metabolically healthy obese individual [317]. Therefore, accumulation of lipids in this adipose tissue region has been suggested to be protective from typical obesity-mediated metabolic impairments.

Adipose tissue depots appear to have distinct, inherent characteristics that may explain different metabolic outcomes. For example, whereas upper body SAT expansion appears to involve hypertrophy, which results in large insulin resistance adipocytes, lower body SAT expansion involves increases in smaller adipocytes that retain insulin sensitivity [318-328].

Hence, some studies suggests that depots characterized by smaller adipocytes impart protection from obesity-related metabolic impairments [3, 53]. However, not all studies support this view [329, 330].

One common element that has been linked to metabolic dysregulation associated with obesity involves the recruitment of new fats cells and regulation of differentiation. In this context, impairments in the differentiation and proliferation of new fat cells can lead to spill over and accumulation of lipids and lipid intermediates in non-adipose tissue cells [331]. Non-adipose tissue or ectopic lipid accumulation has been associated with insulin resistance and increased risk for a number of obesity-related comorbidities. Adipogenesis and maintenance of mature adipocytes is driven by the activation of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor [332, 333]. PPARγ activation in adipocytes lowers circulating insulin levels, improves whole-body insulin sensitivity and adipokine profiles, and reduces serum lipids [334]. Adipose PPARγ activation also suppresses inflammation induced by high fat diet [334]. It is proposed that these improvements occur, in part, through sequestration of excess fatty acids and triglycerides by adipocyte hyperplasia [335]. We have therefore elected to manipulate PPARγ in the context of lipectomy (LipX) or sham surgery to investigate further the relationship between adipose tissue expansion and glucose homeostasis.

PPARγ knockout mice are characterized by reduced adipocyte maturation after initial adipogenic events, reduced hyperplasia leading to hypertrophic mature adipocytes and elevated plasma fatty acids [336]. We have previously demonstrated that removal of discrete adipose tissue depots (LipX) improves glucose tolerance and increases sequestration of fatty acids in non-excised adipose tissue of lean young and old rodent models [337, 338]. We and others have also demonstrated that compensation in non-excised depots was characterized by increased

adipocyte number and adipose tissue mass [339-341]. In the current study, we have employed LipX to induce adipose tissue depot growth in CON and FKOγ mice. We hypothesized that LipX-induced increases in adipose tissue growth would be impaired in PPARγ knockout mice and this impairment would lead to unfavorable outcomes in glucose homeostasis.

Materials and Methods

Mice and Housing

Breeding Colony: Twelve transgenic adult mice were purchased from The Jackson Laboratory (Bar Harbor, Maine), 6 males and 6 females (~24 g), for tissue specific Cre/Lox inducible knockouts. Half the mice from each sex were homozygous for PPARγ flanked with LoxP sites, targeted for its deletion. The other half were hemizygous for the expression of Cre recombinase enzyme under control of FABP4, fatty acid binding protein-4, an adipocyte lipid transport protein. A 2-generation breeding scheme was used to obtain knockouts (KO) and control littermates used in this study. First, homozygous PPARγ loxP-flanked mice were mated with Cre recombinase FABP4 mice. The first generation of offspring heterozygous for the loxP allele and hemizygous/heterozygous for the cre transgene were then mated with the original homozygous loxP-flanked mice. Second generation offspring, homozygous for the loxP-flanked allele and hemizygous/heterozygous for the cre transgene were designated FKOγ KO mice (n=13). Homozygous LoxP offspring were designated as Control C (CON) mice (n=15).

Experimental Mice: A total of 13 male adipose tissue-specific PPARγ knockout transgenic mice (FKOγ) and 15 male homozygous LoxP CON mice were produced from a colony of ~175 mice. An a priori power analysis using previous data from other breeding colonies to determine the expected variance was used to estimate the number of animals

necessary for this experiment. Sample sizes were determined by providing a 90% chance of finding a significant effect of 25% or greater for the between and within-subject variables. It was predicted 8 to 10 mice were needed to provide adequate statistical power. Due to the limitation of breeding numbers, we did not reach the predicted number of mice; however, the animal numbers acquired were adequate to observe a number of statistically significant differences.

Offspring of interest were weaned 21 days post-birth and transferred to individual housing under controlled conditions (12:12 light-dark cycle, 50–60% humidity, and 25° C). Experiments (surgery) started at 3 months of age before metabolic dysregulation was exacerbated in FKOγ mice. For experiment duration, mice were given ad libitum access to a standard chow diet (Harlan Teklad 7002, Madison, WI) and unlimited water. Weekly body mass and food intake were monitored and recorded. Procedures were reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Surgical Procedures

Surgeries were performed while mice were anesthetized with isoflurane. Half of the experimental mice received sham surgery (mid-ventral abdominal incision through skin and muscle without adipose tissue removed), while the other half-received bi-lateral excision of the intra-abdominal epididymal depot (~550 mg; connected to testes). Both groups received abdominal muscle suture closure and skin was closed with wound clips. A subcutaneous injection of meloxicam analgesic (0.025mg/10 g body weight) was given immediately after surgery was completed. The four experimental groups consist of FKOγ or CON with or without surgery (FKO surgery n=6, FKO sham n = 7, CON surgery n= 7 and CON Sham n = 8).

Glucose Tolerance Test

Pre-surgery (one week prior to adipose tissue removal surgery) and terminal (one week prior to termination, 12 weeks post-surgery) glucose tolerance tests (GTTs) were performed on mice. Mice were fasted, but allowed water, for 6 hours after lights on. Blood was collected from the tail vein and glucose concentration was determined using a Freestyle Lite Glucometer (Abbott, Abbott Park, IL). After fasting blood glucose was collected, (time point 0) mice received a 1.5 g/kg dextrose injection in the intraperitoneal cavity and blood glucose was measured from tail vein blood samples at 15, 30, 45, 60 and 120 minutes post-injection.

Termination

Termination occurred 13 weeks post-surgery. Final body weights were collected before mice were fasted for 4 hours for terminal collection. First, following isoflurane anesthetization, systemic blood was collected via decapitation and serum was separated and stored at –80° C. Femoral muscle and liver were removed and snap-frozen in liquid nitrogen and stored at –80° C. Inguinal (IWAT), epididymal (EWAT), perirenal (PWAT), dorsal (DWAT), and visceral (VWAT) white adipose tissue, as well as inter-scapular brown adipose tissue (BAT), were collected and weighed, snap-frozen, and stored at –80°C. IWAT, VWAT and PWAT depots were halved and fixed in osmium for cell size distribution (see below). Subcutaneous lymph nodes were removed from IWAT and visceral lymph nodes were removed from VWAT prior to being frozen.

RNA isolation and cDNA synthesis

Lipid-specific RNAeasy mini-kit columns were used to isolate RNA from adipose tissue using QIAzol Lysis Reagent (QIAGEN, Valencia, CA). Aggregate RNA was converted to complementary-DNA using iScript (Bio-Rad, Hercules, CA) with a normalized quantity of RNA totaling 0.25 μg.

Quantitative Real-Time PCR

Sequences of primers for adipose tissue and liver are shown in Table 3.2. Primers were optimized as previously described [342]. Samples were run in triplicate using an iCycler (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). Expression patterns of genes of interest were normalized to constitutively expressed β2 microglobulin (B2M) and relative expression was quantified as previously described [342]. Genes were selected for their known associations with adipose tissue metabolism and adipogenesis. Genes of interest are reported as relative change compared with control.

Plasma and Tissue Measurements

Systemic plasma at termination was analyzed for insulin, leptin and resistin using a commercial kit (EMD Millipore Corporation, Billerica, MA) and analyzed on a Luminex instrument (LX200; Millipore, Austin, TX). Skeletal muscle and liver lipids were extracted using the procedure of Bligh and Dyer [343]. Muscle and liver triglyceride concentration (Sigma Chemical Co, St. Louis, MO) and plasma non-esterified fatty acids (Wako, Richmond, VA) were determined enzymatically using commercially available kits.

Adipocyte Distribution

Approximately half of the collected total IWAT, VWAT and PWAT depot was fixed in osmium tetroxide according to the method of Hirsch and Gallain [344]. Fixation was completed in a warm water bath for at least 24 hours. Cell number and size distribution were determined by Coulter Counter analysis (Beckman Coulter, Fullerton, CA), as suspended particles were passed through an aperture in the counter to provide a histogram in per unit volume of suspension. Distribution is presented as a percent in cell size bin, which represents a range of 10um hence 25um bin contains cell sizes 25um through 34um.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistics of multiple groups were completed using 2-way between-subject analysis of variance (ANOVA) (IBM SPSS for Windows, release 22; Chicago, IL) with group (CON and FKO) and surgery (Sham and LipX) as factors. This was utilized for the following dependent variables: body and adipose mass, lipids, gene expression, adipocyte mean size and adipokine concentration. Adipocyte cells size distribution and glucose tolerance test were analyzed using 2-way repeated measures ANOVA with adipocyte size as a within subject variable. Percent change in insulin and area under the curve (AUC) as well as total adipocyte number within the visceral, inguinal and perineal depots were analyzed by ANOVA. Post-hoc test of individual groups were accomplished with Tukey's test. Differences among groups was considered significantly different if $P \leq 0.05$. Exact probabilities are shown when applicable.

Results

Food Intake, Body and Adipose Tissue Mass and Lipids

Thirteen weeks post-surgery, cumulative food intake (CON Sham; 334.5g ± 3.8, CON LipX; 341.4g ± 1.8, FKO Sham; 320.2g ± 9.1 and FKO LipX 330.4g ± 7.2) and terminal body mass (Table 3.1) were not different among groups. Both CON and FKO LipX groups had ~550 mg of epididymal adipose tissue removed, but only the FKO LipX mice had significantly smaller epididymal depot mass at termination compared with respective Sham (Table 3.1; $p \leq 0.05$). Despite similar body mass among groups, adipose tissue mass of FKO mice was significantly less than CON. In particular, IWAT (Group main effect, $p = 0.011$), PWAT (Group main effect $p = 0.017$), DWAT (Group main effect $p = 0.006$) and total depot mass (summed IWAT, VWAT, PWAT and DWAT, Group main effect $p = 0.011$) were significantly reduced in FKOγ compared to CON mice. VWAT was also less in FKOγ, though not significant. In CON mice LipX was associated with significant increases in non-excised adipose tissue depot mass including a ~65% increase in IWAT, ~110% increase in VWAT, ~ 50% increase in both PWAT and DWAT depots (Table 3.1; $p \leq 0.05$) compared to CON Sham. This resulted in an overall ~65% increase in total adipose depot mass in CON LipX mice when compared to CON Sham. No increase in adipose tissue mass was observed in FKOγ LipX mice when compared to FKOγ Sham. Circulating and muscle lipids were not significantly different among groups, however FKOγ liver triglycerides tended to be higher than CON (Table 3.1; $p = 0.063$).

Glucose Tolerance Test and Insulin Concentration

One week before termination (12 weeks post-surgery) glucose concentration following a 6 hr fast was not different among groups (Data not shown). Glucose tolerance test results are

reported as percent difference between LipX and Sham at each time point (0, 15, 30, 60, and 120 minutes) for CON and FKOγ mice. LipX decreased the glucose response by ~30% in CON mice, whereas LipX increased the glucose response by ~30% in the FKOγ group, these two groups were significantly different from one another (Figure 3.1A and 3.1B; $p \leq 0.05$). Systemic insulin concentration was significantly higher, ~2 fold, in FKOγ mice (CON Sham = 161 ± 20, CON LipX = 169 ± 18, FKO Sham = 339 ± 58, FKO LipX = 375 ± 75; $p = 0.033$, FKO Sham vs. CON Sham and $p = 0.01$, FKO LipX vs. CON Sham). LipX did not result in significant effects on insulin concentration (Figure 3.1C). Overall, these data demonstrate that LipX resulted in improvements in glucose tolerance in CON mice and impairments in glucose tolerance in FKOγ mice.

Adipocyte Number and Cell Size Distribution

Adipose tissue removal provokes compensation in non-excised depots via differentiation/proliferation, which is typically assessed by the measurement of adipocyte number and adipocyte size distribution [340, 345]. Therefore, we evaluated adipocyte number and size distribution in a subcutaneous depot, inguinal (IWAT), and two intra-abdominal depots, the visceral depot (VWAT) that releases effluent to the portal circulation and the perirenal depot (PWAT) that releases effluent to the systemic circulation. LipX resulted in increased total adipocyte number in CON but not FKOγ mice. In particular, the number of adipocytes in the inguinal and perirenal depots of CON mice were significantly increased by ~45% following LipX (Figure 3.2 A and E inset; $P \leq 0.05$). LipX also induced a significant shift in adipocyte distribution in the inguinal (cell size/surgery interaction: Figure 3.2A; $p = 0.001$) and visceral (cell size/surgery interaction: Figure 3.2C; $p = 0.04$) depot. In CON mice, adipocytes in these

depots shifted toward a higher percent of small cells, but not FKOγ mice. In the inguinal depot of CON mice, LipX caused a significant increase in the percent of 25-44μm (Figure 3.2A; $p \leq 0.05$) adipocytes and a significant decrease in the percent of 45-84μm (Figure 3.2A; $p \leq 0.05$). Similarly, in the visceral depot LipX increased the percent of 25-34μm adipocytes and significantly decreased 45-64μm size adipocytes (Figure 3.2B; $p \leq 0.05$). LipX did not affect perirenal depot adipocyte distribution in either group. These changes in adipocyte number and distribution were prevented in FKOγ mice. This data demonstrates that LipX-induced adipose depot compensation was prevented with the inhibition of PPARγ in adipose tissue.

Adipose Tissue Gene Expression

Given the dramatic changes in adipose tissue mass, adipocyte number and size distribution observed in CON but not FKOγ mice we examined gene markers related to adipose tissue growth/expansion and maturation in both the inguinal and visceral depot. These two depots were further evaluated for gene expression because of their differential associations with metabolic risk. We focused on gene markers of cellular differentiation (peroxisome proliferator-activated receptor γ; *PPARγ* and CCAAT enhancer binding protein α; (*C/EBPα*)), fatty acid uptake and transport (fatty acid binding protein 4; *FABP4* and fatty acid transport protein 4; *FATP4*) and genes encoding proteins secreted from adipose tissue (adiponectin; *adipoQ*). Overall, all of the adipose gene markers were significantly reduced in FKOγ mice compared with CON in both the inguinal and visceral depot. For the inguinal depot *PPARγ, C/EBPα, FABP4, and adipoQ* were significantly lower in FKOγ mice compared with CON (Figure 3.3 A,C,E and I, $p \leq 0.007$). Similarly, these genes along with *FATP4* were significantly lower in the visceral depot of FKOγ mice (Figure 3.3 B, D, F, H and J, $p \leq 0.025$). LipX induced alterations in

inguinal and visceral adipose tissue gene expression, but only in CON mice. In the inguinal depot LipX caused a significant ~5-fold decrease in gene expression of *PPARγ* (Figure 3.3 A, interaction p = 0.009), C/EBPα (Figure 3.3 C, interaction p = 0.005) and FABT4 (Figure 3.3 G, interaction p = 0.000), whereas in the visceral depot LipX resulted in a ~2-fold increase in *adipoQ* (Figure 3.3 J, approaching interaction p = 0.079). Therefore, as predicted, LipX-induced alterations in adipose tissue gene expression were specific to CON mice and did not occur in FKOγ. The direction of most changes, however, were in opposition of what was anticipated.

Circulating Adipokines

Compared with CON mice, FKOγ mice had significantly lower leptin (Table 3.1, Group main effect p = 0.04) and resistin (Table 3.1, Group main effect p = 0.02) concentrations in systemic plasma. LipX significantly decreased leptin concentrations in both CON and FKOγ mice (Table 3.1, Surgery main effect p = 0.019), however resistin was only reduced in CON LipX mice (~45%, Table 3.1, $p \leq 0.05$; compared with sham control).

Liver Gene Expression

FKOγ mice were characterized by a ~2-fold increase expression of selected gene markers of inflammation, caspase1 (*CASP1*) (Figure 3.4 A, p = 0.000) and interleukin 1 α (Figure 3.4 B, p = 0.007) and β (Figure 3.4 C, p = 0.004) (IL1α and ILβ) (Figure 3.4 A-C; $p \leq 0.05$). None of these genes were affected by LipX.

Discussion

The present study was designed to examine the link between fat removal, adipose tissue compensation, and glucose homeostasis. We employed lipectomy to induce compensatory adipose tissue depot growth in Control and FKOγ mice. We hypothesized that lipectomy-induced increases in adipose tissue compensation would be impaired in FKOγ knockout mice and this impairment would be associated with unfavorable outcomes in glucose homeostasis. Our results support the notion that adipose tissue expansion, via PPARγ-mediated mechanisms, plays a role in glucose homeostasis.

The removal of gonadal adipose tissue (epididymal) in CON mice increased total fat mass, adipocyte number in inguinal and perirenal adipose tissue depots, and shifted cell distributions towards smaller adipocytes in the inguinal and visceral adipose tissue depots. This is consistent with other rodent studies where gonadal LipX increases the mass of intra-abdominal and subcutaneous adipose depots [346-348] . In contrast, the removal of gonadal adipose tissue in FKOγ mice did not increase total fat mass, adipocyte number or shift cell size distribution. These data suggest that LipX-induced adipose tissue hyperplasia is mediated, at least in part, by PPARγ. Since LipX did not result in compensatory increases in adipose tissue mass, adipocyte number or decreases in adipocyte size in FKOγ mice, we speculate that limitations placed on adipogenesis and adipocyte proliferation by the absence of PPARγ restrict sequestration of lipids in adipose tissue. Given the simultaneous improvement in glucose homeostasis, our results are consistent with the notion that smaller adipocytes are associated with metabolic protection.

Adipose tissue expandability is often associated with favorable metabolic outcomes and appears to play a fundamental role in the regulation of glucose homeostasis. Indeed, studies demonstrate that the hyperplastic potential of white adipose tissue is directly associated with

insulin sensitivity [349] and improved glucose tolerance [350]. Consistent with this, intra-abdominal LipX resulted in both adipose tissue expansion and improved glucose tolerance in CON mice. Adipose tissue expansion and improvements in glucose tolerance, however, did not occur in FKOγ mice. Rather, LipX in FKOγ mice reduced glucose tolerance. It should be emphasized that LipX-induced improvements are not due to fat removal alone, but rather the consequential compensation. Therefore, we propose that LipX-mediated improvements in glucose homeostasis are linked to the ability of adipose tissue to expand via mechanisms that include increases in cell number and shift in size distribution.

A restriction or impairment of adipose tissue expansion and continued sequestration of lipids can lead to lipid deposition in non-adipose tissues, such as liver and muscle (For review see: [351]. In the present study, FKOγ sham mice were characterized by a 40% increase in liver triglycerides compared to CON sham mice. Although this increase was not statistically significant (approaching $p = 0.063$) the data suggest that the lack of PPARγ and the resultant limitations placed on adipose tissue expansion resulted in ectopic lipid sequestration in the liver. In addition, muscle and liver triglycerides tended to be higher (~25%) in FKOγ mice who received lipectomy compared to their sham counterparts. These data, while not definitive, support the notion that adipose tissue expansion is linked to the degree of ectopic lipid accumulation.

We also analyzed genes involved in adipocyte differentiation and growth to gain insight into the FKOγ phenotype and the effects of lipectomy. Consistent with previous studies [336] we observed that genes involved in cellular differentiation and fatty acid uptake, transport and storage, as well as genes that encode secreted proteins were lower in the white adipose tissue of FKOγ mice compared with CON. These differences were consistent across the inguinal and

visceral adipose tissue depots. In CON mice, LipX altered genes involved in adipocyte differentiation and fatty acid uptake in the inguinal, but not visceral depot. In opposition to our prediction of enhanced gene expression of adipose tissue growth/compensation markers in LipX CON mice, gene expression of PPARγ, C/EBPα and FATP4 were decreased in the inguinal depot of CON LipX mice compared with Sham. Though it is possible that the downregulation of gene expression is due to decreased rate of expansion of the inguinal depot, it is important to acknowledge that this is a single time point and perhaps not indicative of alterations occurring at an early time point. Previous studies in rodents have consistently demonstrated that body fat loss following lipectomy was normalized ~3 months post-surgery [347, 348, 352]; hence, gene markers of compensation should be decreased at the 13 week time point that we chose for our study. LipX did not alter expression of these genes in the inguinal or visceral adipose tissue depot of FKOγ mice.

It is proposed that dysregulated visceral adipose tissue contributes to the development of hepatic steatosis and insulin resistance [353] because of its proximity to and release of lipids and cytokines to the liver [354]. However, in the current study, LipX induced visceral adipose tissue compensation in CON mice and was associated with improved glucose tolerance despite an increase in visceral adiposity, which is proposed to be detrimental. We propose that events following lipectomy enhance the inherent ability of visceral adipocytes to expand and sequester triglycerides, thus decreasing lipid effluent to the liver. At 13 weeks, however, circulating free fatty acids, liver triglycerides, and liver markers of inflammation were not decreased in mice with fat removed relative to their sham counterparts. It is important to note all mice received standard rodent chow and remained lean throughout the experiment, thus some liver measures may already be at a minimum and therefore less flexible toward change.

Despite considerable adipose tissue compensation, lipectomy decreased both leptin and resistin in CON mice. The reduction in leptin was not expected given this adipokine is a lipostatic signal and LipX mice were characterized by increased adiposity. However, leptin can also inhibit cell proliferation; therefore, high levels may be counterproductive during periods that involve adipose tissue compensation [355]. LipX in CON mice also decreased resistin, an adipokine highly associated with obesity [356] and impaired insulin action in the liver [74, 234, 357]. Healthy, insulin sensitive adipocytes produce less resistin [358], thus a depot with a higher distribution of small proliferating cells, as observed in lipectomy-induced compensation, would be expected to produce less resistin, and potentially play a role in improvements in glucose regulation. In general, FKOγ mice had lower circulating concentrations of adipokines compared with CONs. This is consistent with previous studies [336] and suggests that PPARγ is also associated with normal adipocyte function and hormone release related to cell maturation.

In summary, in the present study we have elected to manipulate PPARγ (present or absent in adipose tissue) in the context of lipectomy or sham surgery to further investigate the relationship between adipose tissue expansion and glucose homeostasis. We hypothesized that lipectomy-induced increases in adipose tissue growth would be impaired in FKOγ knockout mice and this impairment would lead to unfavorable outcomes in glucose homeostasis. Our results support the notion that adipose tissue expansion, via PPARγ-mediated mechanisms, plays a role in glucose homeostasis. More specifically data from the present study suggest that increased adipocyte number and changes in size distribution to smaller adipocytes is closely linked to improvements in glucose homeostasis observed in response to lipectomy.

Tables

Table 3.1: Terminal Data Results. Body weight, total and individual (inguinal, visceral, perirenal and dorsal white adipose tissue and inter-scapular brown adipose tissue) adipose tissue mass, adipocyte mean size, systemic circulating adipokines, free fatty acid (FFA) and tissue triglyceride (TG: muscle and liver) concentration 13 weeks post-surgery. Values are reported as mean±SEM

					P value		
	CON Sham	CON LipX	FKO Sham	FKO LipX	Model	Group	Sur
Final Body Mass (g)	34.4±2.4	37.4±2.5	32.7±0.5	32.2±1.6	–	–	–
Adipose Tissue Mass (g)							
EWAT	0.97±0.18	0.66±0.13	0.99±0.10	0.26±0.05*	0.001	–	0.0009
IWAT	0.63±0.09	1.04±0.23*	0.40±0.05	0.42±0.10	0.034	0.011	–
VWAT	0.37±0.04	0.79±0.16*	0.45±0.05	0.43±0.06	0.041	0.075	0.026
PWAT	0.51±0.06	0.80±0.13*	0.36±0.05	0.40±0.07	0.046	0.017	–
DWAT	0.54±0.05	0.81±0.21	0.30±0.05	0.36±0.06	0.018	0.006	–
Total	2.33±0.25	3.70±0.07*	1.70±0.12	1.81±0.30	0.029	0.011	–
BAT	0.28±0.03	0.25±0.05	0.20±0.01	0.20±0.04	–	–	–
Adipocyte mean size (um)							
IWAT	50.4±1.72	49.3±0.76	53.95±2.56	52.23±1.68	–	–	–
VWAT	58.18±1.49	54.50±2.38	58.18±1.24	60.25±2.23	–	–	–
PWAT	53.82±1.90	54.40±2.50	58.94±2.59	56.93±2.59	–	–	–
Adipokine (ug/mL)							
Leptin	4918±621	2728±846*	3269±491	1484±430*	0.041	0.040	0.019
Resistin	1516±147	826±109*	725.5±195	767±287	0.011	0.02	–
Systemic FFA (mmol/L)	0.55±0.04	0.51±0.02	0.57±0.04	0.45±0.03	–	–	–
Muscle TG (mg/g tissue)	25.6±4.9	19.7±3.8	23.2±4.9	28.1±11.2	–	–	–
Liver TG (mg/g tissue)	31.5±6.2	28.3±2.7	43.8±12.5	52.3±12.0	–	0.063	–

CON, Control; LipX, Lipectomy; Sur, Surgery; um, micrometer.
Chart contains P values of model and main effect of group and surgery (– = not significant).
*P ≤ 0.05, compared with respective control.

Figures

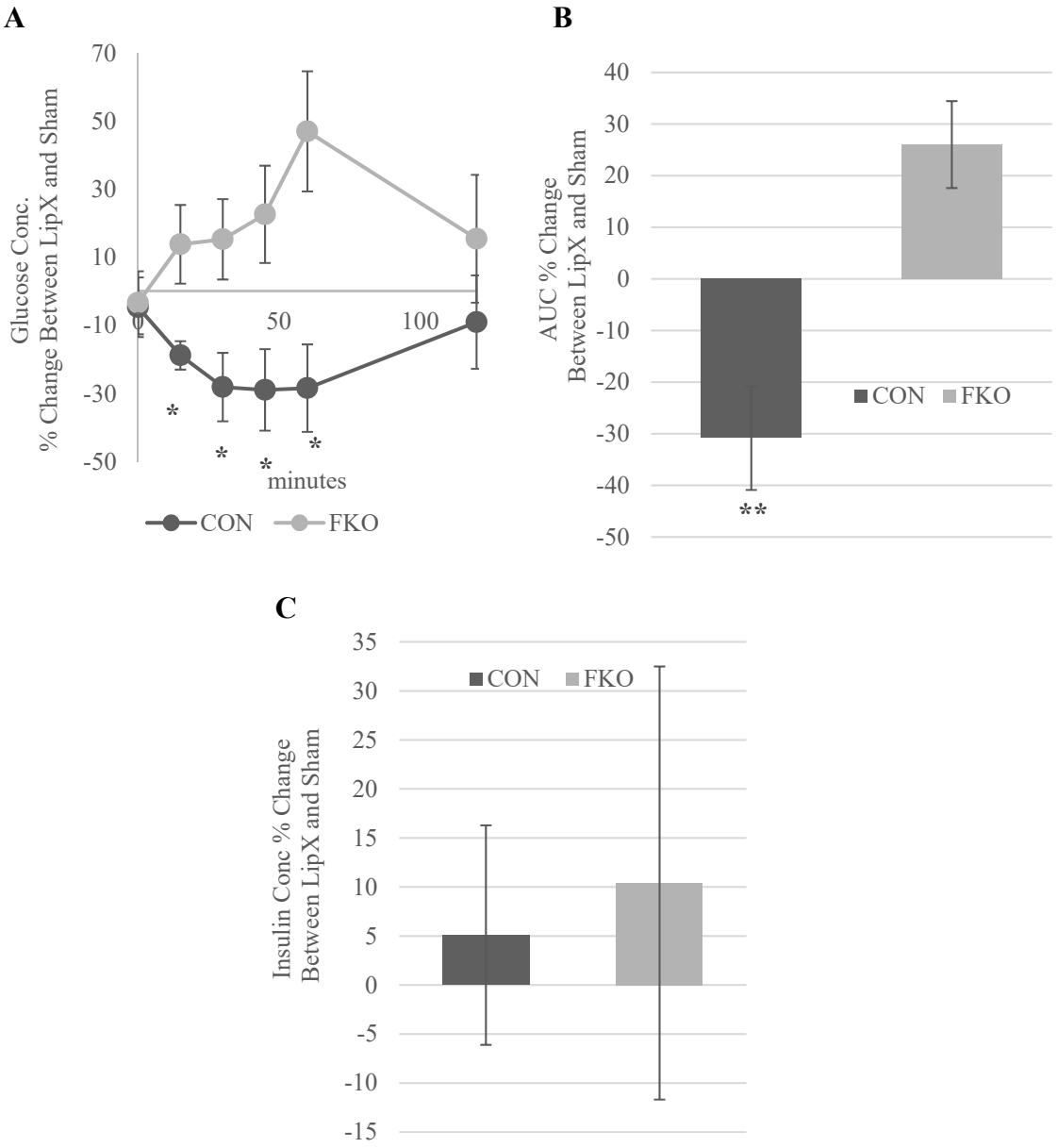
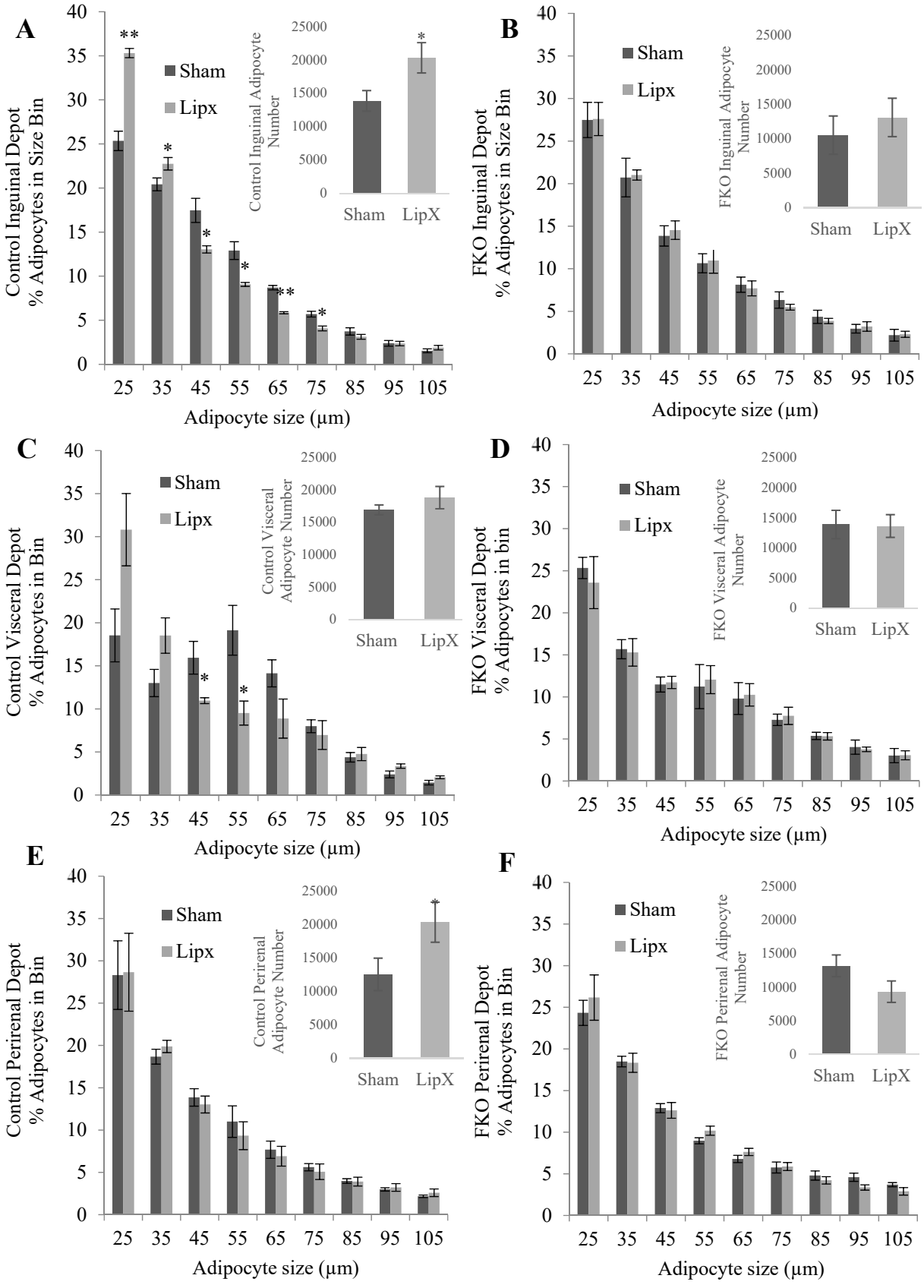


Figure 3.1: Glucose Tolerance Test (GTT) and Insulin Concentration. A) GTT – Percent change in glucose concentration between the LipX and Sham group of CON and FKOγ mice. LipX is associated with decrease glucose concentration in FKOγ mice, whereas as it is associated with an increase in glucose concentration in the CON (* $p \leq 0.05$). B) Percent change in Area Under the Curve between the LipX and Sham group was decreased in control mice demonstrating improved glucose tolerance, whereas it was increased in FKOγ mice which is indicative of decreased glucose tolerance (** $p = 0.002$) C) Insulin concentration expressed as percent change between the LipX and Sham surgery, was not different between CON and FKO mice.


A
Control Inguinal Depot
% Adipocytes in Size Bin
Sham
Lipx
Control Inguinal Adipocyte Number
Sham
LipX
Adipocyte size (µm)
B
FKO Inguinal Depot
% Adipocytes in Size Bin
FKO Inguinal Adipocyte Number
C
Control Visceral Depot
% Adipocytes in Bin
Control Visceral Adipocyte Number
D
FKO Visceral Depot
% Adipocytes in bin
FKO Visceral Adipocyte Number
E
Control Perirenal Depot
% Adipocytes in Bin
Control Perirenal Adipocyte Number
F
FKO Perirenal Depot
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FKO Perirenal Adipocyte Number

Figure 3.2: Adipocyte Number and Cell Size Distribution of Inguinal, Visceral and Perirenal Adipose Tissue. Inset of A and E) LipX in CON mice, but not FKOγ, resulted in a significant increase in adipocyte number in the inguinal and perirenal adipose depots (* = $p \leq 0.05$, compared with Sham). In CON mice, LipX also shifted the A.) inguinal (Cell size/surgery interaction: Figure 2A; $p < 0.05$) and C) visceral (Cell size/surgery interaction: Figure 2C; $p \leq 0.05$) depot adipocyte distribution towards smaller cell size. In the inguinal depot of CON mice LipX resulted in a significant increase in the percent of 25-44μm (** = $p \leq 0.001$, * = $p \leq 0.05$, compared with Sham) adipocytes and significant decrease in the percent of 45-84μm (** = $p \leq 0.000$, * = $p \leq 0.05$, compared with Sham). For the visceral depot LipX increased the percent of 25-34μm adipocytes and significantly decreased 45-64μm (* = $p \leq 0.05$, compared with Sham). LipX did not affect perirenal depot adipocyte distribution or change distribution of cell size in FKOγ mice.

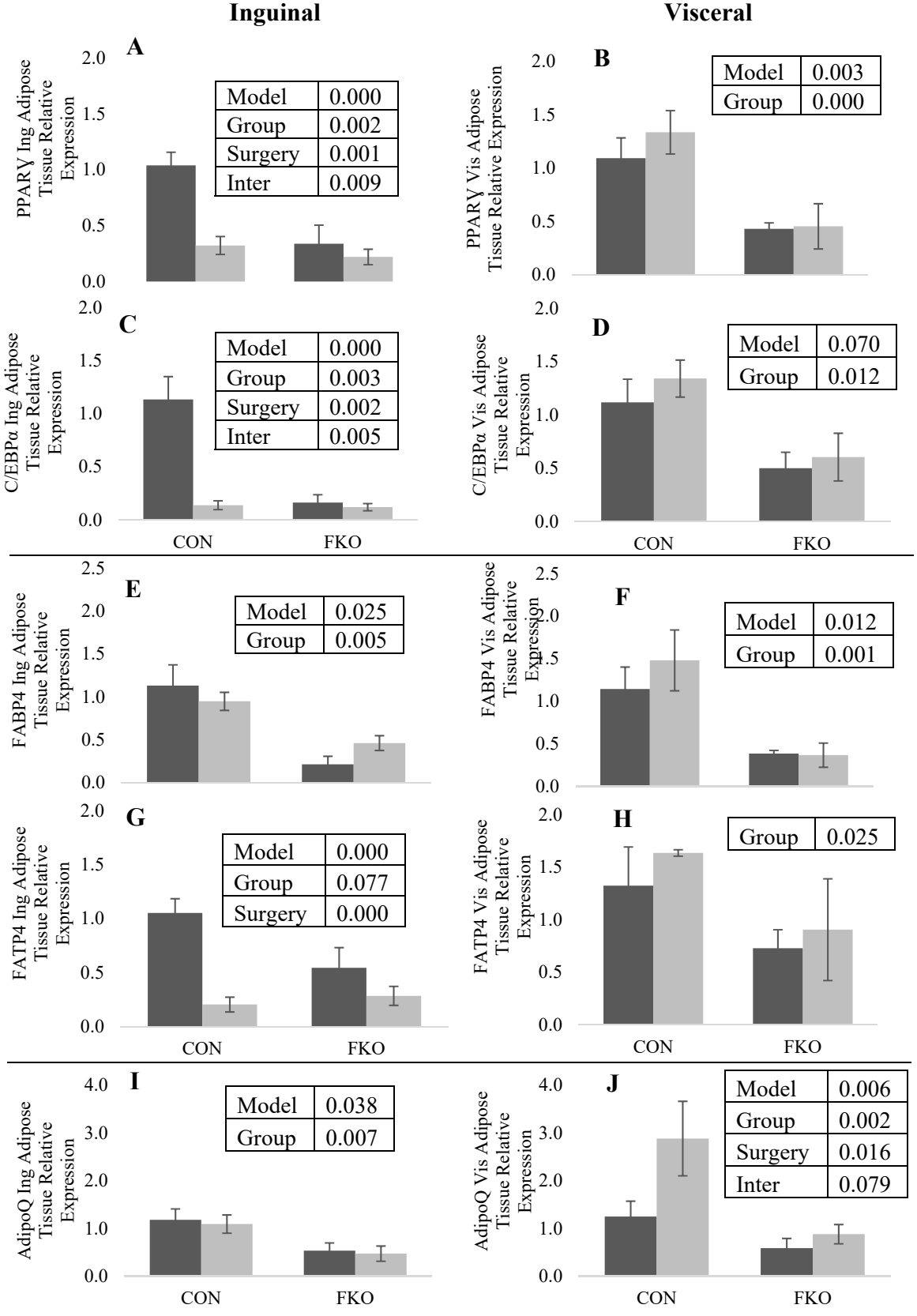

Inguinal
Visceral
A
PPARγ Ing Adipose Tissue Relative Expression
Model 0.000
Group 0.002
Surgery 0.001
Inter 0.009
B
PPARγ Vis Adipose Tissue Relative Expression
Model 0.003
Group 0.000
C
C/EBPα Ing Adipose Tissue Relative Expression
Model 0.000
Group 0.003
Surgery 0.002
Inter 0.005
D
C/EBPα Vis Adipose Tissue Relative Expression
Model 0.070
Group 0.012
E
FABP4 Ing Adipose Tissue Relative Expression
Model 0.025
Group 0.005
F
FABP4 Vis Adipose Tissue Relative Expression
Model 0.012
Group 0.001
G
FATP4 Ing Adipose Tissue Relative Expression
Model 0.000
Group 0.077
Surgery 0.000
H
FATP4 Vis Adipose Tissue Relative Expression
Group 0.025
I
AdipoQ Ing Adipose Tissue Relative Expression
Model 0.038
Group 0.007
J
AdipoQ Vis Adipose Tissue Relative Expression
Model 0.006
Group 0.002
Surgery 0.016
Inter 0.079
CON
FKO

Figure 3.3: Adipose Tissue Gene Expression. Gene markers of cellular differentiation (peroxisome proliferator-activated receptor γ; *Pparγ* and CCAAT enhancer binding protein α; *C/EBPα*), fatty acid uptake and transport (fatty acid binding protein 4; *FABP4* and fatty acid transport protein 4; *FATP4*) and genes encoding proteins secreted from adipose tissue (adiponectin; *adipoQ*). All of the adipose gene markers were significantly reduced in FKOγ compared to CON mice. A) PPAR, C) CEBP, and G) FATP4 were significantly reduced in inguinal but not visceral adipose tissue from LipX vs Sham CON mice. LipX did not result in any significant changes in FKOγ mice. Insets within figure include p values for significance of model, main effects of group (CON vs. FKO) and surgery (LipX and Sham) and interactions when applicable.

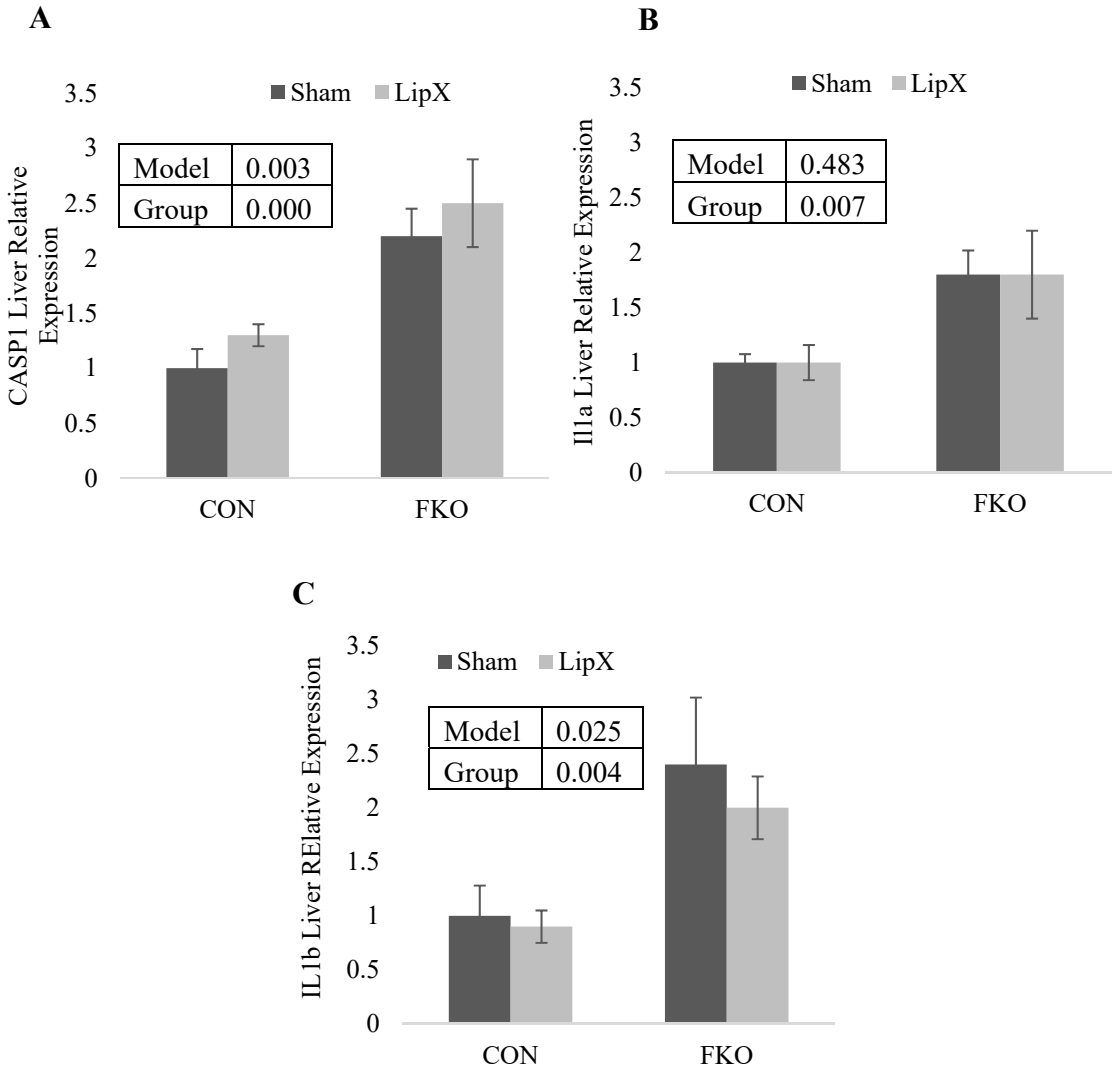


Figure 3.4: Liver Gene Expression. FKOγ mice were characterized by a significant increase in expression of selected gene markers of inflammation, A) caspase1 (*CASP1*) and interleukin B) 1 α and C) β (IL1α and Ilβ). None of these genes were significantly affected by LipX. Insets within figure include p values for significance of model, main effects of group (CON vs. FKO) and surgery (LipX and Sham) and interactions when applicable.

Table 3.2: Sequences of Primers for Adipose Tissue and Liver. Peroxisome proliferator-activated receptor γ, *PPARγ*; CCAAT enhancer binding protein α, *C/EBPα*; fatty acid binding protein 4, *FABP4*; fatty acid transport protein 4, *FATP4*; adiponectin, *adipoQ*; Beta-2-microglobulin, *B2M,* caspase1, *CASP1*; interleukin 1 α and β; *IL1α* and Ilβ.

Gene	Forward	Reverse
PPARγ	GCGGTGAACCACTGATATTCA	TCCGAAGTTGGTGGGCCAGA
C/EBPα	TTCGGGTCGCTGGATCTCTA	TCAAGGAGAAACCACCACGG
FABP4	TGAAATCACCGCAGACGACA	ACACATTCCACCACCAGCTT
FATP4	TGCTCCTGTACTTGGGGTCT	GAGCACCATGCCACCAAAGA
AdipoQ	CGACACCAAAAGGGCTCAG	TGCACAAGTTCCCTTGGGTG
B2M	CGGTCGCTTCAGTCGTCAG	ATGTTCGGCTTCCCATTCTCC
CASP1	AGATGGCACATTTCCAGGAC	GATCCTCCAGCAGCAACTTC
IL1α	CACGGGGACTGCCCTCTAT	TGTCGGGGTGGCTCCACT
IL1β	TCTTTGAAGTTGACGGACCC	TGAGTGATACTGCCTGCCTG

CHAPTER 4: PROGRESSIVE SUBCUTANEOUS ADIPOSE TISSUE REMOVAL IN MICE PRODUCES DOSE-DEPENDENT DETERIORATION IN GLUCOSE REGULATION[8]

Summary

The protective effects of subcutaneous adiposity are proposed to be linked to the depot functioning as a ”metabolic sink” receiving and sequestering excess lipid. This postulate, however, is based primarily on indirect evidence and mechanism that mediate this protection are unknown. We propose to examine this directly with progressive subcutaneous adipose tissue removal. Ad libitum Chow fed mice underwent sham surgery, unilateral or bilateral removal of inguinal adipose tissue or bilateral removal of both inguinal and dorsal adipose tissue. Subsequently mice were separated into 5 week CHOW or 5 or 13 week HFD groups (N = 10 per group). Primary outcome measures included adipocyte distribution, muscle and liver triglycerides, glucose tolerance, circulating adipocytokines, muscle insulin sensitivity, and markers of fat utilization in muscle. Progressive adipose tissue removal resulted in a dose-dependent deterioration in systemic glucose tolerance in 13-week HFD mice. This was associated with decreased femoral muscle insulin sensitivity in the basal state, but not in the insulin-stimulated state. We have demonstrated that subcutaneous adipose tissue is protective in

[8] The aim of this work was to conduct an experimental study to examine systematic removal of subcutaneous adipose tissue on systemic and tissue-specific glucose intolerance. Our lab carried out controlled experiments employing incremental lipectomy to examine differences in glucose homeostasis under standard CHOW and HFD conditions. This article outlines the protective properties of peripheral adipose tissue on metabolic regulation.

This chapter includes a manuscript prepared for submission as original research titled *Progressive Peripheral Subcutaneous Adipose Tissue Removal in Mice Produces Dose-Dependent Deterioration in Glucose/Insulin Regulation* (A.D. Booth, A.M. Magnuson, J Fouts, Y. Wei, D. Wang, M.J. Pagliassotti, and M.T. Foster). My contributions to this experiment included housing, general care, food and weight measurements, GTT, surgical lipectomy, lab assays, and tissue collection. My contribution to this manuscript included full initial text preparation, data calculations, table and figure preparation, statistics, as well as review of content for accuracy and cohesiveness, including editing and corrections for future submission.

Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 4.1.

two independent ways, by maintaining systemic glucose tolerance and basal muscle insulin sensitivity.

Introduction

Obesity has steadily increased over the past 50 years with 40% of Americans being obese (BMI>30) today compared to only 14% in the 1960s [359]. Obesity is linked to numerous co-morbidities including glucose intolerance, insulin resistance, dyslipidemia, and cardiovascular dysfunction, which are largely preventable [6, 360]. However, obesity is not an exclusive predictor of metabolic dysregulation. For example, some obese individuals maintain insulin sensitivity, and have a reduced risk for type-2-diabetes, hypertension and heart disease [361-363], whereas others with seemingly normal adipose tissue levels develop these co-morbidities [364, 365]. Variations in fat distribution, in part, are suggested to mediate these risks [1]. Individuals with apple-shaped (android) phenotype deposit adipose tissue in the upper body, otherwise known as visceral adiposity. In contrast pear-shaped (gynoid) phenotype deposit fat subcutaneously beneath skin in the lower body gluteus-femoral region. These differing types of adipose tissue distribution have opposing metabolic outcomes in overweight and obese individuals.

Visceral adipose tissue is highly associated with metabolic disease, however other studies suggest that subcutaneous adipose tissue (SAT), or lack thereof, may be playing a contributory role [3]. This postulate arises from the relative proportions that visceral and subcutaneous depots contribute to overall adiposity. Specifically, visceral adipose is merely ~10% of total adiposity whereas SAT accounts for ~85% total fat mass. There is evidence that lower body subcutaneous adipose tissue (LBSAT) accumulation is protective relative to visceral. It is postulated that

LBSAT functions as a metabolic sink with enhanced ability, relative to visceral fat, to take up lipid from circulation and store it thereby protecting insulin sensitive tissues from ectopic deposition [3, 366, 367]. Indeed, LBSAT storage is associated with increased glucose tolerance and insulin sensitivity [4]. Additionally, SAT is positively associated with factors related to energy intake regulation (leptin, adiponectin) and negatively associated with inflammatory factors (IL-6, TNFα, MCP-1) [3, 368-371].

Here we postulate that if subcutaneous adipose tissue is protective, then removal of these depots should have deleterious consequences. We have previously demonstrated in mice that removal of the protective lower body adipose depots, specifically inguinal WAT, by lipectomy resulted in systemic and muscle insulin resistance which was exacerbated by high fat diet (HFD) [366]. In the same study, CHOW and HFD fed mice with inguinal fat removal both experienced increases in muscle triglycerides. Metabolic outcomes of peripheral adipose tissue removal has also been examined in humans, but these studies have inherent experimental limitations due to variations in amount of adipose tissue removed, study duration and age range [372-376]. Yet, studies by Hernandez et al. support that subcutaneous adipose tissue is protective because its removal causes redistribution of lipids to the abdomen [374] and worsens postprandial blood lipid concentration [377]. Rodent research supports and extends findings in humans [339, 346-348, 352, 378-382].

The intent of this study is to examine systematically the hypothesis that peripheral adipose tissue promotes glucose homeostasis by protecting muscle from ectopic lipid accumulation, hence acting as a “metabolic sink”. We have previously demonstrated that the removal of lower body subcutaneous adipose tissue results in impairments in glucose tolerance and increased lipid accumulation in skeletal muscle [366]. We propose to utilize the

subcutaneous lipectomy model to comprehensively examine the effects of peripheral adipose tissue both in relation to insulin-mediated regulation of glucose metabolism and non-adipose tissue lipid accumulation. We will expand on our initial observations by carrying out time course studies that include removal of varying amounts of peripheral adipose tissue. Our general strategy was to perform sham surgery, unilateral or bilateral removal of inguinal adipose tissue or bilateral removal of both inguinal and dorsal adipose tissue to evaluate the effects of this on glucose tolerance, adipose tissue compensation and non-adipose tissue lipid accumulation. We predict that progressive peripheral adipose tissue removal will produce dose-dependent increases in non-adipose tissue lipid accumulation that will subsequently cause an associated dose-dependent deterioration in systemic glucose tolerance. This study will fundamentally advance the field of adipose tissue biology by determining whether the correlative evidence linking peripheral adipose tissue to reduced risk for adverse metabolic outcomes has a causal basis.

Methods

Mice and Housing

Male C57BL/6 mice were purchased at 3 months of age from The Jackson Laboratory (Bar Harbor, Maine) and were allowed to acclimate for one week. Mice were individually housed under controlled conditions (12:12 light-dark cycle, 50–60% humidity, and 25° C) and initially had access to ad libitum standard CHOW diet (CHOW:Harlan Teklad LM485, Madison, WI) with unlimited water. Chow diet was 3.1 kcal/g with 18% kcal from fat (6% by weight of diet). Following surgery, they either remained on standard CHOW diet or were given a high fat western style diet with 21% milk fat and 34% sucrose (Envigo TD.08811). Weekly body mass

and food intake were monitored and recorded. Procedures were reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Surgery

Subcutaneous adipose tissue is located between the muscle and skin, predominately on top of the hind legs (inguinal depot) and upper back (dorsal depot). Surgeries were performed while mice were anesthetized with isoflurane and fat was accessed through a small mid-ventral incision. The four surgery groups consisted of sham-operated surgery (Sham), Unilateral (Uni IngX) and Bilateral (Bi IngX) removal of inguinal adipose tissue, and Bilateral removal of both inguinal and dorsal adipose tissue depots (All). Sham surgery consisted of mid-ventral abdominal incision through skin only without adipose tissue removed. Lipectomy groups had ~80% fat excised from each depot removed, which accounts for ~20%, 40%, and 80% of total SAT for Uni IngX, Bi IngX, and All, respectively. Skin was closed with wound clips. A subcutaneous injection of meloxicam analgesic (0.025mg/10 g body weight) was given immediately after surgery was completed.

Glucose Tolerance Test

Pre-surgery (one week prior to adipose tissue removal surgery) and terminal (one week prior to termination) glucose tolerance tests (GTTs) were performed on mice. Mice were fasted, but allowed water, for 6 hours after lights on. Blood was collected from the tail vein and glucose concentration was determined using a Freestyle Lite Glucometer (Abbott, Abbott Park, IL). After fasting blood glucose was collected, (time point 0) mice received a 1.5 g/kg dextrose injection in the intraperitoneal cavity and blood glucose was measured from tail vein blood samples at 15,

30, 45, 60 and 120 minutes post-injection. Additional blood was collected at time point 0, 15 and 120 minutes for insulin concentrations. Insulin concentrations were assessed using an Ultra-Sensitive Mouse Insulin Elisa Kit (Crystal Chem, Downers Grove, IL).

Termination

Final body weights were collected and mice were fasted for 4 hours for terminal collection. First, following isoflurane anesthetization, systemic blood was collected via decapitation and serum was separated and stored at –80° C. Liver was removed and snap-frozen in liquid nitrogen and stored at –80° C. Inguinal (IWAT), epididymal (EWAT), perirenal (PWAT), dorsal (DWAT), and visceral (VWAT) white adipose tissue, as well as inter-scapular brown adipose (BAT), were collected, weighed, snap-frozen, and stored at –80°C. Subcutaneous lymph nodes were removed from IWAT and visceral lymph nodes were removed from VWAT prior to being frozen. Femoral and dorsal muscles were also collected and snap-frozen and stored at –80° C.

Plasma Measurements

Systemic plasma at termination was analyzed for insulin, leptin, resistin, tumor necrosis factor α (TNFα), monocyte chemotactic protein-1 (MCP-1), interleukin 6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1 total) concentrations, determined using commercial kits (EMD Millipore Corporation, Billerica, MA) and analyzed on a Luminex instrument (LX200; Millipore, Austin, TX).

Tissue Lipid Measurements

Skeletal muscle and liver lipids were extracted using the procedure of Bligh and Dyer [343]. Muscle and liver triglyceride concentration (Sigma Chemical Co, St. Louis, MO) were determined enzymatically using commercially available kits.

Adipocyte Distribution

A sample of EWAT or VWAT was fixed in osmium tetroxide according to the method of Hirsch and Gallain [344]. Fixation was completed in a warm water bath for at least 24 hours. Cell number and size distribution were determined by Coulter Counter analysis (Beckman Coulter, Fullerton, CA), as suspended particles are passed through an aperture in the counter to provide a histogram in per unit volume of suspension.

Muscle Insulin Resistance

Following a four-hour fast, mice were either injected (ip) with saline or insulin and then terminated ~15 minutes later. Muscle insulin sensitivity was determined by the ratio of phosphoralated Akt (Ser 473) ((Abcam) Anti-Akt1 (phosphor S473) antibody [EP21094] ab81283, rabbit monoclonal) to total Akt Protein ((Abcam) Anti-Akt1 antibody [9A4] ab89402, mouse monoclonal) measurements were made using gel-based Western Blot procedures using both Odyssey Clx (Licor) and Chemiluminescence. Proteins were extracted from muscle tissue and separated based on size via SDS-PAGE. The divided proteins were transferred onto a PVDF membrane and the membrane is blocked from non-specific binding. Proteins of interest are identified with immunoassay using primary antibodies and visualized with secondary antibodies

(LiCor: IRDye 680RD Goat anti-Rabbit 926-68171 and LiCor: IRDye 800CW Goat anti-Mouse 827-08364). Quantification was done in Image Studio Lite (Licor) and ImageJ software [383].

Muscle Fat Utilization/Storage

Two proteins were measured for factors of muscle fat utilization via western blot using Odyssey Clx (Licor). This included lipoprotein lipase (LPL) ((Abcam) Anti-lipoprotein lipase antibody [LPL.A4] ab21356, mouse monoclonal) for fatty acid cleavage outside of the cell and carnitine-palmitoyltransferase 1 (CPT1) ((Abcam) Anti-CPT1B antibody ab134988, rabbit polyclonal) for transport into the mitochondrial matrix for beta-oxidation. Housekeeping protein for normalization was GAPDH (Cell Signaling #2118 (14C10) rabbit monoclonal).

Statistical Analysis

Comparisons among groups are made using 1-way analysis of variance by surgery group having four factors (Control Sham, Uni IngX. Bi IngX, All), separately for each diet x time cohort. Post-hoc analysis was performed with a two-independent sample t-test, equal variance assumed. Whenever possible, analyses were done separately for femoral muscle on side with fat removed and not removed. Statistically significant differences among groups were recognized as having a p-value ≤ 0.05.

Results

Fat Pad Removal, Food Intake, and Body Weight

Table 4.1 contains the amount of fat removed for each surgery group. The Bi IngX group had approximately twice as much fat removed as the Uni IngX group (avg 110 vs 224

milligrams), while the All (Bi IngX + Bi DorX) group had 3.5 times more fat removed as the Uni IngX group (avg 110 vs 381 milligrams). Approximately 20%, 40%, or 80% of total subcutaneous fat was removed in the unilateral, bilateral or All surgery groups. Small amounts of adipose tissue recovered from lipectomy depots were significantly smaller than depots collected from respective sham controls (Table 4.3; $p \leq 0.05$). Average weekly food intake was not different among any of the surgical groups, however final body weight was significantly lower in All group HFD 13 week mice compared with respective Sham control (Table 4.1; $p = 0.016$). Despite the large amount of fat removal, the mass of other non-excised adipose tissue, in general, did not change (Table 4.3). The only depot to significantly increase (compensate) in size following fat removal was the visceral adipose depot of CHOW 5 week mice with Bilateral inguinal removal (Table 4.3, $p = 0.017$). Because adipocyte compensation (proliferation) can occur without significant increases in mass, we also measured adipocyte size and distribution. Adipose tissue distribution was only examined at 13 weeks because this is the typical time point where adipocyte compensation is complete [339, 346-348, 352, 379-382]. Specifically, we measured distribution in intra-abdominal depots, visceral and epididymal, because of their high association with metabolic disease (Figure 4.1). Surgical fat removal did not alter adipocyte number, average size or distribution in the visceral adipose depot. However, the epididymal adipose depot of mice with Bilateral inguinal removal had a significant decrease in total adipocyte number (Figure 4.1E inset; $p < 0.02$) that resulted from lower adipocyte numbers in all size bins, with significant decreases in the 45-54um and 55-64um bins (Figure 4.1E; $p \leq 0.04$).

Glucose and Insulin Measurements

Here we sought to comprehensively examine if subcutaneous adipose tissue is protective by determining if progressive removal would lead to an accumulative decline in glucose tolerance. In general, surgery did not change fasting glucose or insulin concertation in CHOW or HFD fed mice (Table 4.2). In addition, adipose tissue removal in 5 week Chow and HFD mice did not progressively alter glucose tolerance. Nevertheless, subcutaneous adipose tissue removal in 13 HFD mice exacerbated glucose intolerance in a dose-dependent fashion. Specifically, Bilateral removal of IWAT significantly increased glucose AUC compared with Sham Control (Figure 4.2C; $p = 0.04$). This difference was further exaggerated by the addition of dorsal adipose tissue removal. Hence, All removal was also significantly greater than Sham Control (Figure 4.2C; $p = 0.0005$) and had the highest glucose AUC among the surgical groups. Insulin AUC was not altered by surgery (Figure 4.2C).

Circulating Factors (Adipokines and Cytokines)

Circulating adipokines and cytokines were measured to determine if glucose dysregulation following fat removal was associated with alterations in circulating factors (Table 4.2). In general, leptin, resistin, Plasminogen activator inhibitor-1 (PAI1) and interleukin-6 (IL-6) were not affected by fat removal. Hence, adipose tissue dysregulation following fat removal is not associated with changes in adipocytokines.

Liver and Skeletal Muscle Triglycerides

Subcutaneous adipose tissue is proposed to be "protective" by way of functioning as a "metabolic sink", thus we proposed fat removal might increase lipids in other tissues. Therefore,

we measured liver and femoral muscle triglyceride concentration. Subcutaneous adipose tissue removal was not associated with changes in liver triglyceride concentration. However, fat removal significantly increased triglyceride concentration in femoral muscle in 5 week HFD mice (Figure 4.3B, One-way ANOVA surgery effect; P = 0.036). Unilateral (both removed and non-removed), Bilateral inguinal removal and All removal caused a ~2 fold increase in femoral muscle triglycerides, however only the femoral muscle of the removed side in the Unilateral inguinal surgery group was significantly greater than the respective control by ~3 fold (Figure 4.3B; p = 0.01). Femoral muscle triglyceride concentration from mice fed HFD for 13 weeks was not different among groups, with the exception of the muscle from the non-removed IWAT side from the Unilateral IngX group. The muscle next to adipose tissue that remained had significantly lower triglyceride concentration than its removed counterpart (Figure 4.3C; p = 0.005).

Muscle Insulin Sensitivity

Subcutaneous adipose tissue removal in 13-week HFD mice exacerbated systemic glucose intolerance. Since muscle is a primary site of insulin-dependent glucose disposal, we examined femoral muscle insulin sensitivity to determine if it contributed to elevated systemic glucose. The means of graphs in figure 4.4 represent the ratio of pAkt/Total-Akt of insulin and saline injected mice following a 4 hour fast. Insulin stimulated pAkt/Akt was not different among surgical groups in 13 week HFD mice, with the exception of the 13 week HFD All group having a significant increase in pAkt/Akt (Figure 4.4C; p = 0.0001). The femoral muscle of the ALL group was the only tissue to have any pAkt/Akt response to the insulin injection (Figure 4.4C; p = 0.0001). Instead, effects of adipose tissue removal on pAkt/Akt measures

predominately occurred in saline-injected mice. Adipose tissue removal caused a significant decrease in pAkt/Akt of the femoral muscle of saline-injected (basal) 13 week HFD mice (Figure 4.4C, One-way ANOVA surgery effect; P = 0.005). Specifically, the Unilateral (non-fat removed side) and Bilateral IWAT removal as well as the All group had significantly lower pAkt/Akt compared with control (Figure 4.4C; p = 0.004, p = 0.002, p = 0.003, respectively). Hence, this trended toward the more adipose tissue removed the greater the decrease in insulin sensitivity when compared with the sham control.

Muscle Fat Utilization/Storage

We next examined if progressive adipose tissue removal altered protein factors associated with fatty acid utilization within the adjacent femoral muscle, including lipoprotein lipase (LPL) and carnitine-palmitoyltransferase 1 (CPT-1). LPL cleaves fatty acids from lipoproteins in the lumen of capillaries, thus promotes cellular uptake into myocytes for utilization and/or storage [384]. CPT1 facilitates transport of FAs across the mitochondrial membrane for oxidation, an indicator of FA utilization within myocytes [385]. Adipose tissue removal did not change LPL/GAPDH in muscle of 5 week CHOW mice (Figure 5.5A), however there were significant surgery effects in 5 week HFD mice (Figure 5.5B, One-way ANOVA surgery effect; P = 0.019). 5 week HFD mice with Unilateral IWAT removal (removed side) had significantly increased LPL/GAPDH compared with Sham mice (Figure 5.5B; p = 0.02). Likewise, 5 week HFD mice with All removal had significantly higher LPL/GAPDH than Sham controls (Figure 5.5B; p = 0.04). Although there was no overall effect of surgery in mice fed HFD for 13 weeks, the Unilateral IWAT removal (non-removed side) had significantly increased LPL/GAPDH compared with Sham mice (Figure 5.5C; p = 0.05). A stepwise decrease in CPT1/ GAPDH was

observed in 5 week CHOW mice (Figure 5.5D, One-way ANOVA surgery effect; P = 0.07), with significantly lower CPT1/GAPDH in the Bilateral IWAT and All groups (Figure 5.5D; p=0.02 and p=0.03, respectively). There were no significant changes in CPT1/GAPDH for 5 or 13-week HFD mice with surgery.

Discussion

Fat distribution is a major determinant of metabolic health. In particular, it is well established that central adiposity (intra-abdominal/visceral adipose tissue accumulation) is a risk factor for adverse metabolic outcomes, such as cardiovascular disease and type 2 diabetes [1, 2]. Yet, adipose tissue accumulated within lower body subcutaneous adipose tissue depots is not. In opposition to central adiposity, accumulation of adipose tissue in the thighs, buttocks and hips is associated with protection from obesity risks such as impaired glucose tolerance and dyslipidemia [3]. It is postulated that lower body subcutaneous adipose tissue functions as a "metabolic sink". In support of this, we previously demonstrated that bilateral removal of inguinal white adipose tissue (IWAT) results in glucose intolerance and decreased muscle insulin sensitivity. In the present study, we extend previous research to examine if progressive peripheral adipose tissue removal would produce dose-dependent deterioration in glucose tolerance and dose-dependent increases in non-adipose tissue lipid accumulation. The results of this study demonstrate that 20%-80% removal of subcutaneous adipose tissue in 13 week HFD mice induced a dose-dependent decrease in glucose tolerance with an associated decrease in basal non-insulin stimulated femoral muscle pAkt/Akt. These surgery-mediated outcomes demonstrate subcutaneous adipose tissue is protective via two distinct actions.

We previously demonstrated that lower body subcutaneous adipose tissue serves to protect muscle from excessive triglyceride deposition and ultimately helps to preserve whole body glucose homeostasis [366]. We concluded that systemic glucose intolerance following inguinal adipose tissue removal resulted from an associated decrease in femoral muscle insulin sensitivity. In the present study, we utilized dose-dependent removal of subcutaneous adipose tissue to investigate if incremental adipose tissue removal resulted in cumulative metabolic dysregulation. Unilateral, Bilateral and All subcutaneous adipose tissue removal resulted in ~20%, 40% or 80% decrease in total subcutaneous depots. The present study supports and extends our previous observations [366] that removal of subcutaneous adipose tissue disrupted systemic glucose regulation. This was best demonstrated in 13-week HFD mice, where increasing fat removal was associated with progressive worsening of glucose tolerance compared with 13-week HFD controls.

We next examined whether the metabolic sink concept could explain the progressive decline in glucose tolerance associated with incremental adipose tissue removal. In doing so, we first excluded several additional factors known to play a role in systemic glucose regulation. Specifically, incremental removal of subcutaneous adipose tissue in 13-week HFD mice was not associated with progressive increases in food intake, adipose depot compensation, adipokines, cytokines or liver triglyceride. This demonstrates that the progressive decrease in systemic glucose tolerance induced by adipose tissue removal are likely not due to factors such as 1) incremental increases in total dietary intake of carbohydrates, 2) compensatory growth of other adipose depots that are associated with metabolic disease, or 3) cumulative deposition of liver triglycerides which could additively contribute to exacerbation of glucose intolerance.

Our previous experiment demonstrated that subcutaneous fat removal does cause ectopic fat accumulation in proximal muscle [366]. We predict this occurs because of reduced subcutaneous adipose tissue storage; hence the "metabolic sink" for lipid filling is taken away [386]. Previously we demonstrated an increase in femoral muscle triglyceride as early as 5 weeks post-bilateral IWAT removal in both CHOW and HFD mice [366]. Although this outcome to some extent occurred in the recent experiment, this association in general was not consistently or adequately supported in the current study. Indeed, the incremental decreases in glucose tolerance associated with progressive subcutaneous adipose tissue removal in 13-week HFD mice occurred without differences in femoral muscle triglyceride concentration. In support of this femoral muscle carnitine palmityltransferase (CPT1), involved in fatty acid utilization via beta-oxidation and lipoprotein lipase (LPL), which facilitates uptake of lipids into cells [384], was not incrementally different among surgery groups of 13 weeks HFD mice. Perhaps a HFD duration of 13 weeks is too excessive for the current systematic evaluation. This diet duration may not be conducive in evaluating surgery-induced differences in muscle triglyceride concentration because all groups were excessively high.

HFD-induced muscle triglyceride accumulation is associated with defects in muscle insulin signaling [387, 388] subsequently leading to insulin resistance [388-390]. We postulated that subcutaneous adipose tissue removal-induced exacerbation of systemic glucose intolerance would be linked to insulin resistance in femoral muscle. Therefore, pAkt/Akt was measured to assess insulin sensitivity [391-393], both insulin-stimulated and non-insulin-stimulated. We previously demonstrated in 5 week HFD mice that the systemic glucose intolerance induced by subcutaneous adipose tissue removal was associated with exacerbated muscle insulin resistance [366]. We therefore postulated that this association would also occur in 13-week HFD mice and

be commensurate to the amount of subcutaneous adipose tissue removed. However, muscle insulin sensitivity, with the exception All group, was similar among surgery groups. It is worth noting that this similarity among groups was due to the inherent lack of femoral muscle insulin response in 13-week HFD mice, including control mice without fat removed. Hence, as previously suggested a HFD duration of 13 weeks may be too excessive because the femoral muscle had no pAkt/Akt response to exogenous insulin. Unlike the other surgery groups and sham control, femoral muscle insulin sensitivity was increased in the All group. We postulate this insulin hypersensitivity may occur because of the additional bilateral removal of the dorsal subcutaneous adipose, but this is yet to be elucidated. Although surgery did not alter insulin-stimulated pAkt/Akt, there was a suppressed pAkt/Akt response in the non-insulin stimulated (saline-injected) 13 week HFD mice. Here increases in fat removal resulted in incremental decreases in basal insulin sensitivity. Some studies suggest that increases in basal pAkt in muscle are associated with muscle insulin resistance [394]. Others support that diminished basal pAkt [395] or decreased basal glucose uptake [396] in muscle is associated with metabolic dysregulation independent of the insulin interaction. We speculate that these insulin-independent decreases in pAkt/Akt are related to HFD-induced decreases in muscle contraction mediated AMPK [397, 398] because AMPK is demonstrated to activate the insulin signaling pathway independent of insulin binding [399-402].

Taken together our data demonstrates that subcutaneous adipose tissue is protective. The current study, however, supports that the adipose tissue removal alterations in glucose tolerance cannot fully be attributed to muscle insulin action. Many different systems can account for the glucose excursion, including decreased pancreatic insulin production, unknown adaptations in intra-abdominal adipose tissue, non-Akt-dependent glucose uptake in muscle, and elimination of

adipose tissue-specific glucose and lipid disposal. Randal first described a nutrient-mediated influence on fuel metabolism, thus increased circulating lipids due to prolonged high fat feeding could account for reductions in glucose utilization. The exact interplay of glucose homeostatic mechanisms remains to be established.

In conclusion, prior research supports that visceral adiposity may be a consequence of dysregulated lipid storage within the subcutaneous adipose depot. This seems particularly intuitive because body fat is primarily comprised of subcutaneous adipose tissue. Hence, obesity-induced glucose dysregulation may be due to limitations in the ability of the protective "metabolic sink" to store surplus lipids, leading to ectopic deposition into insulin sensitive tissues. Our data supports that subcutaneous adipose tissue plays a fundamental role in glucose homeostasis in a dose-dependent manner. Subcutaneous adipose tissue protects systemic glucose tolerance via protecting muscle insulin sensitivity by way of controlling lipid infiltration. Here we extend our previous studies to demonstrate that progressive peripheral adipose tissue removal produces a dose-response deterioration in systemic glucose tolerance and muscle non-insulin dependent pAkt/Akt stimulation. Remarkably, these events occur independently of one another. Therefore, we extend the "metabolic sink" postulate to include a partitioning of the reservoir, whereas removal of individual compartments result in an incremental worsening of metabolic dysregulation.

Tables

Table 4.1: Post Surgery Adipose Tissue Removal, Food Intake, and Body Weight. Adipose tissue removal accounted for ~20%, 40%, and 80% total subcutaneous adipose tissue. Food intake and body weight were not significantly different among surgery groups, with the exception of 13 week HFD All removal being slightly smaller.

		Ctrl	Uni IngX	Bi IngX	All	p-value†
Adipose Tissue Removed (mg)						
Chow	5 wks	NA	119.7 ± 11.4^{a}	219.4 ± 14.6^{b}	369.1 ± 21.9^{c}	0.0001
HFD	5 wks	NA	104.0 ± 6.5^{a}	254.4 ± 24.8^{b}	401.5 ± 35.0^{c}	0.0001
	13 wks	NA	100.0 ± 6.7^{a}	211.7 ± 9.5^{b}	308.6 ± 24.6^{c}	0.0001
Average Weekly Food Intake (kcal)						
Chow	5 wks	72.4 ± 2.5	76.7 ± 4.6	74.7 ± 1.8	75.4 ± 3.1	0.81
HFD	5 wks	140.2 ± 3.3	143.9 ± 4.3	143.0 ± 3.6	146.0 ± 8.8	0.90
	13 wks	96.9 ± 2.5	95.7 ± 2.8	93.4 ± 1.9	94.3 ± 2.0	0.72
Final Body Weight (g)						
Chow	5 wks	28.6 ± 0.5	29.2 ± 0.6	29.0 ± 0.7	28.2 ± 0.6	0.69
HFD	5 wks	34.3 ± 1.5	34.7 ± 1.4	35.7 ± 1.6	33.0 ± 1.0	0.58
	13 wks	45.8 ± 1.0^{a}	43.4 ± 1.5^{a}	43.0 ± 1.0^{a}	42.1 ± 0.9^{b}	0.14

†Single Factor ANOVA (factor: surgery group)
Values with a different letter are significantly different from each other ($p<0.01$)

Table 4.2: Fasting Glucose and Insulin, Plasma Adipokines, and Liver TGs. Fasting glucose and insulin were not significantly different between surgery groups, except 5 week HFD bilateral inguinal removal had higher basal insulin levels. Resistin was lower in 5 week CHOW and 13 week HFD All removal groups. IL-6 was higher in 5 week CHOW Bi IngX and lower in 13 week HFD All groups compared to their respective controls. Liver TGs were lower in 13 week HFD All removal. All other circulating factors were not significantly different.

		Ctrl	Uni IngX	Bi IngX	All	p-value†
Fasting Glucose mg/dl						
Chow	5 wks	112 ± 7.1	115 ± 9.8	120 ± 4.0	110 ± 6.7	0.79
HFD	5 wks	122 ± 6.4	132 ± 4.5	137 ± 5.0	126 ± 9.1	0.38
	13 wks	133 ± 9.9	140 ± 6.9	134 ± 8.1	137 ± 5.9	0.92
Fasting Insulin ng/dl						
Chow	5 wks	0.5 ± 0.06	0.6 ± 0.07	0.5 ± 0.04	0.5 ± 0.05	0.68
HFD	5 wks	2.7 ± 0.44	3.5 ± 0.41	4.7 ± 0.68*	3.1 ± 0.49	0.09
	13 wks	3.5 ± 0.54	2.6 ± 0.56	2.6 ± 0.52	2.6 ± 0.30	0.44
Leptin						
Chow	5 wks	723 ± 177	1,273 ± 561	1,329 ± 247	646 ± 51	0.32
HFD	5 wks	16,548 ± 2,023	15,966 ± 1,508	22,550 ±3,719	14,112 ± 5,077	0.37
	13 wks	26,106 ± 4,956	23,625 ± 1,408	25,859 ± 5,715	16,742 ± 5,736	0.50
Resistin						
Chow	5 wks	2,038 ± 93	1,949 ± 177	1,998 ± 95	1,625 ± 99**	0.10
HFD	5 wks	2,719 ± 339	3,228 ± 519	3,018 ± 489	2,379 ± 276	0.53
	13 wks	2,016 ± 382	1,446 ± 339	1,761 ± 215	1,053 ± 212*	0.16
PAI1 tot						
Chow	5 wks	1,000 ± 319	1,559 ± 214	1,773 ± 384	1,488 ± 565	0.57
HFD	5 wks	2,331 ± 296	2,696 ± 348	4,733 ± 1,311	2,201 ± 436	0.09
	13 wks	5,045 ± 1,610	4,914 ± 901	5,631 ± 1,703	3,567 ± 691	0.72
IL-6						
Chow	5 wks	2.4 ± 0.7	2.6 ± 0.4	4.7 ± 0.7*	4.4 ± 1.0	0.06
HFD	5 wks	7.5 ± 0.4	9.5 ± 2.7	7.1 ± 1.4	6.6 ± 3.8	0.80
	13 wks	7.8 ± 0.9	8.5 ± 2.3	6.3 ± 0.7	4.3 ± 0.6**	0.20
Liver TG (mg/g)						
Chow	5 wks	21.0 ± 1.1	21.5 ± 1.1	20.7 ± 1.5	25.6 ± 1.6	0.13
HFD	5 wks	133.8 ± 20.6	126.7 ± 11.2	132.5 ± 21.9	99.9 ± 15.4	0.62
	13 wks	293.7 ± 13.4	291.4 ± 27.2	304.7 ± 19.1	254.3 ± 7.7*	0.36

†Single Factor ANOVA (factor: surgery group)
*post hoc $p<0.05$ compared with control group, ** $p<0.01$

Figures

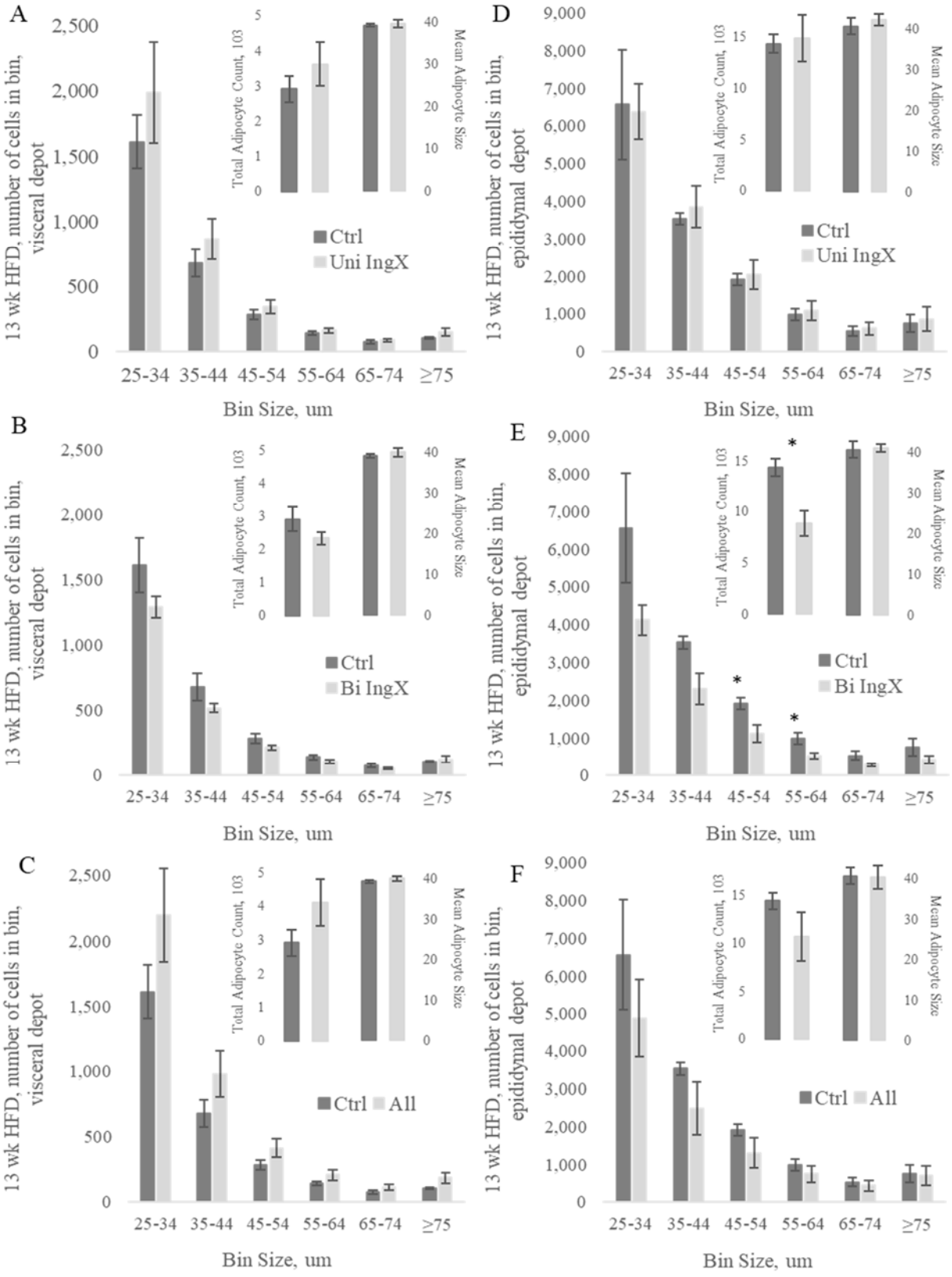

A
13 wk HFD, number of cells in bin, visceral depot
Total Adipocyte Count, 10^3
Mean Adipocyte Size
Ctrl
Uni IngX
25-34 35-44 45-54 55-64 65-74 ≥75
Bin Size, um
B
13 wk HFD, number of cells in bin, visceral depot
Ctrl
Bi IngX
Bin Size, um
C
13 wk HFD, number of cells in bin, visceral depot
Ctrl All
Bin Size, um
D
13 wk HFD, number of cells in bin, epididymal depot
Ctrl
Uni IngX
Bin Size, um
E
13 wk HFD, number of cells in bin, epididymal depot
*
Ctrl
Bi IngX
Bin Size, um
F
13 wk HFD, number of cells in bin, epididymal depot
Ctrl All
Bin Size, um

Figure 4.1: 13 week HFD Mice Cell Size and Abundance. Intra-abdominal adipocyte size distribution. A-C) Visceral adipocyte size and distribution did not change when comparing individual surgery groups with control mice. Amount in bin (measured in um), total adipocyte number, and mean adipocyte size are not significantly different. D-F) Epididymal adipocytes were significantly different for Bi IngX compared to controls. They had less total adipocytes and less between the sizes 45-64 um for the intra-abdominal depot. Uni IngX and All groups were not different from controls.

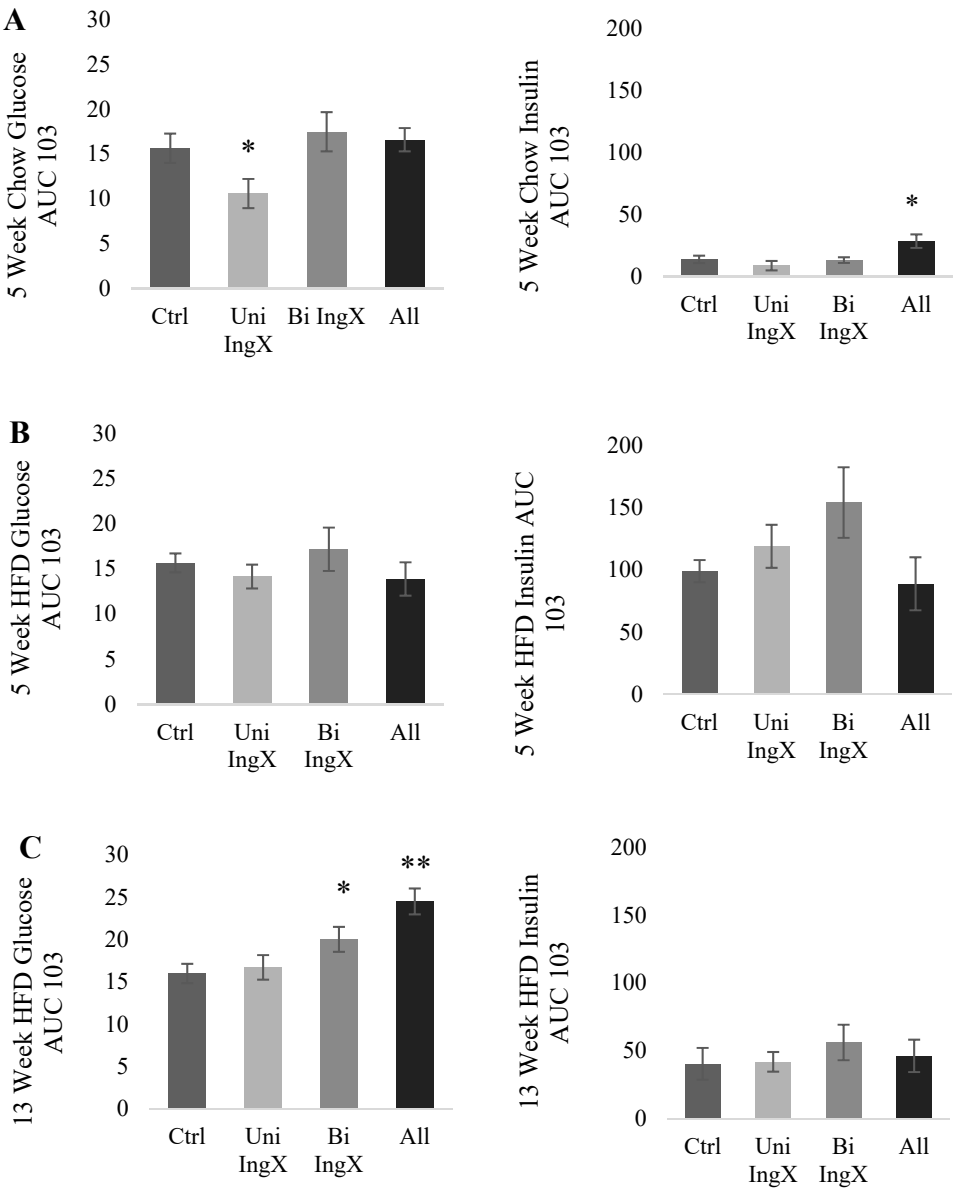


Figure 4.2: Glucose and Insulin Response to GTT Area Under the Curve. Mice participated in a 2-hour glucose challenge one week prior to termination. A 1.5 g/kg bolus of dextrose was injected (ip) and glucose measurements were made at 0, 15, 30, 45, 60, and 120 minutes. Insulin measurements were made at 0, 15, and 120 minutes. Area under the curve (AUC) represents the total glucose/insulin excursion from 0-120 minutes. A) 5 Week Chow mice had lower glucose in the Uni IngX group and higher insulin in the All group compared with controls. B) 5 Week HFD mice did not differ significantly from controls for either measurement. C) 13 Week HFD mice exhibited a dose-response effect of the glucose load with increasing subcutaneous fat removed.

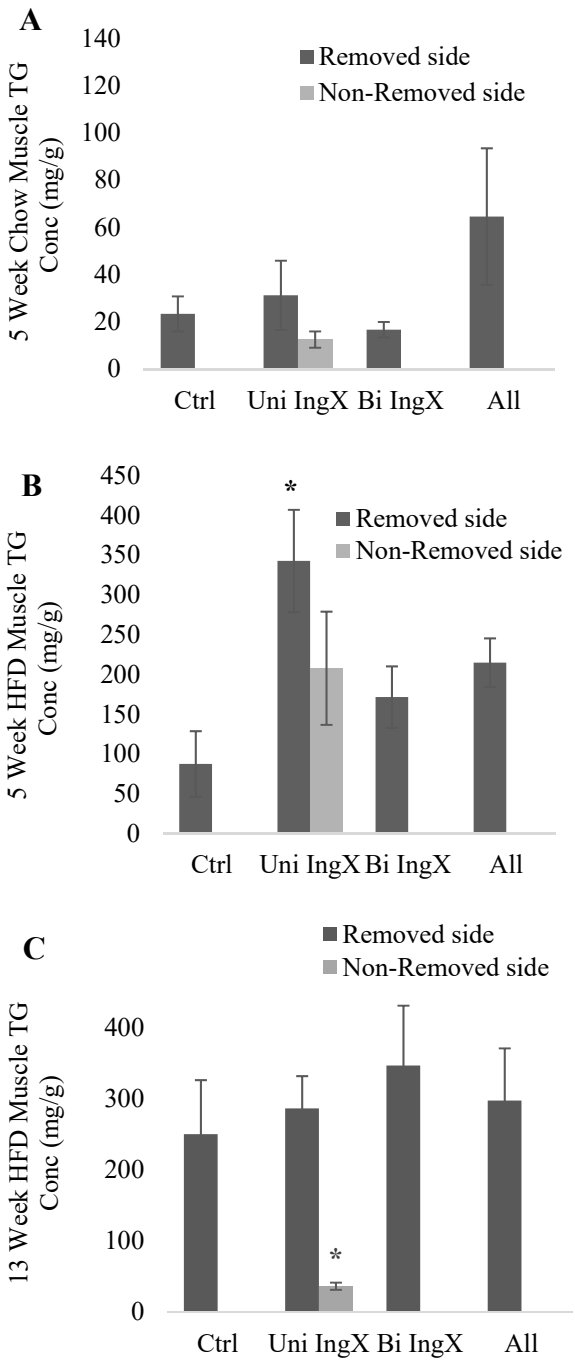


Figure 4.3: Femoral Muscle Triglyceride Concentration. Intramuscular triglycerides were measured in mg/g. For unilateral inguinal removal, muscles were measured separately for removed and non-removed side. A) 5 Week Chow mice did not differ from controls. B) All 5 Week HFD surgery groups had 3+ fold higher muscle TGs than control. Only unilateral removed side was significant. C) 13 Week HFD mice were excessively high for all groups, including controls. Interestingly, non-removed side of unilateral group was significantly lower than its removed side.

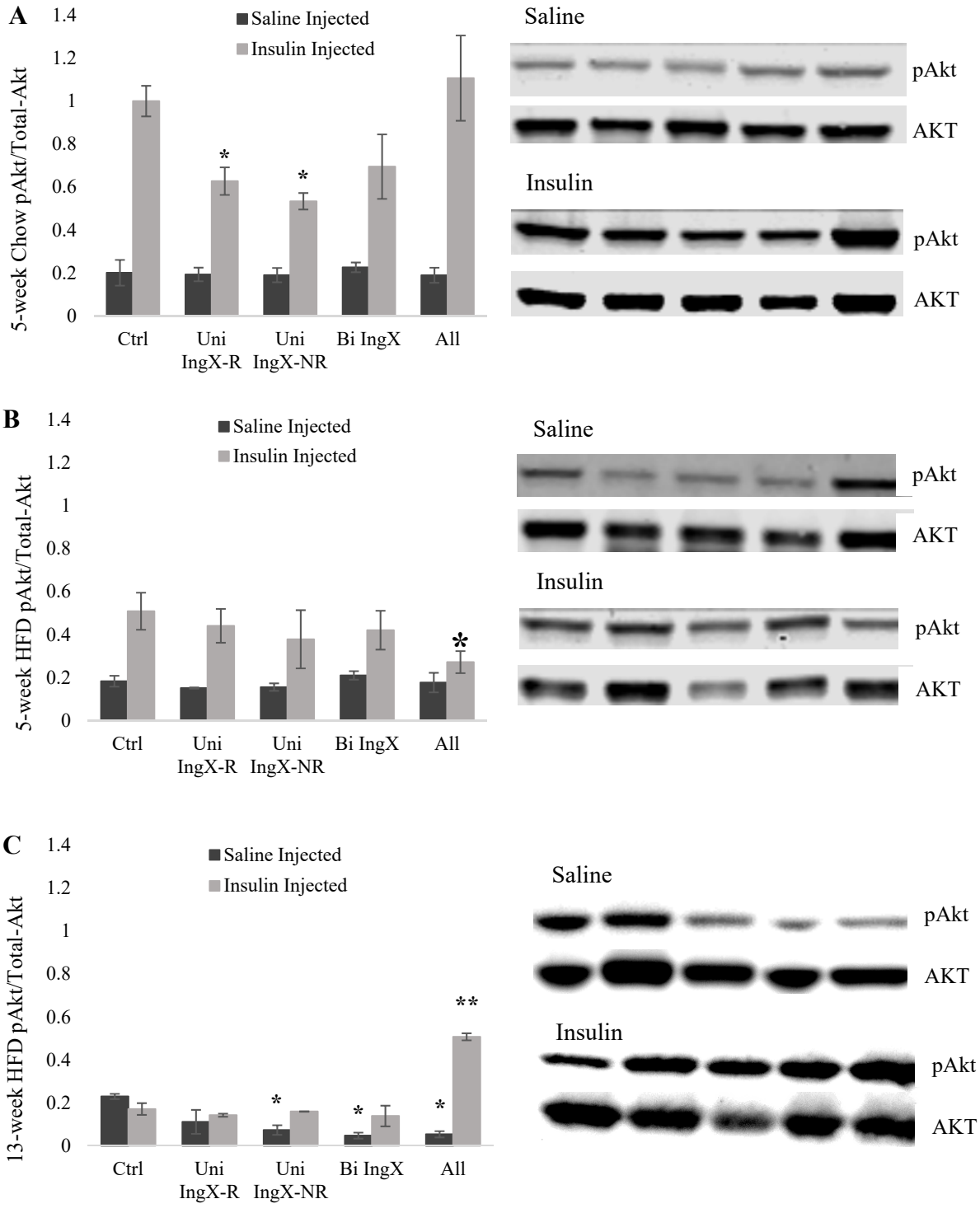
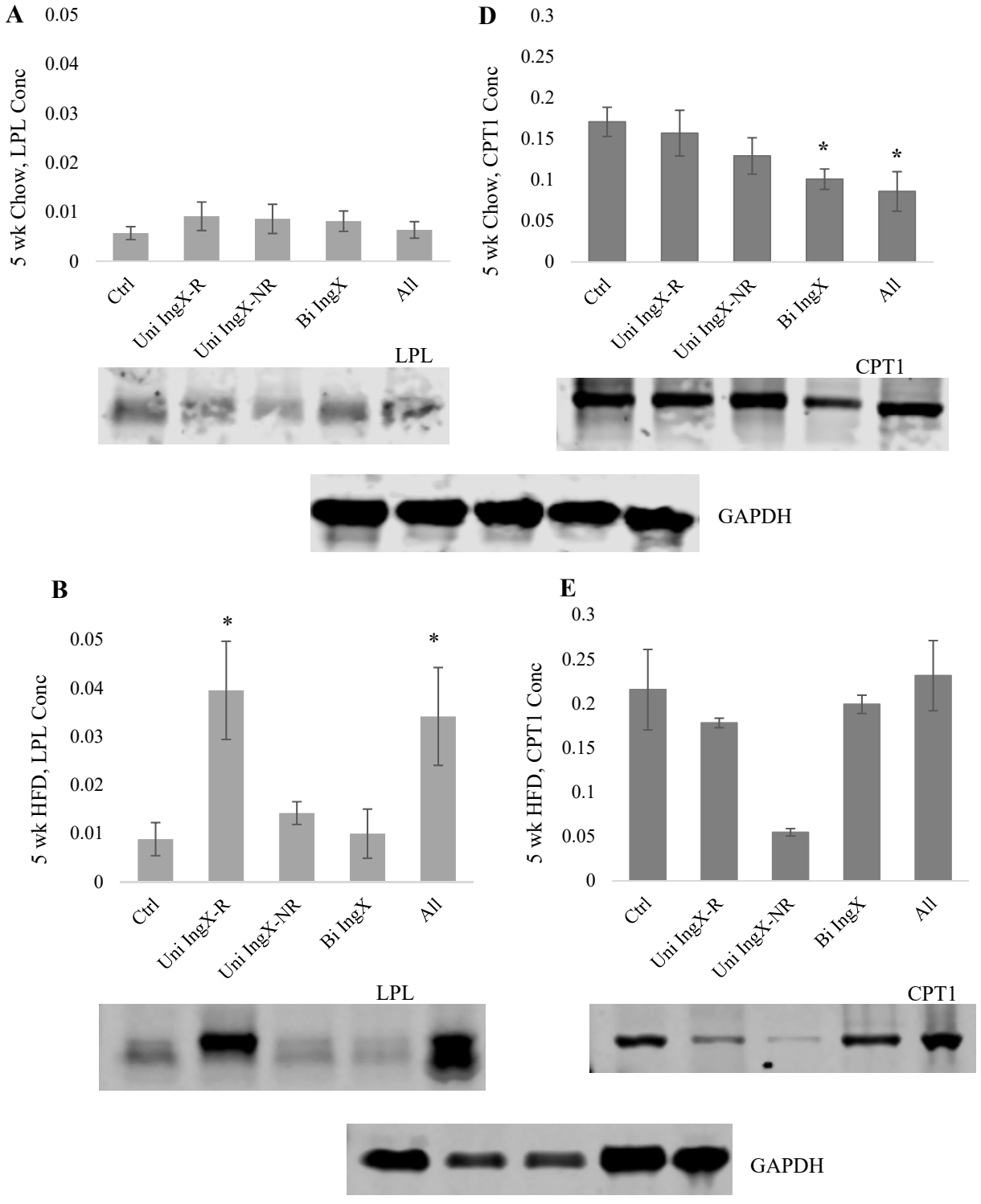

A
5-week Chow pAkt/Total-Akt
Saline Injected
Insulin Injected
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl
Uni IngX-R
Uni IngX-NR
Bi IngX
All
Saline
pAkt
AKT
Insulin
pAkt
AKT
B
5-week HFD pAkt/Total-Akt
Saline Injected
Insulin Injected
Ctrl
Uni IngX-R
Uni IngX-NR
Bi IngX
All
Saline
pAkt
AKT
Insulin
pAkt
AKT
C
13-week HFD pAkt/Total-Akt
Saline Injected
Insulin Injected
Ctrl
Uni IngX-R
Uni IngX-NR
Bi IngX
All
Saline
pAkt
AKT
Insulin
pAkt
AKT

Figure 4.4: Femoral Muscle Insulin Sensitivity. The ratio of phosphorylated Akt to total Akt was used to measure muscle insulin sensitivity. Approximately 15 minutes prior to termination, mice were injected (ip) with either insulin or saline. For unilateral inguinal removal, muscles were measured separately for removed and non-removed side. Corresponding western blots are shown next to each graph. A) 5 Week Chow mice had significantly lower insulin response in the unilateral group for both muscles. B) 5 Week HFD mice decreased slightly from controls and was significant in the All removal group. C) 13 Week HFD mice did not decrease in their response to the insulin injection among the surgery groups. However, the All group had a hypersensitive response to insulin. Basal (saline) muscle insulin activity did decrease in a dose-dependent pattern with increasing amounts of fat removed.


A
5 wk Chow, LPL Conc
0.05
0.04
0.03
0.02
0.01
0
Ctrl
Uni IngX-R
Uni IngX-NR
Bi IngX
All
LPL
D
5 wk Chow, CPT1 Conc
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
Ctrl
Uni IngX-R
Uni IngX-NR
Bi IngX
All
CPT1
GAPDH
B
5 wk HFD, LPL Conc
0.05
0.04
0.03
0.02
0.01
0
*
*
Ctrl
Uni IngX-R
Uni IngX-NR
Bi IngX
All
LPL
E
5 wk HFD, CPT1 Conc
0.3
0.25
0.2
0.15
0.1
0.05
0
Ctrl
Uni IngX-R
Uni IngX-NR
Bi IngX
All
CPT1
GAPDH

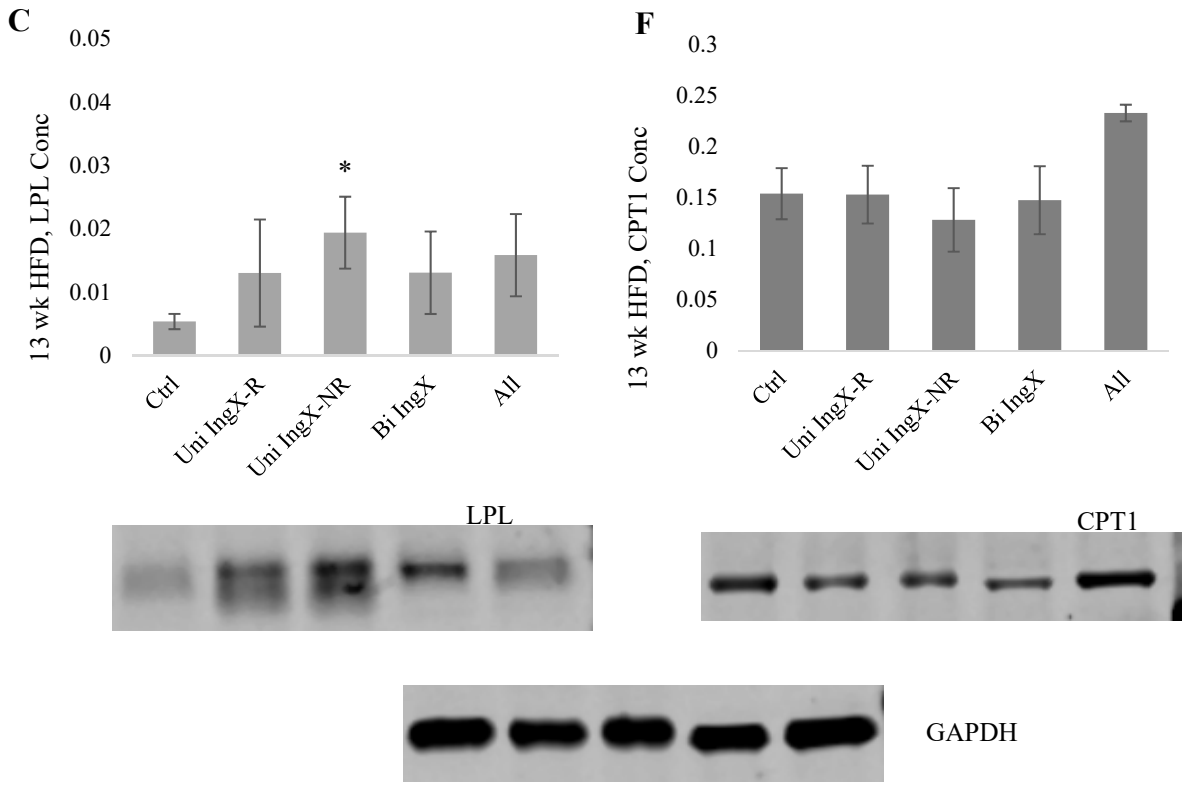


Figure 4.5: Markers of Fatty Acid Oxidation in Femoral Muscle. Lipoprotein lipase (LPL) was measured as an indicator of fatty acid uptake with the femoral muscle tissue. Carnitine palmitoyltransferase 1 (CPT1) was measured as an indicator of beta-oxidation within the femoral muscle cells. A, D) LPL in 5 week CHOW mice did not differ between surgery groups, while CPT1 declined significantly from control levels with bilateral inguinal and All removals. B, E) 5 week HFD mice had a significant spike in muscle LPL activity with unilateral (removed side) and All removal, however CPT1 levels did not differ significantly. C,F) 13 week HFD mice were not different from controls in either LPL or CPT1 activity, with the exception of increased LPL with unilateral fat removed (non-removed side).

Table 4.3: Adipose Tissue Depot Mass at Termination. All masses are reported in grams. EWAT=epididymal white adipose tissue, VWAT=visceral white adipose tissue, PWAT=perirenal white adipose tissue, IWAT=inguinal white adipose tissue, BAT=brown adipose tissue, DWAT=dorsal white adipose tissue. Values with a different letter are significantly different from each other ($p<0.05$). Excised depots are proportionally lower than control mice, thus there was no significant regrowth of fat during the study period. Visceral adipose tissue was higher in unilateral removal mice on CHOW diet for 5 weeks. Overall, there was no adipose tissue compensation in non-excised depots.

		Ctrl	Uni IngX	Bi IngX	All	p-value
Chow						
	N	10	10	10	10	
	EWAT	0.38 ± 0.03	0.48 ± 0.05	0.46 ± 0.05	0.43 ± 0.03	0.36
	IWAT	0.25 ± 0.02[a]	0.16 ± 0.02[b]	0.04 ± 0.01[c]	0.06 ± 0.01[c]	<0.0001
5 wks	VWAT	0.16 ± 0.01[a]	0.23 ± 0.03[b]	0.20 ± 0.02[a,b]	0.17 ± 0.01[a,b]	0.049
	BAT	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.69
	DWAT	0.25 ± 0.02[a]	0.29 ± 0.02[a]	0.26 ± 0.03[a]	0.08 ± 0.02[b]	<0.0001
	PWAT	0.13 ± 0.01	0.18 ± 0.02	0.18 ± 0.03	0.16 ± 0.02	0.26
HFD						
	N	10	10	10	10	
	EWAT	1.60 ± 0.19	1.73 ± 0.07	1.74 ± 0.18	1.40 ± 0.14	0.38
	IWAT	1.03 ± 0.12[a]	0.70 ± 0.05[b]	0.40 ± 0.08[c]	0.28 ± 0.05[c]	<0.0001
5 wks	VWAT	0.68 ± 0.08	0.77 ± 0.07	0.80 ± 0.13	0.62 ± 0.07	0.50
	BAT	0.32 ± 0.03	0.43 ± 0.04	0.39 ± 0.06	0.40 ± 0.04	0.37
	DWAT	0.98 ± 0.11[a]	1.18 ± 0.10[a]	1.17 ± 0.14[a]	0.47 ± 0.07[b]	<0.0001
	PWAT	0.75 ± 0.09	0.87 ± 0.05	0.88 ± 0.10	0.70 ± 0.08	0.33
	N	10	9	9	9	
	EWAT	2.46 ± 0.17	2.36 ± 0.10	2.39 ± 0.06	2.31 ± 0.10	0.82
	IWAT	1.41 ± 0.07[a]	0.91 ± 0.09[b]	0.32 ± 0.04[c]	0.50 ± 0.17[c]	<0.0001
13 wks	VWAT	1.02 ± 0.07	0.87 ± 0.08	0.93 ± 0.06	0.88 ± 0.06	0.39
	BAT	0.51 ± 0.03	0.45 ± 0.06	0.46 ± 0.03	0.48 ± 0.04	0.76
	DWAT	1.26 ± 0.08[a]	1.16 ± 0.09[a]	1.22 ± 0.08[a]	0.57 ± 0.08[b]	<0.0001
	PWAT	1.08 ± 0.06	1.00 ± 0.09	1.01 ± 0.07	0.84 ± 0.11	0.25

CHAPTER 5: DEVELOPING A MUSCLE LIPID PROFILE ASSOCIATED WITH DIET-INDUCED OBESITY AND INSULIN RESISTANCE[9]

Summary

Skeletal muscle accounts for ~75% of post-prandial blood glucose disposal and plays a significant role in systemic glucose regulation. Increased triglyceride concentration in muscle tissue, as seen with obesity, is associated with inhibition of insulin action and decreased glucose uptake. Muscle insulin resistance has been suggested as the primary defect in the pathogenesis of obesity-related diseases like type 2 diabetes. Here we use liquid chromatography paired with mass spectrometry (LCMS) to identify patterns of lipid species in femoral muscle of mice associated with diet-induced glucose dysregulation. Mice were fed a standard CHOW diet for 5 weeks or HFD for 5 or 13 weeks. Femoral muscle triglyceride concentration increased and insulin sensitivity decreased for each cohort accordingly. HFD mice had increased caloric intake, body weight, liver triglycerides, adipose depots and circulating insulin compared to CHOW. 217 lipid species were identified and quantified based on analysis of variance and fold change for main diet effect of relative abundance values. CHOW diet was associated with long-chain unsaturated triglycerides, phosphatidylserine, and phosphatidylinositol. 5 or 13 weeks on HFD was associated with systemic and muscle insulin resistance, along with shorter chain saturated

[9] The aim of this work was to conduct an experimental study to determine a muscle lipid signature associated with diet-induced insulin resistance. Our lab carried out controlled experiments in mice with CHOW and HFD for 5 and 13 weeks. We utilized the CSU core facility, Proteomics and Metabolomics Facility, who carried out LCMS for the project. This article outlines a cluster of lipids associated with healthy and insulin resistance femoral muscles.

This chapter includes an in-preparation manuscript for submission as original research. (Authors: A.D. Booth, A.M. Magnuson, C Broekling, and M.T. Foster). My contributions to this experiment included housing, general care, food and weight measurements, GTT, lab assays, tissue collection, and compound annotation. My contribution to this manuscript included initial text preparation, data analysis, data calculations, table and figure preparation, and statistics.

Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 5.1.

triglycerides, diacylglycerides, and sphingomyelin in femoral muscle. Future lipid studies should consider groups of compounds and not individual species.

Introduction

According to the World Health Organization, excessive adiposity leading to weight gain and obesity is a global epidemic driven primarily by excessive energy intake [403]. Consequently, increasing weight status links with comorbidities such as, but not limited to, type 2-diabetes, and insulin resistance [404, 405]. Insulin resistance is a fundamental characteristic of type-2-diabetes, thus plays a major role in the pathogenesis of the disease [406, 407]. It is suggested that during the progression of obesity, skeletal muscle insulin resistance is the initiating or primary defect associated with declines in glucose tolerance, well before pancreatic deficiency develops [408, 409]. Skeletal muscle is an essential site for insulin-mediated postprandial glucose uptake [410], hence metabolic events that interfere with muscle insulin sensitivity will also likely lead to interruptions in systemic glucose homeostasis.

It is well established that muscle insulin resistance is highly associated with obesity and affiliated type-2-diabetes [408, 409, 411]. Further lipid accumulation within muscle, as occurs with Western diet high fat and sugar intake, is positively correlated with its insulin resistance [412-414]. Because of this, much focus has been placed on linking and understanding the role of muscle lipid accumulation in the development of IR. Numerous reports in both rodents [415] and humans [390, 416] suggest lipids, for example triglycerides, link to muscle insulin resistance. In opposition, others demonstrate increases in muscle lipid triglycerides do not result in aberrant insulin sensitivity [366, 417-419]. This contradiction shifted the field to decrease the relevance of triglyceride amounts and focus more on dysregulation and storage of lipid intermediates that

may interfere with muscle insulin signaling. Diacylglycerols and ceramides emerged as the toxic lipid intermediates that link increased triglyceride deposition to insulin resistance [420, 421]. Convincing evidence in both cell and animal studies strongly support that elevated diacylglycerols [420, 422] and ceramides [421, 423] are associated with impaired insulin signaling and insulin resistance. Intracellular accumulation of these lipid intermediates is also associated with chronic inflammation and numerous other metabolic diseases [422, 423]. Previous studies, however, were limited because only a few selected lipids were analyzed instead of a whole lipid profile. Evaluation of a full spectrum lipid species could give a comprehensive description of numerous other lipids that play a role in muscle insulin resistance.

A lipidome refers to a global set of lipid species within a system and lipidomics is the process of identifying such species to identify significant patterns and/or contributors to an outcome. Omics are often used to examine differences in species populations given exposure to a modifiable treatment. Isolation of lipids from muscle tissue followed by liquid chromatography and mass spectrometry (LCMS) is a powerful technique in chemical analytics that can identify and quantify individual compounds within a sample [424]. Is this study, we will utilize lipidomics to characterize femoral muscle lipid species that correlate with a Western HFD in mice. The study contains three groups of mice, CHOW or HFD fed mice for 5 weeks and a HFD fed 13-week mouse group. These groups will allow us to analyze differential muscle lipidomics between CHOW and HFD mouse, but will also extend to determine if a longer HFD duration continues to change lipid species abundance compared with a shorter duration. Results from this current study may suggest lipid signatures, and not individual lipid components, better associate to measure of muscle insulin resistance.

Methods

Mice and Housing

Male C57BL/6 mice were purchased at 3 months of age from Jackson Laboratory (Bar Harbor, Maine) and were allowed to acclimate for one week. Mice were individually housed under controlled conditions (12:12 light-dark cycle, 50–60% humidity, and 25° C) and initially had ad libitum access to standard CHOW diet (Envigo Teklad 6% fat 7002, Madison, WI) with unlimited water. Following a baseline glucose tolerance test (GTT) , they were then grouped according to GTT and weight into a standard CHOW (5 week only) or Western (high-fat, high-sugar; 21% milk fat and 34% sucrose (Envigo TD.08811); 5 and 13 week) diet group. Weekly body mass and food intake were monitored and recorded. Procedures were reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Glucose Tolerance Test (GTT)

A second GTT occurred one week prior to respective study termination, at 4 weeks for CHOW and HFD group and at 12 weeks for the other HFD group. The procedure followed a 6 hour fast that started at the light on cycle. Blood was collected from the tail vein and glucose concentration was determined using a Freestyle Lite Glucometer (Abbott, Abbott Park, IL). After fasting blood glucose was collected (time point 0), mice received a 1.5 g/kg dextrose injection in the intraperitoneal cavity and blood glucose was measured from tail vein blood samples at 15, 30, 45, 60 and 120 minutes post-injection. Additional blood was collected at time points 0, 15 and 120 minutes for insulin measurements. Insulin concentrations were assessed using an Ultra-Sensitive Mouse Insulin Elisa Kit (Crystal Chem, Downers Grove, IL).

Termination

Mice were terminated after 5 weeks on CHOW diet and either 5 or 13 weeks on HFD. Final body weights were collected and mice were fasted for 4 hours prior to tissue collection. Mice were first anesthetized with isoflurane and systemic blood was collected via decapitation. Serum was separated and stored at – 80°C. Six white adipose tissue (epididymal, inguinal, visceral, dorsal, perirenal) and interscapular brown adipose tissue depots were collected, along with liver and bilateral femoral muscles. All tissues were snap-frozen and stored at – 80°C.

Triglyceride Concentration

Skeletal muscle and liver lipids were extracted using the procedure of Bligh and Dyer [343]. Muscle and liver triglyceride concentration (Sigma Chemical Co, St. Louis, MO) were determined enzymatically using commercially available kits.

Lipid Extraction

Approximately 20 mg of muscle tissue was homogenized in a glass homogenizer with 1.5 ml of 2:1 Chloroform:Methanol and then brought to 4 ml using the same ratio. The mixture was poured through a 2V grade qualitative 12.5 cm Whatman filter into a clean 10 ml glass tube. The volume in the tube was again brought up to 4 ml with the same 2:1 solution as above. One ml of water was added to the tube, vortexed for 20 seconds, and then centrifuged for 10 minutes at 2500 rpm. The top non-lipid portion was removed and the lower lipid-containing layer was dried under nitrogen.

UPLC-MS

Extracts were resuspended in 100 uL of 2:1 Chloroform:Methanol. 3 µL of extract was injected twice (n=2 replicates) onto a Waters Acquity UPLC system in discrete, randomized blocks, and separated using a Waters Acquity UPLC CSH Phenyl Hexyl column (1.7 µM, 1.0 x 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (Acetonitrile, 0.1% formic acid). Injections were made in 100% A, held at 100% A for 1 min, ramped to 98% B over 12 minutes, held at 98% B for 3 minutes, and then returned to starting conditions over 0.05 minutes and allowed to re-equilibrate for 3.95 minutes, with a 200 µL/min constant flow rate. The column and samples were held at 65 °C and 6 °C, respectively. The column eluent was infused into a Waters Xevo G2 TOF-MS with an electrospray source in positive mode, scanning 50-2000 m/z at 0.2 seconds per scan, alternating between MS (6 V collision energy) and MS^E mode (15-30 V ramp). Calibration was performed using sodium iodide with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source temp at 150 °C, and nitrogen desolvation temp at 350 °C with a flow rate of 800 L/hr.

Muscle Insulin Sensitivity and FA Handling

Following a four-hour fast, mice were either injected (ip) with saline or insulin and then terminated ~15 minutes later. Muscle insulin sensitivity was determined by the ratio of phosphoralated Akt (Ser 473) to total Akt. Protein measurements were made using gel-based Western Blot procedures with both Odyssey Clx (Licor) and Chemiluminescence (antibodies: Cell Signaling #4060 P-Akt and #4691 Akt pan). Quantification was done in Image Studio Lite (Licor) and ImageJ software [383]. Two proteins were measured for factors of fatty acid utilization via western blot using Odyssey Clx (Licor). This included lipoprotein lipase (LPL) for

fatty acid cleavage outside of the cell and carnitine-palmitoyltransferase 1 (CPT1) for transport into the mitochondrial matrix for beta-oxidation (antibodies: abcam #21356 LPL and #134988 CPT1B). The density of these proteins were normalized to GAPDH (Cell Signaling #2118).

Data Analysis and Statistics

For each sample, raw data files were converted to .cdf format, and matrix of molecular features as defined by retention time and mass (m/z) was generated using XCMS software in R [425] for feature detection and alignment. Raw peak areas were normalized to total ion signal in R, outlier injections were detected based on total signal and PC1 of principle component analysis, and the mean area of the chromatographic peak was calculated among replicate injections (n=2). Features were grouped based on a novel clustering tool, RAMClustR [426], which groups features into spectra based coelution and covariance across the full dataset, whereby spectra are used to determine the identity of observed compounds in the experiment. Compounds were annotated based on spectral matching to in-house, NISTv12, predicted MS1 and retention time spectral databases using RAMsearch [427] and the Metlin compound and spectral database. The peak areas for each feature in a spectrum were condensed via the weighted mean of all features in a spectrum into a single value for each compound. Analysis of variance was conducted on each compound using the aov function in R, and p-values were adjusted for false positives using the Bonferroni-Hochberg method in the p.adjust function in R [428]. PCA was conducted on mean-centered and pareto variance-scaled data using the pcaMethods package in R.

Comparisons among cohorts were made using 1-way analysis of variance for food intake, body weight, adiposity measures, glucose and insulin measures, liver and muscle TG

concentrations, muscle insulin sensitivity, and protein concentrations. Pairwise post-hoc analysis was performed with a two-independent sample t-test, equal variance assumed. Statistically significant differences among groups were recognized as having a p-value ≤ 0.05.

Results

Food Intake, Body Mass and Terminal Measures

Food intake, body mass and terminal measures are included in Table 5.11. HFD mice had a greater average weekly food intake than CHOW mice (ANOVA $p = 0.001$; CHOW vs HFD5 and HFD13 $p<0.0001$). Likewise, HFD mice also weighed more than CHOW mice (ANOVA: $p=0.001$), 13 week HFD mice weighed the most (CHOW vs HFD5 $p=0.002$, CHOW and HFD5 vs HFD13 $p<0.0001$). Accordingly, individual adipose tissue depots were increasingly larger due to both diet type and time on diet (ANOVA: $p=0.001$), however the adipose tissue mass among six depots was only marginally increased for mice on HFD for 5 weeks compared to 13 weeks ($p=0.06$ for combined weight). 5 weeks on HFD significantly increased liver triglyceride accumulation by more than 6-fold increase over CHOW diet ($p = 0.001$). The longer duration of HFD intake, 13 week, further increased triglyceride accumulation 2.2-fold over the shorter period, this too was significant (HFD5 vs HFD13, $p=0.0003$).

Fasting basal glucose (as measured by time 0 of the GTT) was not significantly different among the groups (Figure 5.1A), nor was the remainder of the glucose tolerance test or area under the curves (AUC). However, fasting insulin (measured at same time point as fasting glucose) was significantly increased in both 5 and 13 weeks HFD mice compared with CHOW (CHOW vs HFD5 $p=0.0002$, CHOW vs HFD13 $p<0.0001$). Insulin response to the glucose injection was also significantly different between the two diets (Figure 5.1B). Both HFD groups

had greater insulin concentration at 15 and 120 minutes post glucose injection compared with CHOW (15 min: CHOW vs HFD5 and HFD13 $p=0.0001$, 120 min: CHOW vs HFD5 and HFD13 $p<0.0001$). Insulin AUC was also increased for both 5 and 13 HFD mice (CHOW vs HFD5 $p<0.0001$, CHOW vs HFD13 $p=0.009$).

Femoral Muscle Triglyceride and Insulin Sensitivity

Triglyceride accumulation in femoral muscle was increased in HFD mice (Figure 5.2A; ANOVA $p=0.02$). 13 weeks on HFD significantly increased triglyceride concentration compared with control (CHOW vs HFD13 $p=0.018$).

Muscle insulin sensitivity is directly related to glucose clearance from the blood [429] and has been inversely linked to lipid accumulation in the muscle [416]. In this study, insulin sensitivity in femoral muscle was measured by the rate of Protein Kinase B (Akt) phosphorylation presented as a ratio of phospho-Akt to Total Akt. At termination, half of the mice were stimulated with an insulin injection and half were given saline injection. Basal rates of pAkt/Akt were not different between cohorts (Figure 5.2B). However, insulin stimulated pAkt/Akt was significantly lower in HFD mice (ANOVA: $p=0.001$), where CHOW > 5 week HFD > 13 weeks HFD (CHOW 5 vs HFD5: $p=0.007$, CHOW 5 vs HFD13: $p<0.0001$, HFD5 vs HFD13: $p=0.008$). Factors involved in muscle lipid storage LPL/GAPDH and CPT1/GAPDH were not significantly different between cohorts (Figure 5.3).

Lipidomics

Principle Component Analysis, Annotation Selection and Data Read-out

Principle components reveal a highly significant effect of diet, mostly attributable to the first component (Figure 5.4A; PC1 $p<0.001$). Lipid species were quantified as relative abundance within muscle tissue. As described in Figure 5.4B, a total of 2,181 different lipid compounds were identified by LCMS. 806 of them were significantly different by diet at the $p = 0.05$ level, 450 were highly significant at the $p = 0.01$ level, and 189 were very highly significant at the $p = 0.001$ level. Only 50 of the 2,181 had a significant diet x time interaction at the $p = 0.05$ level. 240 were selected for annotation and 217 were annotated from national online libraries of mass spectra. Selections were based on ANOVA p-value for main effect of diet < 0.001, ANOVA p-value for diet*time interaction < 0.05, and diet fold change > 20 at two time points.

For each compound, fold change of the average group abundance was used to compare values by diet and time. For diet comparisons, the sample average of 5 week HFD was divided by the sample average of 5 week CHOW. Thus, fold change values greater than one means the compound is more abundant in animals on HFD, whereas less than one indicate greater in CHOW. Likewise, for time comparisons the sample average of 13 week HFD was divided by the sample average of 5 week HFD. Values greater than 1 here indicate more abundance in 13-week HFD mice. Bar graphs were generated to organize lipid species according to their fatty acid composition. The vertical axis lists combined fatty acid content, first by total carbons and second by number of double bonds, from smallest to largest. The horizontal axis represents a transformation of the fold change, specifically log base 2, where the value 0 indicates equivalence.

Triglycerides

Figure 5.5A depicts fold change of femoral muscle triglycerides between 5 week HFD and CHOW mice. It is strikingly evident that triglycerides that contain higher numbers of carbon (indicative of longer chain length) and contain double bonds (indicative of less saturation) are more abundant in the femoral muscle of CHOW animals. Specifically, triglycerides that contain a total number of carbons greater than or equal to 53 are exclusively more abundant in 5-week CHOW mice than the respective HFD mice. A greater number of double bonds within a specific carbon number group does not necessarily confer an accumulative association with the CHOW group. Conversely, compounds with the least amount of carbons and zero or one double bond are more abundant in femoral muscle of HFD-fed animals. Triglycerides exclusively greater in HFD mice include those with less than or equal to 46 carbons that are predominately saturated. The region of ambiguity and thus a crucial crossover point occurs between 48 and 52 carbons. It is important to note these graphics reflect total carbons and double bonds of the three fatty acids within a triglyceride. We are unable to decipher lengths of individual fatty acids or placement of double bonds. Therefore, it is unknown whether the unsaturated triglycerides are comprised of n-3, n-6, n-9, etc.

In the same graph format, we examine a time effect with HFD-fed animals at 13 weeks divided by HFD-fed animals at 5 weeks (Figure 5.5B). Therefore, this graph demonstrates if an increase in diet duration would be associated with a greater abundance of particular triglyceride types. An increase in diet duration did not consistently associate with a specific carbon number or saturation. The same graph layout presented for triglycerides will continue for most other lipid species unless otherwise stated.

Phospholipids

Significant phospholipids were identified and presented in Figure 5.6. Two species of phospholipids presented to be highly associated with the CHOW group (Figure 5.7A and 5.7C). This included most all phosphatidylserine (PS) and phosphatidylinositol (PI), despite differences in number of carbons or double bonds. With respect to HFD (Figure 5.7B and 5.7D), both lipids are found in higher abundance with the longer time period of 13 weeks compared to 5 weeks. Total number of carbons or double bonds were not differentially associated.

Additional phospholipids were identified, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) (Figure 5.9). These lipids, however, did not associate with a particular diet type.

Diacylglycerides

Diacylglycerides (DAG) have greater abundance in the femoral muscle of mice fed HFD for 5 weeks compared with CHOW diet (Figure 5.7A). An increase in diet duration does not cause further alterations in abundance within the femoral muscle (Figure 5.7B).

Ceramides and Sphingomyelin

Five ceramides were significantly different between 5 week HFD and CHOW mice (Figure 5.7C). Despite significant differences among ceramide specie types, there was no clear indication of ceramides having a greater link to one specific diet. Increased diet duration also did not associate with an increase in the abundance of ceramides.

Sphingomyelins, however, which contains a ceramide backbone, were consistently higher in femoral muscle of HFD animals at 5 weeks compared with respective CHOW (Figure 5.7E). Abundance was greater with increasing diet duration was well (Figure 5.7F).

Vitamins and Others

Fat-soluble vitamins A and D were more abundant in HFD, while vitamin E was found in higher abundance with CHOW (Figure 5.10A). A wax ester (the combination of a fatty acid and a fatty alcohol molecule), eicosenoic acid (an omega fatty acid), and cardiolipin (2 phosphatidic acid moieties connect with a glycerol backbone) were all more abundant in femoral muscle of HFD-fed mice at 5 weeks (Figure 5.11A). Phosphatidic acid (glycerol, phosphoric acid, 2 fatty acids), monoglycerol (glycerol linked to a fatty acid), cholesterol, and diacyltrehalose (2 molecules of sugar with fatty acids attached) were higher in CHOW.

Lipid Signature

Based on species abundance levels, a distinct lipid signature was identified for two diet types; relative proportions are presented in figure 5.8. Chow diet was predominately associated with long-chain triglycerides, unsaturation, phosphatidylserine, and phosphatidylinositol. HFD was dominated by shorter-chain triglycerides, saturation, diacylglycerides, and sphingomyelin.

Discussion

Study Results

This study provides a thorough analysis of femoral muscle lipid species that may be involved with a reduction in insulin sensitivity. It represents a comprehensive comparison

between not only CHOW and HFD mice, but also investigates if an extended HFD duration causes increases in abundance of lipids already noted to be deleterious. Links between increased abundance of saturated fatty acids and diacylglycerols and insulin resistance are well supported in the literature [422, 430]. Although our literature supports this, we do not demonstrate an association with ceramides as previous suggested [423], instead we demonstrate a possible role of sphingomeylins and muscle insulin resistance. Our literature also extends previous studies by demonstrating a complimentary link of triglycerides with long unsaturated fatty acid chain with insulin sensitivity. Overall, this data provides a novel femoral muscle lipid signature linked to HFD intake that may drive the manifestation of insulin resistance induced type-2-diabetes.

Triglycerides

This study identifies a distinction among the femoral muscle triglyceride types that are abundant in CHOW mice versus those in HFD. The difference predominately pertains to two characteristics of the triglyceride that include chain length (number of carbons) and saturation (number of double bonds). In a typical rodent diet, the three fatty acids within a triglyceride typically range from 14-22 carbons and are categorized as long-chain. The level of saturation among our triglycerides range from saturated without any bonds to several polyunsaturated with a total of 12 bonds.

The types of fatty acids deposited in muscle and esterified into triglycerides play a key role in development of insulin resistance syndrome [431]. The standard rodent CHOW consist of an assortment of fatty acids where linoleic > oleic > palmitic > linolenic > stearic. These fatty acids are oxidized or can be modified into fatty acids with varying composition. The fatty acids contained in the HFD, however, were predominately from milk fat. Milkfat is characterized by

an extremely high-saturated fatty acid content. The dietary saturated fatty acids in the HFD are as short as 4:0 carbons and extend to 18:0. The greatest portion represented in the current diet is palmitate (16:0) followed by stearate (18:0) and myristate (14;0). Our HFD also mimicked Western diet containing a significantly amount of sucrose (34% by weight). With excess sucrose consumption, sugar is converted to palmitate through de novo lipogenesis, thus adding additional palmitate beyond the dietary contribution.

Overall, femoral muscle of CHOW diet animals exhibited an abundance of unsaturated triglycerides containing high numbers of carbon (> 53 total carbons). While we are unable to identify the exact structure and composition of fatty acids within the detected TGs, we did speculate according to our annotation analysis. LCMS spectra produce multiple matches from national databases and the selection process is subjective. For example, a list of triglycerides are returned that have equal number of carbons and double bonds, but there are multiple variations to choose from in chain length and bond placement within each fatty acid. We have confidence in reporting the total carbon and bond numbers in this analysis, even though it is less specific. However, with a high number of total carbons and double bonds, we can assume one or more fatty acids in the triglyceride are long and unsaturated.

Intermyocellular lipids are within extracellular muscle tissues and thought to have similar effects as visceral fat. It is associated with inflammation and increases with age [432]. Conversely, intramyocellular lipids are within muscle cells and not always bad as with the case of athletes [433]. The present study focuses on femoral muscle lipid accumulation without distinction between intramyocellular and intermyocellular placement.

Our results demonstrate that unsaturated triglycerides that contain long chain fatty acids are more abundant in the femoral muscle of CHOW mice. In opposition, saturated triglycerides

associated with medium chain fatty acids are more abundant in the femoral muscle of HFD mice. This confers that muscle insulin sensitivity may be influenced by lipid types. Indeed, research supports that chain length and degree of saturation influence lipid metabolism and subsequently insulin-dependent glucose regulation [434, 435].

Skeletal muscle long-chain fatty acids are associated with improved insulin action [388, 435-438]. They are detected in lower abundance in a non-obese, insulin resistance rat model [436]. Long-chain fatty acids have been shown to reduce inflammation leading to insulin resistance by suppression of pro-inflammatory cytokines IL-6 and TNFα in C2C12 myotubes [435]. The increase in insulin senstivity is thought to be driven by phosphorylation of Akt permitting enhanced glucose uptake, possibly by inhibition of protein kinase C [435]. Long-chain fatty acids are also found to increase fatty acid oxidation and decrease intramuscular lipid storage, which is the basis of the athletes paradox [439, 440]. Elongase enzymes are one factor in generating endogenous long chain fatty acids and are found to be lower in individuals with unfavorable metabolic profiles [441]. Altogether, long chain fatty acids may reverse the effects of insulin resistance and prevent its progression to type 2 diabetes [442]. Our data resemble these findings in that CHOW fed mice without insulin resistance had a higher abundance of femoral muscle long-chain triglycerides than mice fed HFD for 5 weeks.

There is a great deal of evidence that fatty acid saturation is closely associated with lipid-induced insulin resistance in skeletal muscle [434, 443-445]. Saturated fatty acids reduce insulin-dependent GLUT4 translocation and activate inflammatory pathways, which influence glucose tolerance both systemically and within skeletal muscle [443]. The NK-κB inflammation pathway is mediated by activation of toll-like receptor 4 (TLR4), which can be attributed to saturated fatty acids [446, 447]. Activation of TLR4 is upregulated in adipocytes and macrophages with obesity

and is a contributing factor in diet-induced insulin resistance [445]. Palmitate is an abundant saturated fatty acid that has been linked to increased DAG and ceramide accumulation, and reduced glucose uptake by inhibiting Akt phosphorylation [442]. Both of these lipid intermediates have been implicated in muscle insulin resistance. Expression of desaturase enzymes are positively correlated with insulin activity and glucose uptake, which are one factor in generating endogenous unsaturated fatty acids by adding double bonds [441, 448]. Defects in delta5 and delta6 desaturases are related to development of insulin resistance through decreased amount of unsaturated fatty acids [449]. Our data are consistent with the literature in that insulin sensitive CHOW fed mice had higher amounts of highly unsaturated triglycerides within femoral muscle, which was not the case with HFD fed mice.

Overall, our data indicate that healthy CHOW fed mice have femoral muscle triglycerides with longer chains and more double bonds. Conversely, HFD mice are indicative of shorter chain length and saturated femoral muscle triglycerides. Our findings reveal no cumulative effect of high fat feeding over long term, but rather type of fat is more significant.

Phospholipids

Phospholipids (PL) are phosphorous-containing lipid species with two fatty acid chains that are present in all cells in the body. Phospholipids in cells, such as myocytes, play a fundamental role in the physiochemical properties of the bi-lipid membrane. Recent studies have shown a relationship between phospholipid composition with obesity and insulin sensitivity [450-453]. Here the lipid type associated with phospholipids governs membrane fluidity, rigidity and protein placement and function [454, 455]. Of particular interest is the relation between phospholipid type and number and action of the insulin receptor. Studies support the influence of

cellular components on reduced insulin receptor turnover and degradation [456-458]. Major phosphate groups that occur in phospholipids are bound to nitrogen-containing compounds such as choline, ethanolamine, glycerol, serine, and inositol.

Lipids make up approximately 50% of a typical cell plasma membrane by weight, but close to 99% by number [459], making it the fundamental structural element of membranes. Phospholipids are the primary lipid making up the bilayer that control protein placement and regulate entry of products into the cell. Phosphatidylcholine (PC) resides exclusively on the outer leaflet, while phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are found within the inner leaflet of the membrane [459]. Phosphatidylglycerol (PG) is the primary lipid component of surfactant and predominately found in alveolar cells with little evidence of a role in myocytes [460]. Given the placement and abundance of PC, it is readily available to participate in cell recognition, paracrine, and endocrine functions. PS and PI have anionic head groups that result in a negatively charged interface with the cytosolic portion of the cell. PI is found in significantly lower quantities than other phospholipids, but plays a significant role in membrane cell signaling [461]. Studies show that PE is involved with membrane formation and adaptability, including protein activity and fusion [462]. Altogether, we recognize that phospholipids have different functions within cell membranes, despite them being similar in structure.

Previous studies have demonstrated that phospholipids play a role in obesity and insulin sensitivity of skeletal muscle [453, 463]. CEPT1 knockout mice, an enzyme that functions in the synthesis of PC and PE, remained insulin sensitive when given HFD, which was not observed with control mice [453]. Diet-induced obese control mice had decreased insulin sensitivity and increased expression of CEPT1 in muscle [453]. With p53 knockout, a tumor suppressor gene

that also affects lipid synthesis, PC and PS were upregulated in calf muscle of mice compared to wild type [463]. This change in phospholipids of muscle tissue is associated with mitochondrial dysfunction, thus PC and PS could be mediators of insulin resistance. Indeed, individual results are tissue specific with respect to abundance and function of individual phospholipids. A study of morbidly obese Caucasians found PCs to be higher in adipocytes and lower in plasma and extracellular environment of insulin resistance participants [464]. In plasma of young Australian adults, five PCs were positively associated with waist circumference (WC), two PCs were negatively associated with WC, and one PC was associated with insulin resistance [451]. The last three PCs mentioned above had only one fatty acid attached. Our study had similar results of no consistent pattern with PCs, as their diet association was dependent on total chain length and double bonds. PC is the most abundant phospholipid in mammals and highly recognized in the literature, thus it is likely involved in many different processes. While it is unclear the exact role of PE in muscle, the *PEMT* gene converts PE to PC, thus indicating a possible staging lipid for the outer membrane.

In the present study, PS and PI are consistently higher in femoral muscle of mice fed a healthy CHOW diet compared to an unhealthy westernized HFD diet. Indeed, phospholipids have functions other than that of cell membrane structure and function. PS is involved in programmed cell death with high expression found on the surface of apoptotic lymphocytes serving as a recognition molecule to recruit macrophages to phagocytose and dispose of the dying cell [465]. Therefore, PS is associated with identification and clearance of diseased cells, making room for healthy ones to proliferate [466]. Our data also indicate greater abundance of PS in mice who were given HFD for 13 weeks compared to only 5 weeks. It is possible PS becomes detrimental over long periods when exposed to a poor diet. Obese individuals were

found to have high exposure of PS on the membranes of erythrocytes, which was associated with red blood cell aggregation [467]. High levels of PS could lead to hypercoagulability and thrombosis in overweight and obesity. PI is directly related to insulin signaling with its conversion of PIP2 to PIP3 [461]. Skeletal muscle accounts for the majority of insulin-mediated glucose disposal, therefore PI is essential in maintaining systemic glucose regulation. Our data agree given that a higher abundance was observed in muscles of healthy, insulin sensitive mice.

In addition to head group, skeletal muscle insulin sensitivity is affected by the fatty acid composition of phospholipids [450, 468]. Decreased insulin sensitivity is correlated with a low percent of long chain (20-22 carbons) polyunsaturated fatty acids attached to phospholipids in thigh muscle of normal weight men [450]. However, fatty acid composition can be modified by diet and exercise [442]. Individuals placed on a standard diet and then allocated to a daily exercise program had better peripheral insulin sensitivity than those who were given the same diet but did not exercise [468]. Simultaneously, phospholipid bound palmitic acid decreased and oleic acid increased in the exercise group, thus altering the composition of fatty acids within a membrane [468]. We too found that PC, PE, and PG did not follow a clear pattern associated with diet. Perhaps they are more dependent on total chain length and saturation level of fatty acids than PS and PI in determining their influence on metabolic health.

Lipid Intermediates

Our data indicate a higher abundance of diacylglycerids (DAGs) and sphingomyelin (SM) in femoral muscle of HFD dysregulated mice compared to healthy controls. However, we did not see a consistent pattern in ceramide content. DAGs and ceramides have long been implicated in the pathogenesis of insulin resistance. DAGs act as a second messenger that

activates protein kinase C (PKC) in the plasma membrane [420]. PKC is then able to phosphorylate target substrates, one of which is thought to be a serine residue on the insulin receptor substrate (IRS). This improper phosphorylation event attenuates the insulin-signaling cascade from downstream targets and infers resistance to the insulin hormone. Ceramide too interferes with insulin signaling by blocking activation of protein kinase B, not allowing it to phosphorylate its target proteins [421]. It is unclear what mechanism the ceramide molecule employs, though it is thought to be downstream of IRS-1 and PI3K. A lipidomics study in obese humans that analyzed hundreds of compounds identified only ceramide C18:0 as a potential player in muscle insulin resistance [469]. Cells treated with palmitate result in accumulation of DAG and exhibit impaired glucose uptake, which was not seen with oleate [470, 471]. Nonetheless, DAG levels can be decreased and insulin sensitivity restored with weight loss and exercise training [472]. In our study, we found femoral muscle DAGs to be associated with insulin resistance regardless of FA composition, though no clear associations were seen between ceramide abundance and HFD mice. SM is a lipid molecule closely related to ceramide with phosphorylcholine attached to a ceramide backbone. Recent studies link SM to loss of insulin sensitivity and suggest using it as a potential therapeutic target [473, 474]. SM was higher in intracellular and extracellular adipose tissue, but lower in plasma of morbidly obese insulin resistant Caucasians [464]. Twelve SMs varying in chain length and double bonds were correlated with waist circumference and obesity in plasma of young Australian adults [451]. Our data indicate a connection between HFD-induced insulin resistance and SM abundance.

Miscellaneous

Several forms of vitamin A were identified and differed between diets, while vitamin D was highly abundant with HFD and vitamin E with CHOW. There is no simple connection between fat-soluble vitamins and insulin resistance, but it is probably tissue-specific. Wax esters from fish oil has been shown to reduce obesity and glucose intolerance in mice [475]. Our data show an increase of wax ester in HFD dysregulated mice, possibly as a counter-regulatory mechanism. Cardiolipin is a dimeric PG located in the inner mitochondrial membrane and has been implicated in diabetes and heart disease [476]. We show higher abundance of cardiolipin in animals given HFD over CHOW for 5 weeks, and is increased further with HFD for 13 weeks.

Conclusions

We identified thousands of lipid species in femoral muscle of CHOW and HFD mice, establishing a distinct lipid signature associated with both a healthy and dysregulated state. Mice given standard CHOW diet have a muscle lipid environment characterized by long-chain, highly unsaturated fatty acid composition within triglycerides. They have phospholipids attached to serine and inositol molecules. To a lesser extent, they are associated with vitamins E, as well as several specific lipid species (phosphatidic acid, monoglycerol, cholesterol, diacyltrehalose). Mice who were given a westernized HFD have muscle lipids characterized by triglycerides with fewer carbons and more saturation with hydrogen atoms. They also include diacylglycerides and sphingomyelin in their environment. Less influential compounds were vitamin D, wax ester, eicosenoic acid, and cardiolipid. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and vitamin A were seen in both diets. The amount of time on westernized diet (5 versus 13 weeks) did not appear to play a role. Limitations of this analysis include

incomplete compound annotation and limited spectra matching databases. However, future lipid research should not focus on individual compounds, but rather consider clusters or groups of compounds working in cooperation with each other.

Tables and Figures

Table 5.1: Food Intake, Body Mass and Terminal Measures. Values with a different letter are significantly different from each other ($p<0.01$). HFD mice ate significantly more calories each week than CHOW mice; however, appetite began to regulate with the longer time period on HFD. Body weight at termination increased accordingly for each cohort. Liver TG concentration increased over 6-fold when on HFD for the same amount of time as CHOW. In addition, Liver TGs more than doubled when on HFD for 5 weeks compared with 13 weeks. Individual adipose depot weights were significantly higher at termination for HFD (both times) compared with CHOW controls.

	5 wk Chow	5 wk HFD	13 wk HFD	P-value†
Average Weekly Food Intake (kcal)	72.4 ± 2.5^{a}	140.2 ± 3.3^{b}	96.9 ± 2.5^{c}	<0.001
Final Body Weight (g)	28.6 ± 0.5^{a}	34.3 ± 1.5^{b}	45.8 ± 1.0^{c}	<0.001
Liver TG (mg/g)	21.0 ± 1.3^{a}	133.8 ± 22.6^{b}	293.7 ± 15.0^{c}	<0.001
Adipose Depot Mass (g)				
N	5	6	5	
EWAT	0.32 ± 0.04^{a}	1.76 ± 0.24^{b}	2.42 ± 0.29^{b}	<0.001
IWAT	0.20 ± 0.02^{a}	1.14 ± 0.13^{b}	1.45 ± 0.09^{b}	<0.001
VWAT	0.14 ± 0.00^{a}	0.75 ± 0.09^{b}	1.10 ± 0.13^{b}	<0.001
BAT	0.12 ± 0.01^{a}	0.33 ± 0.04^{b}	0.50 ± 0.06^{b}	<0.001
DWAT	0.20 ± 0.02^{a}	1.08 ± 0.15^{b}	1.31 ± 0.11^{b}	<0.001
PWAT	0.10 ± 0.01^{a}	0.84 ± 0.10^{b}	1.10 ± 0.11^{b}	<0.001
Total AT depot mass	1.08 ± 0.11^{a}	5.90 ± 0.73^{b}	7.88 ± 0.78^{b}	<0.001

†Single Factor ANOVA (cohort)

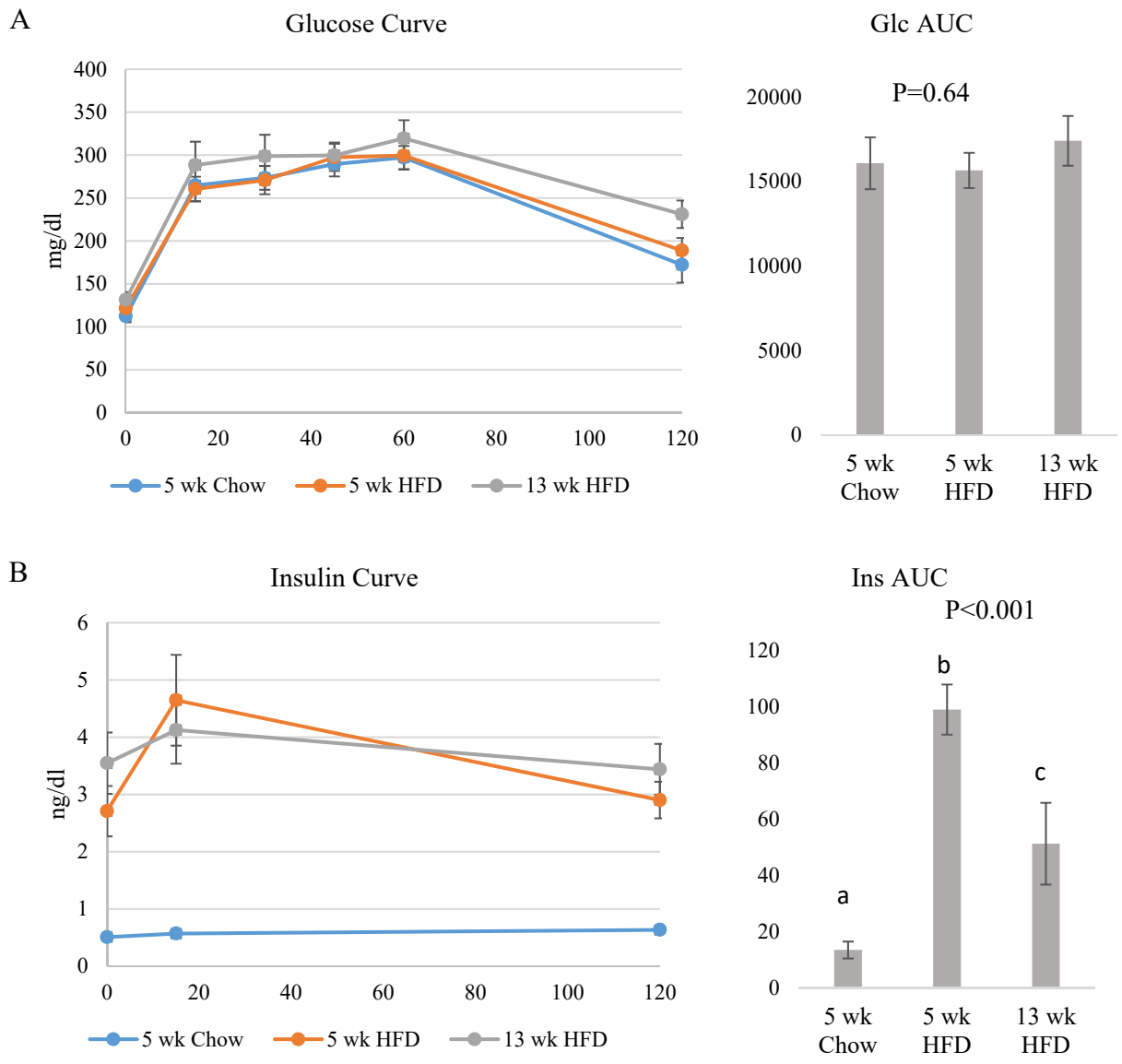


Figure 5.1: Glucose and Insulin Response to GTT. Mice participated in a 2-hour glucose challenge one week prior to termination (4 or 12 weeks post-surgery). A 1.5 g/kg bolus of dextrose was injected (ip) and glucose measurements were made at 0, 15, 30, 45, 60, and 120 minutes. Insulin measurements were made at 0, 15, and 120 minutes. Average values for each time-point are plotted and area under the curve (AUC) was calculated as the total glucose/insulin excursion from 0-120 minutes. A) Glucose values did not differ between cohorts in response to a glucose injection. B) Insulin values were significantly higher in HFD mice than CHOW at all three time points. HFD mice had significantly higher AUC (greater insulin excursion) in response to glucose bolus.

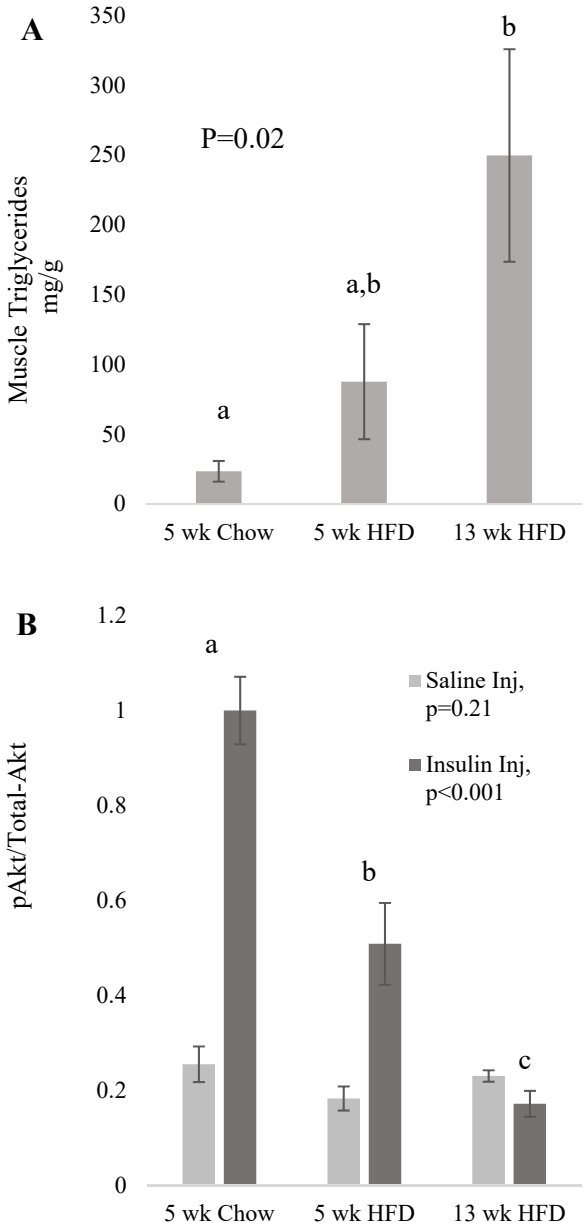


Figure 5.2: Femoral Muscle Triglyceride Concentration and Insulin Sensitivity. Intramuscular triglycerides were measured in mg/g tissue. The ratio of phosphorylated Akt to total Akt was used to measure muscle insulin sensitivity. Approximately 15 minutes prior to termination, mice were injected (ip) with either insulin or saline. A) Muscle TGs deposition increased progressively with each cohort. B) Likewise, muscle pAkt response to insulin decreased progressively by cohort. Muscle triglycerides and insulin sensitivity were significantly and inversely related to diet type and time on HFD.

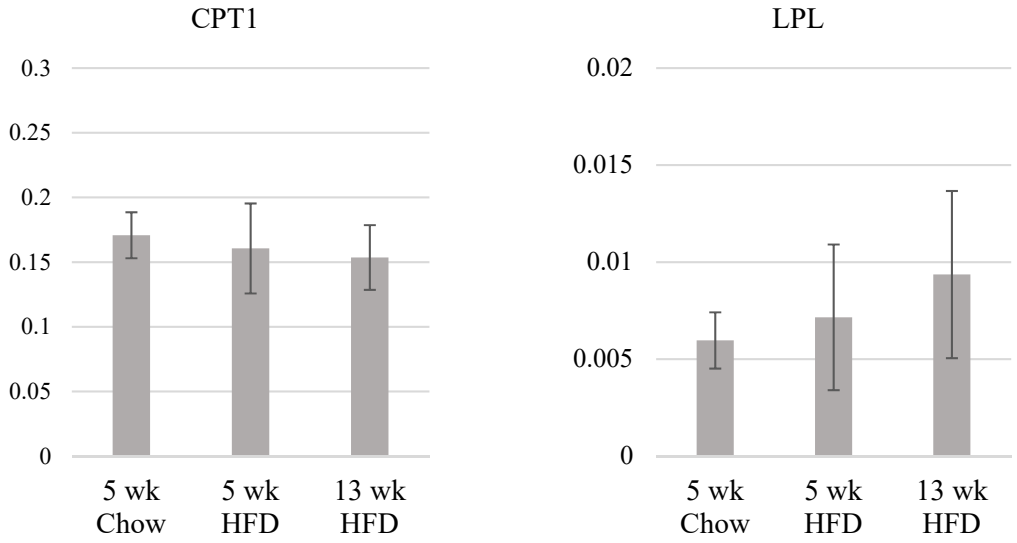


Figure 5.3: Measures of FA Oxidation in Femoral Muscle. A) Carnitine palmitoyltransferase 1 (CPT1) was measured as an indicator of beta-oxidation within the femoral muscle cells. B) Lipoprotein lipase (LPL) was measured as an indicator of fatty acid uptake with the femoral muscle tissue. Neither CPT1 nor LPL were differentially expressed between any of the cohorts.

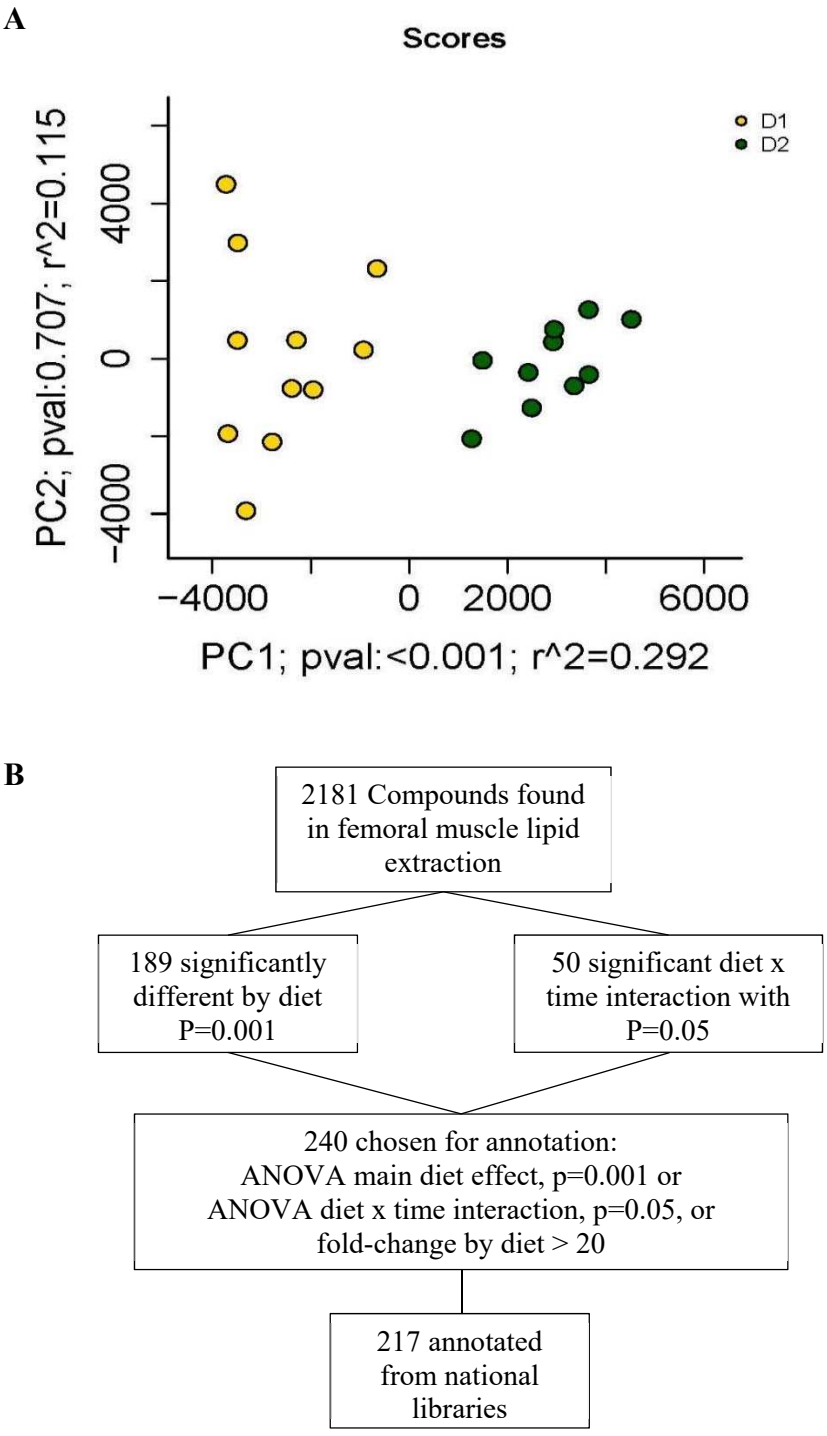
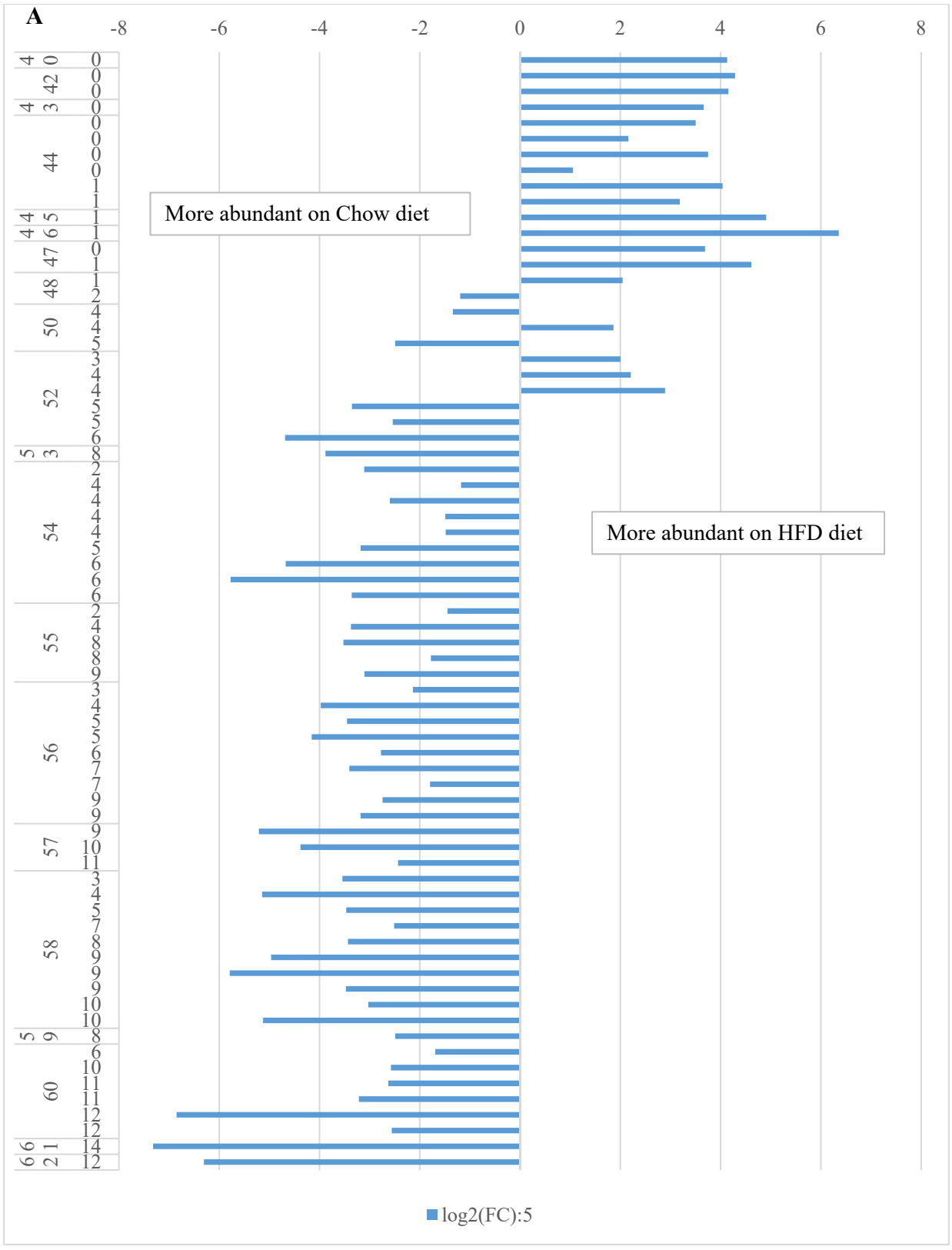


Figure 5.4: LCMS Results. Principle components analysis scatter plot (A) reveal a significant PC1 but not PC2 effect (no other PCs were significant). PC1 explains ~30% of variation in the total data set and represents an effect of diet. B) A schematic of how compounds were chosen from the 2181 total compounds identified in femoral muscles of mice.


A
-8
-6
-4
-2
0
2
4
6
8
More abundant on Chow diet
More abundant on HFD diet
log2(FC):5

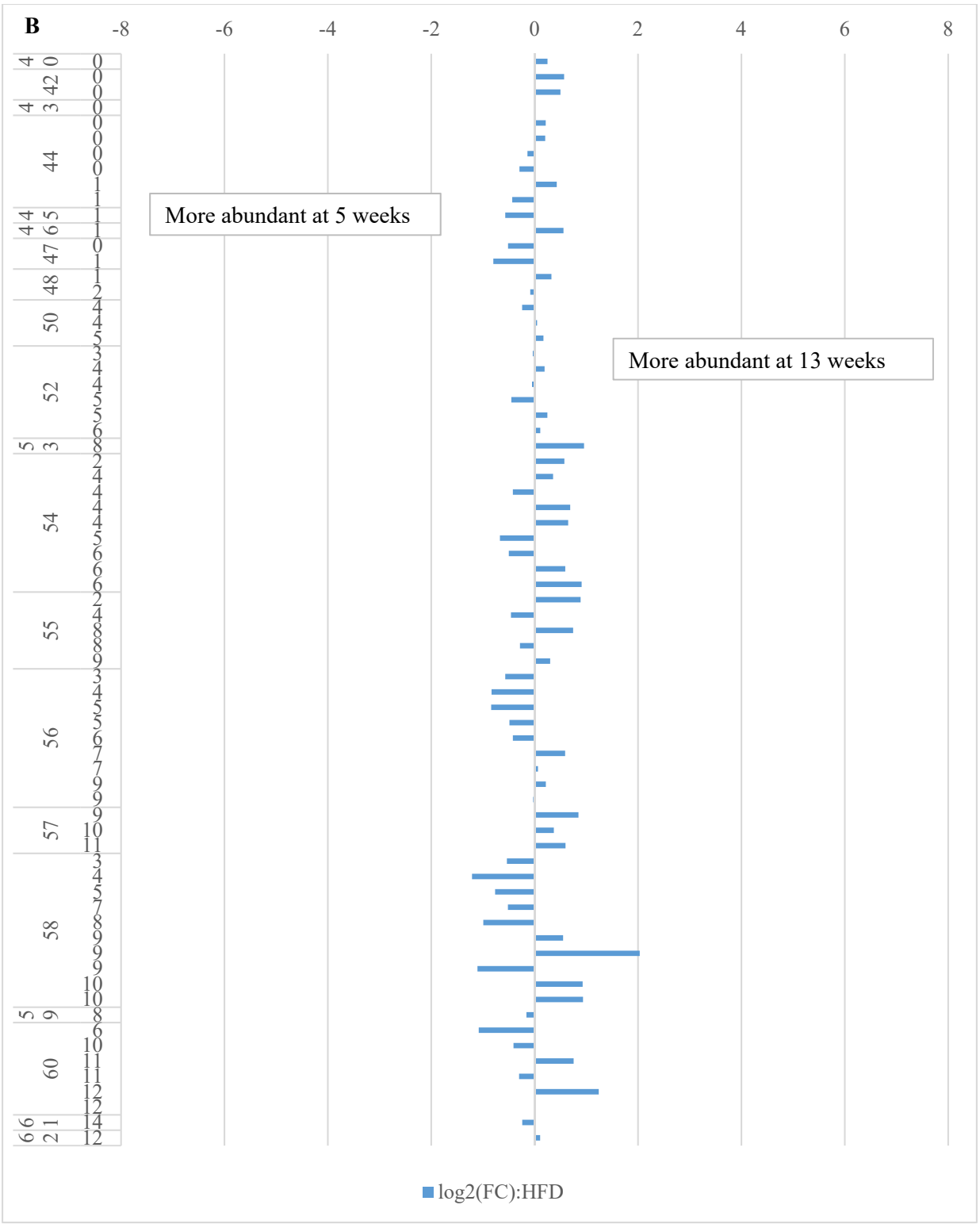


Figure 5.5: Femoral Muscle Triglycerides. Chow mice were associated with long-chain (53-62 carbons) unsaturated triglycerides, while HFD mice were associated with shorter chain and saturated (0 or 1 double bond) triglycerides (A). There was no effect of time on HFD (B).

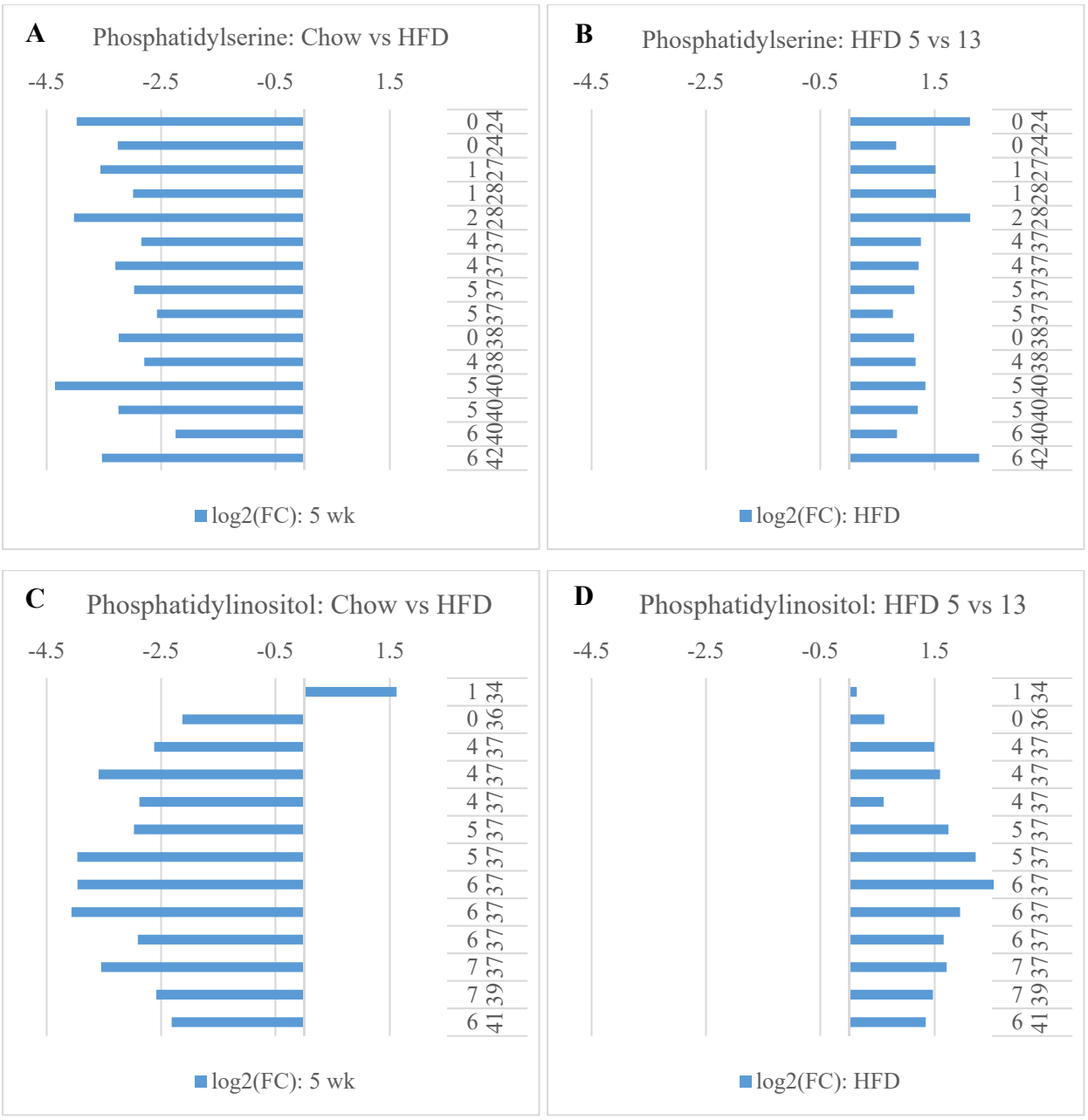


Figure 5.6: Femoral Muscle Phospholipids. Phosphatidylserine and Phosphatidylinositol were strikingly more abundant in mice given a CHOW diet compared to HFD (A, C). However, both phospholipids were more abundant in HFD when it was given for 13 weeks compared to only 5 weeks (B, D).

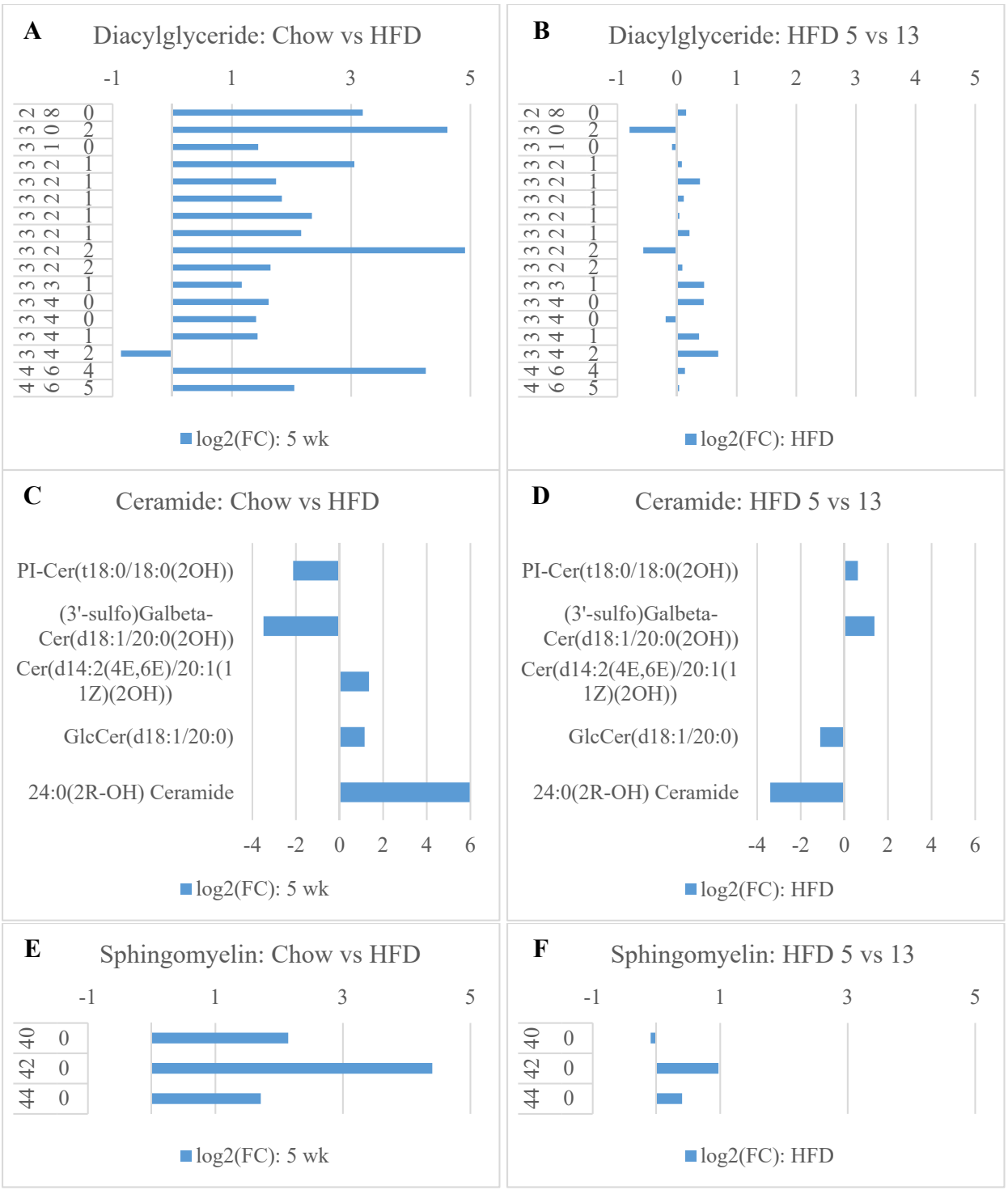


Figure 5.7: Femoral Muscle Lipid Intermediates. Diacylglycerol and ceramide are known intermediates to interfere with insulin signaling. Our data agree that diglycerides are higher in mice on HFD (A), however we did not see the same effect with ceramides (C). Rather, we observed that sphingomyelins were higher in HFD animals (E), which have a ceramide backbone. Time did not appear to have a significant effect on abundance of lipid intermediates (B, D, F).

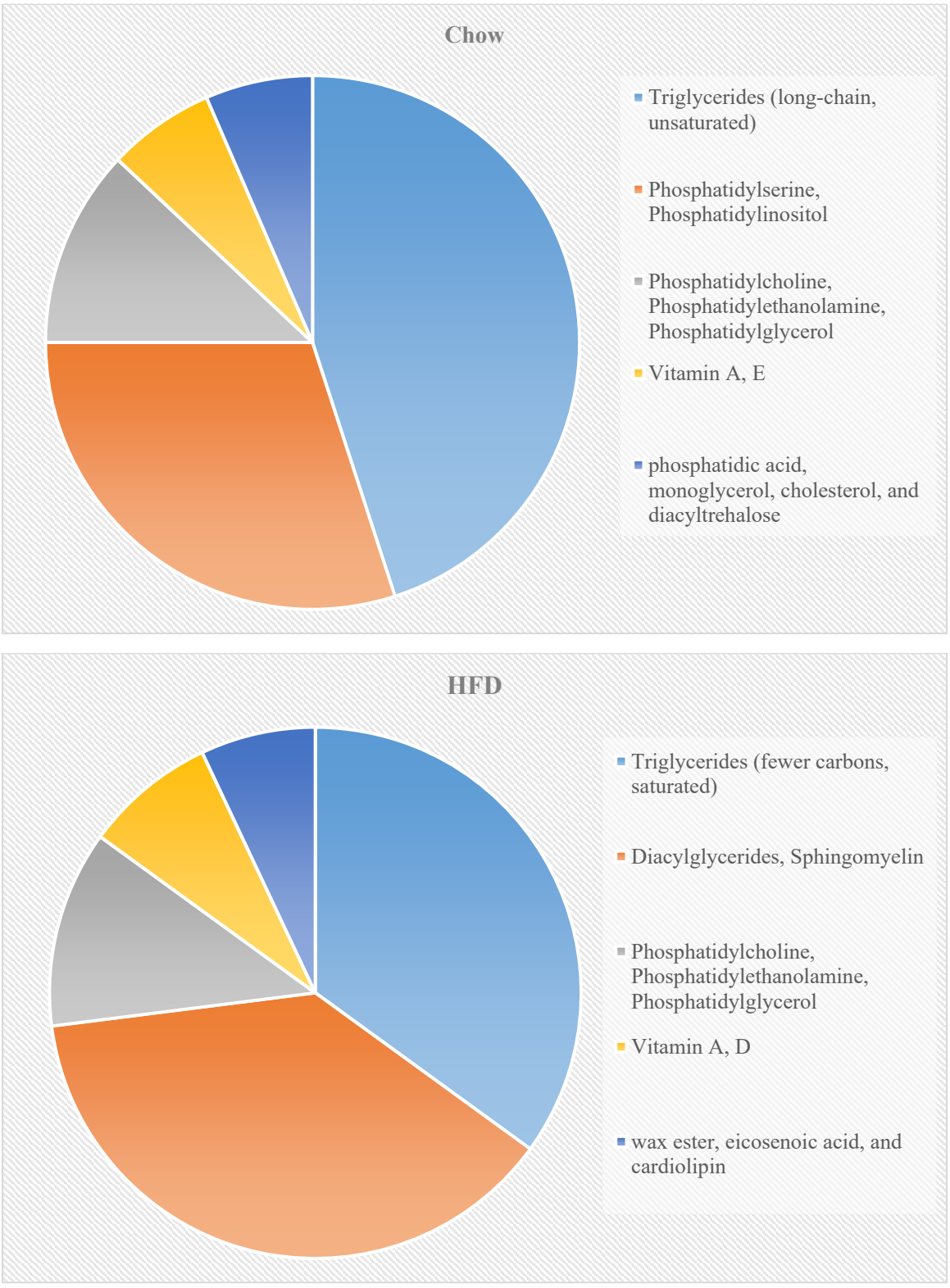
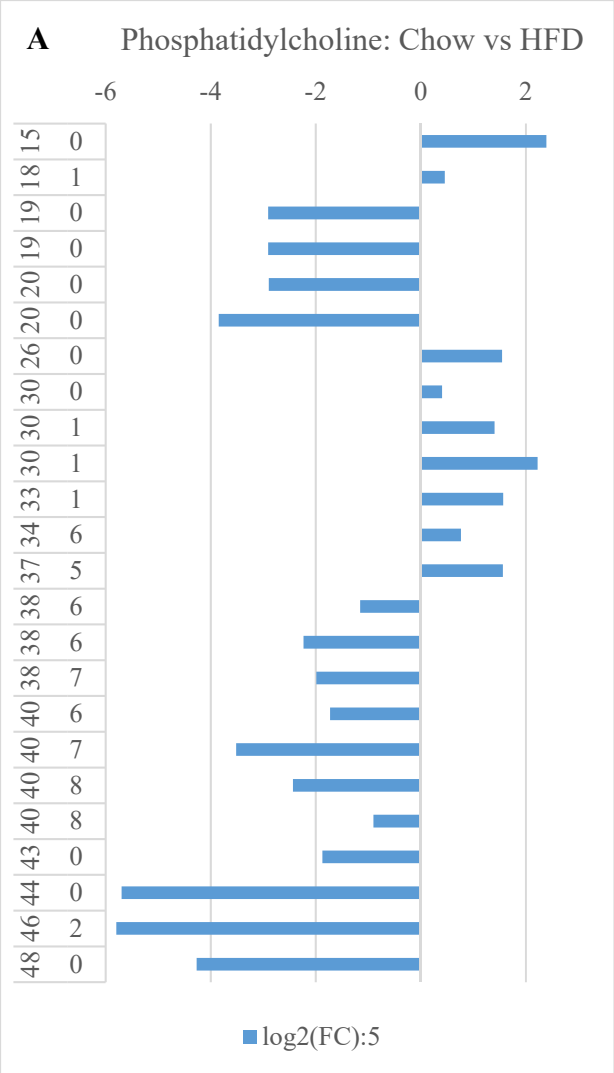
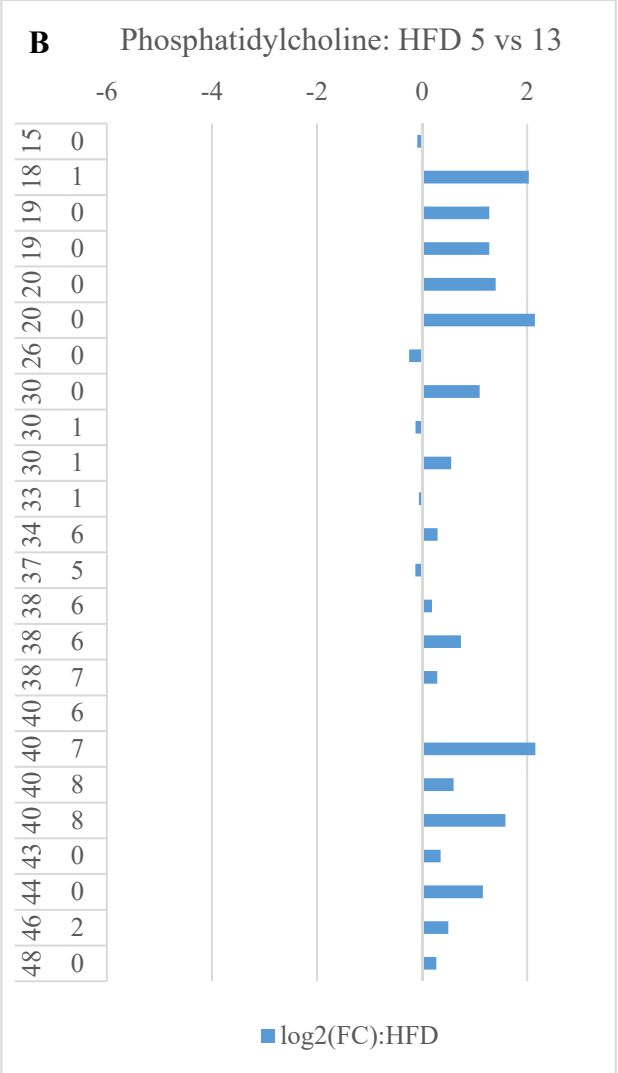
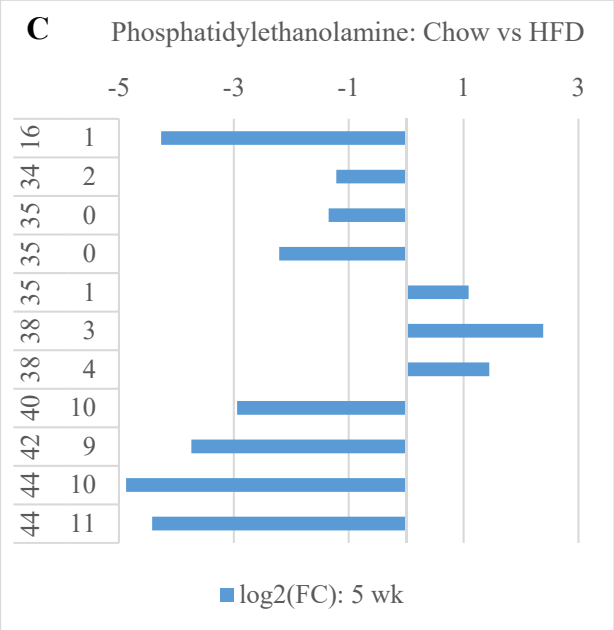
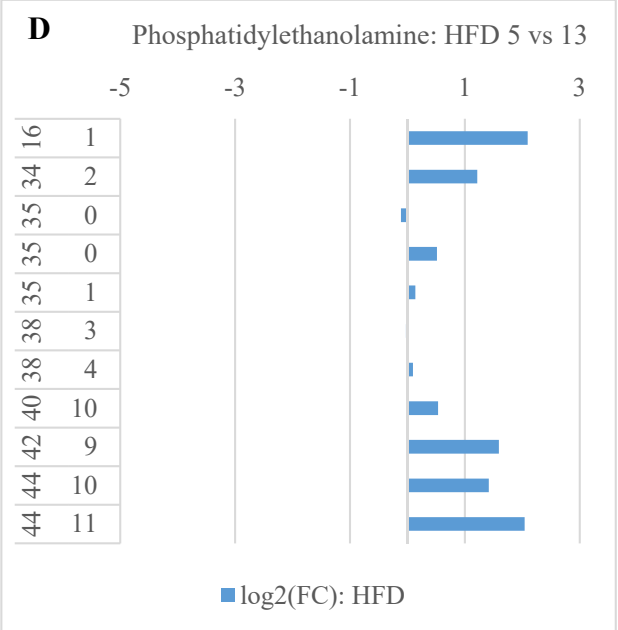


Figure 5.8: Femoral Muscle Lipid Signatures. Diet-associated relative proportions of lipid species in femoral muscles are summarized. Future studies involving lipids with respect to metabolic dysregulation should evaluated groups of lipids instead of only one or a few.


A
Phosphatidylcholine: Chow vs HFD
-6
-4
-2
0
2
15 0
18 1
19 0
19 0
20 0
20 0
26 0
30 0
30 1
30 1
33 1
34 6
37 5
38 6
38 6
38 7
40 6
40 7
40 8
40 8
43 0
44 0
46 2
48 0
log2(FC):5


B
Phosphatidylcholine: HFD 5 vs 13
-6
-4
-2
0
2
15 0
18 1
19 0
19 0
20 0
20 0
26 0
30 0
30 1
30 1
33 1
34 6
37 5
38 6
38 6
38 7
40 6
40 7
40 8
40 8
43 0
44 0
46 2
48 0
log2(FC):HFD


C
Phosphatidylethanolamine: Chow vs HFD
-5
-3
-1
1
3
16 1
34 2
35 0
35 0
35 1
38 3
38 4
40 10
42 9
44 10
44 11
log2(FC): 5 wk


D
Phosphatidylethanolamine: HFD 5 vs 13
-5
-3
-1
1
3
16 1
34 2
35 0
35 0
35 1
38 3
38 4
40 10
42 9
44 10
44 11
log2(FC): HFD

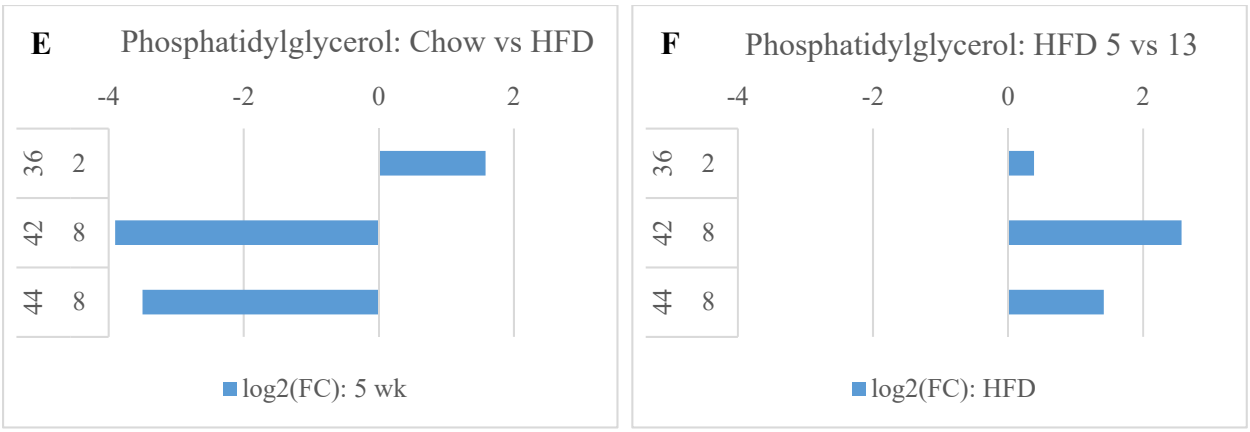


Figure 5.9: Additional Femoral Muscle Phospholipids. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol were also identified and their abundances were graphed. They appear to be dependent on fatty acid chain length and degree of saturation with no clear patterns with diet (A, C, E) or time (B, D, F).

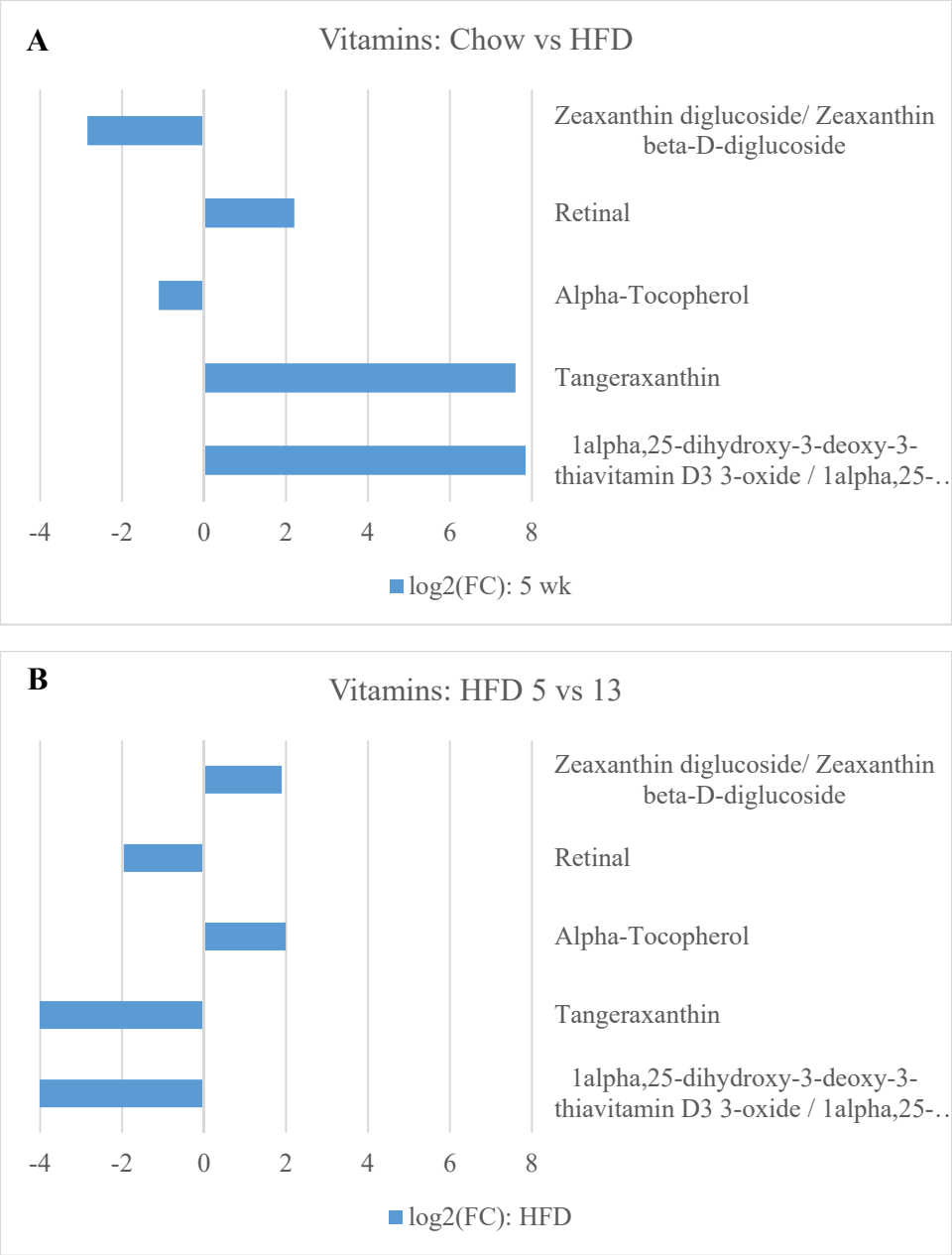


Figure 5.10: Femoral Muscle Vitamins. Individual vitamin species were identified in lipidomics, however do not have a clear influence on diet (A) or time on diet (B) in our data. They are likely dependent on an interacting lipid.

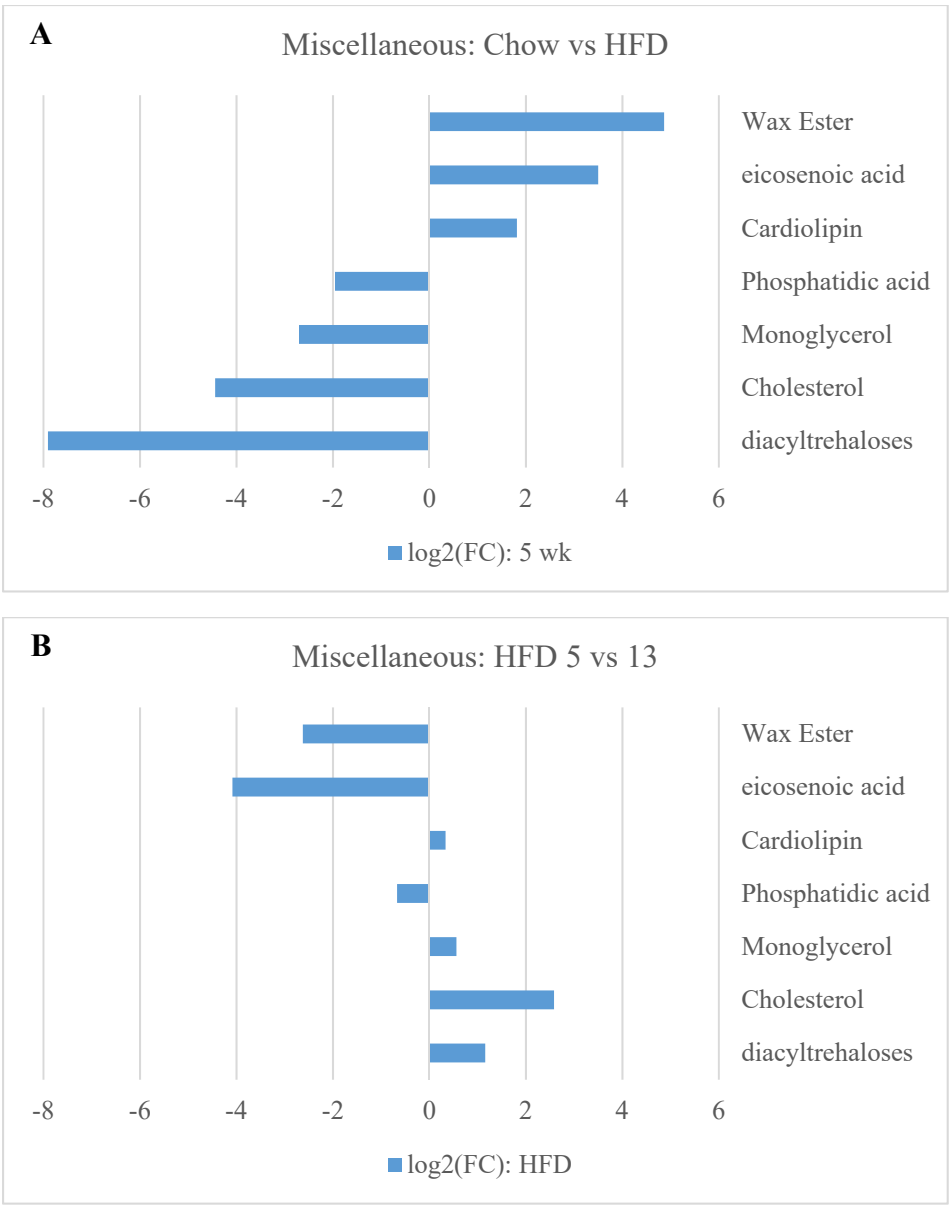


Figure 5.11: Femoral Muscle Miscellaneous Compounds. Several additional compounds were identified that did not fit into a specific lipid class. These too do not have a convincing preference for diet (A) or time (B). Each species should be investigated further for contributing factor to the insulin resistance we observed in mice.

CHAPTER 6: CONCLUSIONS

The purpose of this dissertation was to systematically examine the relation between subcutaneous adipose tissue and glucose homeostasis. The goal was to 1) determine how subcutaneous adipose tissue functioned as a metabolic sink, 2) examine how removal of subcutaneous adipose tissue impairs glucose tolerance, and 3) identify a lipid signature that correlates with impaired glucose tolerance. These studies follow-up observations from the Foster lab that removal of protective fat depots, aka "metabolic sinks", resulted in exacerbation of systemic glucose intolerance and muscle insulin resistance associated with diet-induced obesity.

Two main characteristics of adipose tissue comprise its association with obesity-induced co-morbidities, location and intrinsic properties. Upper body (android) fat accumulation is associated with obesity-related adverse health outcomes, while lower body (gynoid) deposition is considered protective. Previous studies in the Foster Lab examine fundamental mechanisms that lead visceral deposition to be "bad" [337, 338, 477]. The present studies, in opposition, focus on properties of subcutaneous adipose tissue (SAT) accumulation as being "good". This "good" fat is defined as being insulin sensitive, adipogenic, and anti-inflammatory [32, 322]. A beneficial quality of lower body subcutaneous adipose tissue (LBSAT) is its ability to expand and proliferate. Growth of pre-adipocytes, regulated by PPARγ, produce healthy insulin-sensitive, lipid filling cells [334]. This depot also has an increased expression of genes related to adipogenesis and angiogenesis compared to visceral, permitting tissue hyperplasia [478]. Free fatty acids enter and leave SAT via systemic circulation, serving as a buffer for daily lipid flux. In contrast, visceral adipocytes drain into portal circulation and flood the liver with lipid, a damaging consequence of central obesity. Adipokine secretions are a fundamental component of

the regulatory role of adipose tissue, including appetite control and glucose homeostasis. LBSAT is more sensitive to the antilipolytic effects of insulin than visceral adipose tissue [127], therefore maintaining appropriate blood glucose and lipid concentrations, as well as transitioning cells into an anabolic state. Two hormones secreted predominately from adipose tissue that improve glucose uptake and insulin sensitivity are leptin, a lipostatic signal proportional to fat mass, and adiponectin, a regulatory protein inversely correlated to weight status. Both hormones are found in higher abundance with subcutaneous fat mass compared to visceral [479]. In addition, chronic inflammation that occurs with central obesity is associated with pro-inflammatory cytokines IL-6, TNFα, MCP-1, and polarized macrophages [61].

Mounting evidence support visceral adipose tissue as the quintessential factor in the pathogenesis of obesity-induced metabolic disease. However, SAT may have a greater influence on disease suppression given that it accounts for ~85% of total body fat, has a greater ability to expand, and is more insulin sensitive. For this reason, we chose to focus on SAT for the majority of this dissertation.

As previously discussed, adipose tissue proliferation is an important factor in maintaining glucose regulation and insulin sensitivity. It has been shown that removal of intra-abdominal fat results in a compensatory increase in total fat mass [337]. The stimulatory growth of new adipocytes following lipectomy is a protective response to preserve glucose tolerance with the loss of a lipid-storing depot. PPARγ is a receptor that stimulates uptake of lipid by fat cells and activates pre-adipocytes to mature [480]. As such, we demonstrated that lipectomy-induced growth of new adipocytes was inhibited in PPARγ-knockout mice and was associated with compromised glucose tolerance. This outcome points to the importance of recruitment and differentiation of new fat cells in the regulation of glucose homeostasis, which is an inherent

feature of SAT [481]. These findings also support the notion that smaller adipocytes are healthy cells that can readily take up lipid to prevent ectopic deposition and avoid metabolic alterations [482]. With the loss of PPARγ, mice exhibited significant inflammation in the liver, which occurred regardless of whether intra-abdominal fat was removed. This study enhances the concept of LBSAT is protective through proliferation and points to the need for growth of healthy new adipocytes to maintain metabolic regulation. Subcutaneous fat is therefore able to expand more readily than visceral fat, suggesting severe consequences with its absence.

This dissertation supports that prevention of subcutaneous fat expansion is detrimental and removal of lower body subcutaneous fat is equally damaging. He we have demonstrated that LBSAT removal or PPARγ-related inhibition of SAT reduces its ability to maintain glucose homeostasis and protect insulin-sensitivity in specific tissues. In both cases, we observed an increase in systemic glucose intolerance, as well as liver inflammation with SAT suppression and muscle triglyceride accumulation with SAT removal. However, we questioned the exact degree of influence SAT has on metabolic deviations. Next, we expanded on previous observations and examined systematic removal of subcutaneous fat on systemic glucose homeostasis and tissue function. We hypothesized that incremental subcutaneous fat removal would produce a dose-response effect on glucose regulation and that nearby muscle would become dysregulated. We did in fact show that systemic glucose tolerance deteriorated in a dose-dependent manner with increasing amounts of subcutaneous fat removal in animals on HFD for 13 weeks. We also observed a dose-dependent decrease in basal insulin sensitivity in femoral muscle, but not in the insulin-stimulated state. Therefore, we cannot directly attribute the glucose intolerance to a decline in muscle insulin sensitivity in this case. Indeed, other systems could have been affected that we did not examine, including insulin secretion or adipose tissue-mediated glucose disposal.

Peripheral fat removal differed from intra-abdominal fat removal in that total body weight and compensation in non-excised adipose depots did not occur. This is consistent with previous studies of subcutaneous lipectomy [366]. There was significant increases in muscle triglyceride concentration across all surgery groups, thus our long time point of 13 weeks might be too long for mice on HFD. Overall, we demonstrated that incremental removal of SAT causes a dose-dependent deterioration in systemic glucose tolerance and basal femoral muscle insulin sensitivity, independently. This study further confirms the postulate that SAT functions as a "metabolic sink" and protects nearby muscle from ectopic lipid deposition. We show that peripheral fat is systematically related to the development of obesity-induced metabolic disease, thereby forming a direct link between body fat distribution and metabolic risk. Now we are interested in uncovering the details of ectopic lipid deposition itself and its contribution to the anticipated dysregulation.

Lipids are a primary offender of obesity-related insulin resistance [483], however individual lipid molecules are extremely diverse in structure and function. We sought to organize lipid species according to diet-mediated muscle insulin action. Therefore, patterns were examined in femoral muscles of HFD mice with insulin resistance and healthy CHOW-fed mice. Many studies have shown that high muscle triglyceride concentration associated with obesity is linked to insulin resistance syndrome [390]. Conversely, marathon athletes typically have very high muscle triglyceride concentration, while being very insulin sensitive [417]. We hypothesize the discrepancy is due to types of lipids being stored and not to overall concentration. With HFD, femoral muscle lipids were consistent with triglycerides that are saturated and shorter in chain length, diacylglycerides, and sphingomyelin. Chow diet was consistent with femoral muscle lipids that included long-chain, highly unsaturated triglycerides, phosphatidylserine, and

phosphatidylinositol. We also detected phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, ceraminds, vitamins, and some miscellaneous compounds. The additional phospholipids were dependent on fatty acid composition with respect to diet and other compounds were not consistently associated with either diet. Previous literature focused mainly on ceramides and DAGs as being detrimental lipid intermediates to the insulin-signaling cascade. Other studies report fatty acid composition of triglycerides and phospholipids as the primary determinant in muscle glucose uptake. We have identified a broad lipid signature that correlates with both healthy and dysregulated femoral muscles in mice. With our non-targeted lipidomic approach, we were able to capture many types of lipid species and infer their contribution to metabolic dysregulation. This comprehensive approach extends our knowledge of muscle lipid handling and highlights the interconnectivity of lipid metabolites.

This dissertation expands on global knowledge in adipose tissue research. While focusing on positive qualities of subcutaneous fat, we confirm that LBSAT in particular functions as a "metabolic sink" and is protective of obesity-related dysregulation. We further illustrate that the metabolic sink is partitioned into individual compartments that incrementally exacerbate glucose intolerance with removal of each additional piece. This was a novel approach in determining a dose-dependent relationship between subcutaneous fat storage depots and glucose homeostasis. We are the first lab to investigate incremental effects of subcutaneous adipose tissue under healthy dietary conditions and with Westernized high-fat/high-sugar diet. Additionally, we developed a distinct lipid signature associated with healthy and dysregulated femoral muscle in mice. Our results employed a comprehensive approach to describe how clusters of lipid species are related to both standard and unhealthy diets. Future lipid research should no longer be limited

to only a few compounds, but rather investigate how groups of lipids correlate with diet and tissue-related outcomes.

Next Steps

Subcutaneous adipose tissue is undeniably a protective depot when examining individual characteristics of this tissue. However, mechanisms involved in deterioration of glucose tolerance following SAT removal remain to be elucidated. We employed GTTs to determine systemic glucose tolerance and circulating insulin values under fasted conditions. However, utilization of a hyperinsulinemic-euglycemic clamp would be an ideal measure of whole body insulin resistance and the gold standard of glucose tolerance, mimicking the effect of a high carbohydrate meal. We also investigated muscle insulin sensitivity using insulin-stimulated phosphorylation of Akt at serine 473 reside as an end product of the insulin signaling cascade. Future studies of muscle insulin sensitivity should examine the second site of phosphorylation, threonine 308 residue, to determine full Akt activation, as well as downstream targets including AS160 phosphorylation and Rab-mediated GLUT4 translocation. Investigations should also include GLUT4 protein expression itself. With respect to basal muscle insulin sensitivity, AMPK activation from contraction mechanisms should be examined for systemic glucose clearance under fasting conditions. Other measurements that would be useful with a mouse model of peripheral adipose tissue removal would be a hyperglycemic clamp to measure beta cell function with prolonged hyperglycemia to evaluate a corresponding response of insulin secretion to elevated blood glucose.

As a follow-up to the PPARγ knock out study, further proliferation should be examined using a PPARγ agonist from the thiazolidinedione family. Specifically, examine adipose tissue

expansion in all depots following either intra-abdominal or peripheral lipectomy to determine proficiency in pre-adipocytes growth to maintain glucose regulation with new, healthy mature adipocytes. Conversely, a PPARγ antagonist could provide insight into the detrimental effects of hypertrophied adipocytes when faced with a Westernized high-fat/high-sugar diet.

Human studies provide much insight into practical implications of obesity on metabolic disease risk factors. Measuring physical and clinical characteristics of individuals who are metabolically healthy but obese compared to metabolically unhealthy and normal weight or obese would better our understanding of the differential manifestations of metabolic disease. Specifically, comparisons of diet, circulating factors, whole body insulin resistance, and fat and muscle biopsies would advance our knowledge of this phenotypical paradox.

Finally, we would like to expand on our lipidomics studies to incorporate the effect of incremental fat removal on abundance of femoral muscle lipid species. We saw that HFD was associated with decreased muscle insulin sensitivity and shorter chain saturated fatty acids, diacylglycerides, and sphingomylins. We are interested in determining if progressive subcutaneous fat removal will exacerbate those relative abundances within muscle tissue or remain consistent, as well as identification of new significant lipid species.

Overall, peripheral adipose tissue has proven to be protective of systemic and tissue-specific regulation. However, further mechanisms remain to be elucidated with respect to HFD and lipectomy-induced glucose excursions.

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