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The role of adiponectin on body composition, adipocyte differenetiation, preadipocyte proliferation and its modulation of inflammation with high fat diets

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ABSTRACT

The anti-inflammatory and insulin sensitizing attributes of high circulating adiponectin concentrations in the mouse were recently suggested to occur as a result of subcutaneous adipose tissue expansion. Although there is a plethora of data describing the affects adiponectin has on metabolism and inflammation, there are limited data on its role in the growth of adipose tissue. Our data in adiponectin null mice indicate, the consumption of a high fat diet does not significantly change body composition from that of diet matched wild type mice. Furthermore, there was no observable difference in the inflammatory profile of our adiponectin null mice fed high fat diet.

In the absence of the expected increase in adipose tissue, we sought to identify the role of adiponectin in the proliferation and differentiation of stromal vascular cells obtained from *ob/ob* obese mice or wild type controls. Our study identified a significant reduction in the differentiation of cells derived from *ob/ob* animals when treated with adiponectin. Conversely, there was no observable change in differentiation of the wild type cells with treatment of adiponectin, leptin or the combination of the two. Furthermore, we report a significant decrease in proliferation in adiponectin treated wild type cells and in leptin rescued *ob/ob* cells treated with adiponectin.

We also pursued the use of different dietary fatty acids as a means for inducing adiponectin production. We fed swine a diet enriched in the bioactive n-3 fatty acid αLA to examine the effects of this fatty acid on adiponectin and to determine if eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) can be derived from αLA *in vivo*. We identified a significant increase in the tissue enrichment of αLA, EPA and

DPA in a diet dependent manner. However, there was no statistical difference in serum adiponectin concentrations or adiponectin transcript abundance among diets.

Collectively, these studies indicate adiponectin is not instrumental in the regulation of adipose tissue deposition in normal mice. However, adiponectin does retard the pro-differentiation phenotype of leptin-deficient stromal vascular cells. Our data also indicates that, although αLA is readily converted to EPA and DPA *in vivo*, the concentrations seem insufficient to alter serum adiponectin concentrations in swine.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

In the United States, 30% of all adults are classified as obese [1]. If the current trend continues, 50% of the U.S. population will be obese by 2040. This obese population is at an increased risk for the development of low grade inflammation that contributes to the development of cardiovascular disease (CVD) and type II diabetes mellitus (DM) [2-4].

Clinical data and findings from various animal models of obesity indicate a strong positive correlation between adipose tissue mass and the development of obesity-related complications [2]. This association was further strengthened with the identification of adipose tissue as an endocrine organ [3]. The endocrine function of adipose tissue includes the secretion of pro-inflammatory, anti-inflammatory, and the metabolic/appetite-regulating cytokines [4, 5] . Multiple studies have shown that an increase in the proinflammatory cytokines tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), and monocyte chemotactic protein 1 (MCP1) in adipose tissue are involved in the progression of obesity-associated systemic inflammation [6-8] . The development of adipose tissue inflammation is also positively correlated with a reduction in the concentration of the anti-inflammatory cytokine, adiponectin [9]. Although the exact mechanism for the induction of adipose tissue inflammation is not known, the leading hypothesis implicates adipocyte cellular stress. The adipocyte can become stressed with lipid overload [10, 11], hypoxia [12], and increased protein synthesis, all of

which activate a stress response in the endoplasmic reticulum (ER). The induction of ER stress leads to the activation of pro-inflammatory pathways in the adipocyte.

To avoid lipid overload and its associated cellular stress, adipose tissue responds by increasing lipid storage through adipocyte hypertrophy. The lipid storage capacity of adipose tissue is also increased through hyperplasia and differentiation of preadipocytes into mature adipocytes. Furthermore, adipose tissue can also decrease circulating lipid concentrations through the production of hormones that induce lipid oxidation and suppress appetite [13]. When adipose tissue is no longer able to accommodate increased lipid storage or stimulate the removal of lipid through oxidation, stress-induced inflammation occurs. If this cellular stress is not alleviated, cell death quickly follows, and macrophage recruitment and infiltration into adipose tissue is increased [14].

Current literature indicates adipose tissue inflammation can be alleviated by the anti-inflammatory cytokine adiponectin. When adiponectin concentrations are restored to a normal concentration in obese animals, low grade inflammation is reduced. Correction of hypoadiponectinemia or the induction of hyperadiponectinemia both increase PPARα activation and subsequent transcription of lipid-oxidizing enzymes in obese animals and in *in vitro* [15-17]. Adiponectin serum concentrations can be altered with administration of exogenous adiponectin and through the activation of the transcription factor PPARγ [18]. Activation of PPARγ can be accomplished by administration of the synthetic ligands, thiazolidinediones (TZD); however, TZD adversely promote the expansion of adipose tissue [19, 20]. A more desirable means for activation of PPARγ is through consumption of its natural activators, fatty acids.

Neschen et al. showed an increase in PPARγ activation and subsequent increase in adiponectin with the consumption of n-3 fatty acids [21]. Previous literature also indicates that n-3 fatty acids have anti-inflammatory properties independent of adiponectin. Therefore, n-3 fatty acid tissue enrichment through supplementation appears to be a promising treatment option for inflammation. However, it remains to be determined if consumption of high concentrations of αLA results in the desired effect or if DHA and EPA consumption is required. Furthermore, data on the role of αLA conversion to EPA and DHA in non-hepatic tissue is sparse and requires further investigation in non-rodent models.

The accepted dogma for correction of obesity-associated inflammation by adiponectin revolves around the inhibition of TNFα signaling and production, nuclear factor kappa B (NFκB) activity, and removal of adipocyte stress through increasing lipid oxidation. However, in 2007 Kim and colleagues published data indicating the overexpression of adiponectin decreases inflammation and lipid mediated stress by promoting adipose tissue proliferation in leptin-deficient mice [22]. Since 2007, there have been limited follow up data to support the role of adiponectin in adipocyte proliferation, and it is not definitively known if adiponectin is directly involved in adipose tissue growth.

The previously described data indicate adipose tissue is a key player in the exacerbation of low grade systemic inflammation present in obesity. Furthermore, these studies indicate adiponectin is an important in the resolution and prevention of inflammation. However, the current methods of increasing adiponectin production in vivo

rely on pharmaceutical means that have significant side effects. *The identification of n-3 fatty acids as natural ligands for the PPARγ receptor indicates dietary supplementation may be a feasible method for increasing serum adiponectin concentrations. To address the current gaps in the understanding of the influence adiponectin has in body composition, we examined the body composition of adiponectin null mice after being fed a high fat diet. Furthermore, we tested the role of adiponectin on proliferation and differentiation of subcutaneously derived leptin-deficient preadipocytes.* The last key point addressed in our research was the feasibility of n-3 fatty acid tissue enrichment and modulation of adiponectin concentrations with canola oil supplementation.

Objectives and Aims

Our objective was to identify the role of adiponectin in adipose tissue expansion and to identify the role of dietary n-3 FAs in the induction of adiponectin. This work was focused around the *central hypothesis* that adiponectin stimulates preadipocyte proliferation and differentiation and that the dietary supplementation of αLA will increase tissue enrichment of EPA and DHA which in turn stimulate adiponectin production. Together these experiments contribute to the knowledge base on adiponectin function and may lead to the recommendation for increasing n-3 fatty acid in the diet as a means to increase adiponectin concentrations and to establish healthier adipose tissue. My *specific aims* (**I**) determine if dietary αLA is converted to EPA and DHA *in vivo* and if these products are significantly enriched in tissue. We also wish to determine if these fatty acids result in an increase in transcriptional abundance or circulating concentrations of adiponectin. The second aim was to (**II**) identify the role of adiponectin in body

composition of mice, with specific emphasis on lipid storage capacity. Lastly, we sought to (**III**) identify the role of adiponectin in the activation of proliferation and differentiation in primary leptin-deficient mouse stromal vascular cells.

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CHAPTER 2. REVIEW OF LITERATURE

Co-morbidities and the Link to Adipose Tissue

Obesity is associated with the development of type II diabetes, cardiovascular disease, and cancer [1, 2]. Although these co-morbidities are multifactoral disease states, their high prevalence in obesity suggests that excess adipose tissue is a contributing factor. Examination of adipose tissue in obese humans and in animals with one or more co-morbidities indicates that this tissue can develop low grade inflammation.

Persistent inflammation and subsequent greater rates of lipolysis in adipose tissue can lead to systemic metabolic dysfunction [1, 3, 4]. During metabolic dysfunction, glycolytic tissue becomes insulin resistant and type II diabetes results. Furthermore, the unabated systemic inflammation present during metabolic stress can lead to the propagation of cardiovascular disease [5]. It is clear from the literature that obesity is a serious health concern and that adipose tissue is a key factor in the progression of this disease state and its co-morbidities.

The Role of Adipose Tissue in Homeostasis

Adipose tissue is an essential organ system for the storage of energy in animals that experience periods of starvation. Even when food is plentiful, adipose tissue is necessary for the maintenance of metabolic homeostasis. Without adipose tissue, metabolic crises develop. For instance, lipodystrophy mouse models develop severe diabetes when allowed to over consume calories. There are multiple mouse models that are lipodystrophic, with all having serum concentrations of glucose that are 2 -3 times

normal and insulin 2-400 times normal [6-11]. Furthermore, lipodystrophy is associated with increased serum triacylglycerol and free fatty acids. The metabolic profile of the autosomal recessive lipodystrophy mouse model is similar to and indicative of insulin resistance.

When adipose tissue is transplanted from a healthy donor mouse, serum glucose and insulin concentrations return to normal in lipodystrophy mice. Likewise, serum triacylglycerols and free fatty acids are significantly reduced compared to lipodystrophy animals without adipose tissue transplantation but do not equal wild type concentrations after 6 weeks post surgery [12]. The increase in serum glucose, insulin, triglycerides and free fatty acids seen in lipodystrophy mice is similar to the description of lipotoxicity as described by R. Unger in multiple reviews [13-18].

Lipotoxicity is characterized as the deleterious effects of lipid accumulation in non-adipose tissue depots. Lipid accumulation in the key metabolic tissues of liver, muscle, and pancreas leads to insulin resistance. Unlike other tissues, the unique physiology of adipose tissue allows for lipid accumulation without the development of metabolic disturbance. A classical model of lipid-induced disease in non-adipose tissue is the deposition of fatty acid in liver, which is accompanied by a rise in the activation of the transcription factor, nuclear factor κ B (NFκB) [19]. Active NFκB translocates to the nucleus and initiates the transcription of inflammatory cytokines. Tumor necrosis factor α (TNFα) is an inflammatory cytokine under the control of NFκB that increases in the liver during lipid overload [19]. TNFα inhibits insulin receptor substrate (IRS) by promotion of phosphorylation on serine residues, which leads to insulin resistance [20].

Insulin resistance in liver tissue exacerbates dyslipidemia and increases the susceptibility for the development of cardiovascular disease [21]. Furthermore, uncontrolled lipid accumulation in the liver increases the activation of an apoptotic stress response, which leads to fibrosis and cirrhosis [22].

Similarly, fatty acid accumulation in muscle tissue disturbs insulin receptor signaling as observed in skeletal muscle cells treated with saturated fatty acids, likely through the production of ceramide [23]. Ceramide interferes with metabolism by decreasing insulin receptor signaling through inhibition of protein kinase B (AKT/PKB) and inhibition of mitochondrial function. Ceramide also increases apoptosis through induction of the pro-apoptotic protein BAX [24]. Additively, lipid accumulation in muscle corresponds with an increase in TNFα and its metabolic effects. Cumulatively, lipid overload in skeletal muscle causes insulin resistance that reduces glucose uptake that may cause serum concentrations of glucose to escalate.

The most metabolically devastating effect of ectopic lipid accumulation is in the pancreas. Excess lipid in pancreatic β-cells decreases the production of essential proteins involved in insulin secretion, such as GLUT2 and glucokinase [25]. As described in other tissues, lipotoxicity can progress to cell death in the pancreas. Pancreatic β-cell death results in insulin-dependent diabetes, which leads to death if exogenous insulin is not provided [26]. Although the previously described tissue-specific effects of lipid accumulation were not examined in lipodystrophy models, the systemic metabolic profile of the lipodystrophy mouse model exemplifies the protective effects of adipose tissue against ectopic lipotoxicity and insulin resistance [27].

Adipose Tissue Physiology

The cell types present in adipose tissue can be separated into two distinct cell populations: adipocytes and cells of the stromal vascular fraction (SVF) [28, 29]. Adipocytes are the largest cells in adipose tissue and are responsible for lipid storage. Stromal vascular cells consist of myeloid immune cells, pre-adipocytes, and endothelial cells [29, 30]. The cells of the SVF of adipose tissue also includes multipotent mesenchymal stem cells and hematopoetic stem cells derived from bone marrow [31, 32]. Resident stem cells in the SVF of adipose tissue are able to differentiate into adipocytes, chondrocytes, osteoblasts, and myocytes [31, 33-35]. The presence of precursor cells in adipose tissue provides a suitable environment for rapid tissue growth.

The adipocytes and the cells of the SVF communicate through extensive paracrine signals. When mature adipocytes hypertrophy to maximal size, pro-adipogenic paracrine signals are secreted to recruit the expansion and differentiation of the preadipocyte population. Likewise, adipocytes and SVF cells communicate through the secretion of inflammatory hormones. The paracrine system of cellular cross talk in adipose tissue allows adipose tissue to adapt through hypertrophy (adipocyte expansion), hyperplasia (expansion of preadipocyte population), and differentiation (commitment of precursor cells to adipocyte phenotype).

Adipose Tissue Hypertrophy, Hyperplasia, and Differentiation

Traditionally, the number of adipocytes present in an adipose tissue depot were thought to remain unchanged after establishment early in life [36]. However,

adipose tissue is now accepted as a highly adaptable tissue [37]. In times of excess energy balance, adipocyte hypertrophy is utilized to increase lipid storage as triacylglycerol (TAG) [38]. Adipocyte hypertrophy involves the increase in lipid droplet growth and overall cell size. It is suspected that adipocytes have limited size potential. When expansion of the adipocyte encroaches on the upper limit of size, the adipocytes begin to secrete hormonal signals to promote the proliferation (mitotic replication) of preadipocytes and terminal differentiation into adipocytes [39-44].

The information on preadipocyte determination and proliferation is scarce. Only recently has part of the transcriptional control of determination been investigated and linked to the transcription factor Zfp423 [45]. Contrary to the paucity of data on the signaling pathways of determination and proliferation, preadipocyte differentiation is well documented in the literature. Differentiation of preadipocytes begins with cellular growth arrest and clonal expansion of preadipocytes [46-48]. Previous literature indicates preadipocyte cell lines require clonal expansion for terminal adipocyte differentiation [49-54]. Following clonal expansion the cells begin to produce adipocyte specific proteins and develop the characteristic lipid droplets.

The adipocyte phenotype is associated with proteins that mediate lipid metabolism and transport, insulin sensitivity, and increase the production of adipocyte specific proteins. Because of the broad range of proteins that are indicative of adipocyte differentiation, it is easier to measure the concentrations of the transcription factors controlling the expression of these proteins. The key transcription factors involved in differentiation are: Ccaat-element-binding protein alpha (C/EBPα), Ccaat- element-

binding protein delta (C/EBPδ), Ccaatt-element-binding protein beta (C/EBPβ), and peroxisome proliferator activated receptor gamma (PPARγ) [46, 55]. Previous literature has highlighted the involvement of many other transcription factor families in the regulation of adipogenesis. The list of pro-adipogenic transcription factors and co-activators includes: sterol response element-binding protein-1c (SREBP1c), Kruppel-like factors (KLF15, KLF5, and KLF6), TRAP220, TATA-binding protein-associated factor-8 (TAF8), and cyclin D3-cyclin-dependent kinases (CDK6 and CDK4). On the other hand, anti-adipogenic factors include: PREF1, transcription factor homologous to CCAAT-enhancer binding protein (CHOP), C/EBPγ, KLF2, KLF7, and GATA2/3. Interestingly, all of the previously mentioned factors influence differentiation through the modulation of the PPAR and C/EBP families of transcription factors. For instance KLF2 and GATA2/3 inhibit PPARγ, KL5 and KLF15 increase PPARγ activation [56]. Due to the breadth of literature on transcriptional control of differentiation, only the main transcription factors C/EBPα, C/EBPδ, C/EBPβ, and PPARγ are discussed in detail.

Terminal adipocyte differentiation is the result of the combined suppression of anti-adipogenic signals and the induction of proadipogenesis and as such occurs in multiple steps where the activation of one factor is dependent on the prior activation of another. Adipocyte differentiation can be separated into early and late stages. Early stages of differentiation are associated with an increase in the transcription factors C/EBPβ and C/EBPδ [57, 58]. These early phase transcription factors are responsible for the production of the late phase transcription factors PPARγ and C/EBPα. Once C/EBPβ/δ increase the late phase factors, their concentrations begin to subside. Elevation of

C/EBPα and PPARγ concentrations are essential to the terminal differentiation of preadipocytes as indicated by knockout models. C/EBPα null mice fail to develop adipose tissue where as the use of C/EBPα antisense mRNA inhibits differentiation [59, 60]. Similarly, PPARγ null embryonic stem cells fail to differentiate under normal induction techniques [61]. Conversely, overexpression of PPARγ results in differentiation of cells in the absence of hormonal signal [62].

The characterization of preadipocyte differentiation transcriptional regulation and morphology has been investigated mostly in *in vitro* models which require hormonal induction of the adipogenic phenotype [63, 64]. The most commonly used hormones for the development of an adipogeneic phenotype are: glucocorticoids, phosphodiesterase inhibitors, insulin, and thiazolidinediones [63-65]. Although the hormonal cocktail for differentiation of preadipocytes *in vitro* is well established, the *in vivo* hormonal cascade is poorly characterized.

Endocrine Function of Adipose Tissue

Prior to the discovery of the adipose tissue specific protein leptin in 1994, adipose tissue was viewed exclusively as a storage depot for lipid. Leptin was the first cytokine discovery of many in adipose tissue that led to the establishment of this tissue as an endocrine organ [66]. The list of bioactive molecules originating from adipose tissue is currently well over 100 and now includes bioactive lipids in addition to cytokines and chemokines [67, 68]. The generalized physiological effects of adipose tissue-derived hormones include the regulation of appetite, inflammation, and metabolism.

Many of the molecules derived exclusively in adipose tissue exhibit strong relationships with obesity and adipose tissue mass. Three key adipokines that are inflammatory and are increase during obesity are: TNFα, Interleukin-6 (IL6), and monocyte chemotactic protein 1(MCP1). TNFα is secreted from the stromal vascular fraction of adipose tissue, primarily from adipose tissue macrophages [69]. TNFα is a potent insulin signal disruptor through mediation of phosphorylation on serine of the insulin receptor substrate and activation of phosphotyrosine phosphatases [70]. TNFα signals through the secondary signaling proteins mitogen-activated protein kinases (MAPK), p38 MAPK, and extracellular signal-regulated kinases (ERK) [71-73]. TNFα also decreases the production of anti-lipolytic proteins and causes adipocytes to lose their lipid droplet and stop expressing PPARγ and C/EBPα, which creates a fibroblast like non-adipocyte cell [74]. The combined effects of IRS inhibition, removal of lipolytic signals, and its role in adipocyte dedifferentiation make TNFα an inducer of lipotoxicity.

IL6 is a proinflammatory cytokine secreted from adipocytes and the SVF of adipose tissue [75]. IL6 signals through JAK/STAT receptors and disrupts insulin signaling in muscle, liver and adipocytes [76]. The activation of the JAK/STAT receptor complex by IL6 increases protein kinase-C (PKC) and suppressor of cytokine signaling-3 (SOCS3), which stimulates the phosphorylation of inhibitory serines on the IRS and prevents the phosphorylation of tyrosine on the IR, respectively [77-79]. Furthermore, IL6 has been shown to have lipolytic action in adipose tissue a condition that contributes to the development of lipotoxicity [80].

Adipose tissue also secretes the chemotactic protein MCP1, a monocyte recruitment signal [81]. Like IL6 and TNFα, an increase in MCP1 concentrations is associated with a decrease in insulin sensitivity. However, MCP1 is not directly responsible for insulin resistance; rather the subsequent infiltration of macrophages is a more likely culprit [82]. Infiltration of adipose tissue by cytokine-secreting mature macrophages recruited by MCP1 perpetuates the inflammatory milieu in adipose tissue.

Leptin, the first cytokine characterized in adipose tissue, has a role in appetite, metabolism, and inflammation [83-87]. The primary location of leptin action is the hypothalamus where it suppresses appetite and activates the central nervous system [88, 89]. Thus, leptin concentrations are directly correlated to adipose tissue mass and its concentrations increase during obesity in an effort to suppress appetite [90, 91]. Leptin also has physiological functions in peripheral tissues that include metabolism and immunity. In peripheral tissue, leptin influences metabolism through phosphorylation of AMPK and regulation of gene expression through STAT3 [92-94]. Leptin-activated AMPK increases the metabolism of lipid (i.e., increased fatty acid oxidation) [92] and its storage by promoting a pro-adipogenic environment [95].

Adiponectin - Discovery and characterization

Adiponectin was identified by four independent groups between 1995-1996 and appears in the literature under multiple names (Acrp30, apM1, AdipoQ, GBP28, adiponectin) [96-99]. The crystal structure of adiponectin was reported in 1998, and, from this structure three distinct domains were identified: a collagenous domain, a variable N-terminal, and a C-terminal globular domain [99]. Previous literature indicates that the

collagenous domain of adiponectin is subject to post-translational modifications (glycosylation and hydroxylation) [100, 101]. Following these modifications, adiponectin monomers oligomerize to form a trimeric complex through non-covalent interactions in the collagenous domains. Higher order adiponectin isoforms (up to 20 monomers or 7 trimers) are formed by the aggregation of trimers via disulfide-bonds at Cys-39 of the N-terminus domain [100, 102]. Both post-translational modification and oligomerization of adiponectin is reliant on the retention of adiponectin in the adipocyte via the binding protein Erp44 [103]. Following multimerization, adiponectin is released from Erp44 by Ero1-Lα and secreted. Due to the requirement for intracellular retention in the adipocyte, high molecular weight (HMW) isoforms of adiponectin do not develop from monomers or trimers in circulation [104]. Interestingly, circulating concentrations of HMW adiponectin can be increased with the treatment of PPARγ agonists [105, 106]. The increase in adiponectin by PPARγ agonists was previously attributed to the induction of transcription (PPARy is a transcription factor that regulates adiponectin expression). However, recent evidence suggests that TZDs (PPARγ agonists) promote an increase in adiponectin secretion from adipocytes by decreasing the intracellular binding protein ERp44 while increasing the cleavage protein Ero1-Lα [103].

As a result of the complex post-translational oligormerization of adiponectin, multiple molecular weight isoforms of adiponectin are present in the sera. The four commonly described isoforms consist of the most abundant and biologically active high molecular weight isoform (HMW, >250Kda); as well as the medium molecular weight (MMW, hexamer, 180kDa), low molecular weight (LMW, dimer and trimer,70-90kda)

and the globular form [107]. The globular isomer, which makes up less than 1% of circulating adiponectin, is a proteolytic cleavage product consisting of the c-terminal end of an adiponectin trimer [108]. Although there are many circulating isoforms of adiponectin, there are only two adiponectin receptors (AdipoR1 and AdipoR2) responsible for their biological activity[109].

Adiponectin Receptors and Signaling

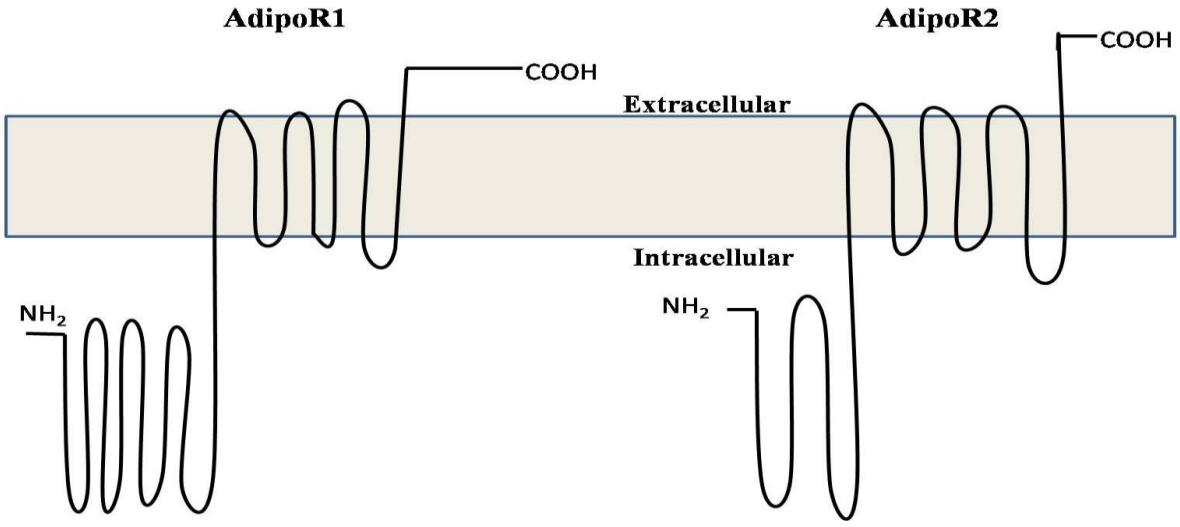

Figure 1. *Illustration of the adiponectin receptors*.

Adiponectin binds to unique seven transmembrane receptors that exist in two highly homologous (66.7% identity) isoforms, AdipoR1 and AdipoR2 [110]. Unlike the distantly related family of traditional G-coupled protein receptors (GCPRs), the adiponectin receptors have a unique rotated configuration in the plasma membrane. The carboxy terminal of the receptor is located in the extracellular space, whereas the N-terminal is on the cytosolic side of the plasma membrane (Figure 1) [110].

Activation of the adiponectin receptors occurs when adiponectin binds the carboxy end of the receptor [111]. The activation of AdipoRs does not result in the interaction with G proteins like traditional G-coupled protein recpetors (GCPR). Until recently the secondary signaling molecule for the adiponectin receptor was unknown.

The adiponectin receptor signaling pathway was recently expanded to include APPL1 (adaptor protein containing phosphotyrosine binding, pleckstrin homology domains, and leucine zipper 1), the first adaptor protein to interact with the ligand bound AdipoRs [111]. APPL1 is the bridge between the AdipoRs and downstream effector proteins, like Rab5 (a small GTPase) and AMPK. Rab5 is involved in the phosphorylation of AKT and the subsequent translocation of GLUT4 to the cell membrane [112]. APPL1-mediated phosphorylation of AMPK results in the inhibition of IKK of the NFkB pathway [113], increase in GLUT4 translocation, increase in insulin sensitivity, and promotion of fatty acid oxidation. APPL1 is also speculated as promoting cellular proliferation through AKT-mediated reduction in BAD/Bcl2 apoptotic signals, as well as activation of the cell proliferation signal ERK 1/2 [114].

Although APPL1 interacts with both AdipoR1 and AdipoR2, activation of either receptor isoform results in a distinct physiological outcome. AdipoR1 activation is generally associated with insulin-sensitizing effects mediated through the phosphorylation of AMPKα, AdipoR2 is commonly attributed to the activation of PPARα and the subsequent production of lipid-oxidizing enzymes [115, 116].

A third receptor for adiponectin was proposed to be T-cadherin by the Lodish group. This group demonstrated successful binding of trimeric adiponectin to the

adhesion molecule T-cadherin [117]. It is unknown if the binding of adiponectin results in intracellular signaling given that T-cadherin is an adhesion molecule with a limited intracellular domain. A more likely function of T-cadherin is to hold adiponectin closely to the cell surface where it can interact with either adiponectin receptor 1 or receptor 2.

Physiological Effects of Adiponectin Signaling Varies by Tissue

The diverse physiological effects mediated by adiponectin are the result of the tissue distribution of the AdipoRs. AdipoR1 is ubiquitously distributed in tissues and highly expressed in skeletal muscle. The AdipoR2 is primarily expressed in abundance in the liver [110]. To understand adiponectins metabolic effects it is important to characterize its effects in the three key tissues where adiponectin has the greatest physiological impact in regards to obesity: muscle, liver, and adipose tissue.

Adiponectin and Muscle. Adiponectin increases glucose uptake and fatty acid oxidation in muscular tissue [93, 118]. Increased glucose uptake in muscle is the result of AMPKα phosphorylation through AdipoR1 activation by globular adiponectin [116]. Phosphorylated AMPKα increases glucose uptake through a reduction in the inhibitory phosphorylation of serine on insulin receptor substrate 1 (IRS1) and activation of glucose transporter 4 (GLUT4) [119]. Activation of adiponectin receptors in muscle also leads to the activation of p38 mitogen-activated protein kinase (MAPK) by AMPK phosphorylation [120]. p38 MAPK phosphorylates and activates PPARα [110, 121]. Active PPARα translocates to the nucleus and increases the transcription of fatty acid oxidative enzymes (acetyl-CoA oxidase (ACO) and carnitine palmitoyltransferase 1

(CPT1)) [115]. Therefore, the main effects of adiponectin in skeletal muscle tissue are to promote insulin stimulated glucose uptake and increase fatty acid oxidation.

Adiponectin and Liver. In the liver, full length adiponectin reduces glucose production through two distinct pathways [93, 122]. Specifically, the AdipoRs phosphorylate AMPK, which causes an attenuation of phosphoenolpyruvate carboxykinase and glucose-6 phosphatase expression [123]. Furthermore, active AMPK phosphorylates acetyl-CoA carboxylase (ACC). Phosphorylated ACC results in the inhibition of ACC enzyme function, inhibiting *de novo* lipogenesis [93, 124]. Interestingly, the liver has a high concentration of AdipoR2 isoform, which is associated with the activation of PPARα. As described for skeletal muscle, PPARα increases the oxidative potential of liver tissue. Therefore, the main metabolic effect of adiponectin in the liver is to decrease gluconeogenesis and *de novo* lipogenesis while increasing fatty acid oxidation.

Adiponectin and Adipose Tissue. Similar to muscle and liver tissue, the activation of AdipoR1 by globular adiponectin results in an increase in glucose uptake through AMPK phosphorylation [125]. Adiponectin-stimulated AMPK increases the translocation of GLUT4 to the membrane of adipocytes [112]. AMPK phosphorylation in adipose tissue promotes the phosphorylation and inhibition of ACC, thus reducing *de novo* lipogenesis[125]. Accompanying the decrease in *de novo* lipogenesis, adiponectin signals through p38 MAPK to activate PPARγ coactivator-1 (Pgc-1). Activation of PGC1 increases FA oxidation [126]. Adiponectin signaling in adipose tissue also activates PPARα inducing β-oxidation in the adipocyte. Depending on the species, adiponectin

may also decrease the *de novo* lipogenesis in adipose tissue as seen in liver tissue. For instance, swine adipose tissue is responsible for almost 95% of *de novo* lipogenesis whereas humans rely on adipose tissue for less than 5% of *de novo* lipogenesis.

Serum Adiponectin Concentrations. Clinical investigations have identified a negative correlation between serum adiponectin concentrations and body weight [127]. In healthy non-obese humans, serum adiponectin circulates at 2.0 µg/ml to 17.0 µg/ml, depending on gender and age, with an average of 9.0 µg/ml. [127-130]. These concentrations drop to an average of 4.0 µg/ml in obese individuals and are associated with increased disease risk [127]. Mouse adiponectin concentrations are also greater in female, lean, and younger animals. Healthy mice have serum adiponectin concentrations ranging from 3-15 µg/ml [105, 131, 132]. Likewise, swine serum adiponectin concentrations are close to humans with concentrations of 5-30 µg/ml (unpublished data from our laboratory).

Adipose Tissue Depot Differences

Each of the multiple adipose tissue depots have distinct physiological profiles and should be considered distinct tissues. The two general locations of adipose tissue commonly described in the literature are subcutaneous and visceral. Within the visceral compartment, adipose tissue depots can be separated into omental or mesenteric. Physiological differences in adipose tissue depots can range from lipolytic sensitivity to increased risk of developing insulin resistance [133-137].

The most notable difference in adipose tissue depots is their cytokine secretion capabilities. In regards to the investigation of obesity-related inflammatory cytokines, a distinct profile exists for each adipose depot. Human omental fat secretes almost 3 times the amount of IL6 than cells from the subcutaneous depot [138]. Likewise, MCP1 is secreted in greater concentrations from visceral adipose tissue than subcutaneous adipose tissue in humans [139]. Also, the production and secretion of the two key adipokines leptin and adiponectin in humans are greater in subcutaneous adipose tissue compared with visceral tissue [140-143].

The plasticity of adipose tissue depots can also differ. Wang et al. and Lau et al. identified a greater propensity for adipogenesis in perirenal depots versus epididymal adipose tissue [144, 145]. Likewise, the expansion of subcutaneous adipose tissue is mediated primarily by adipogenesis whereas expansion of visceral adipose tissue is associated more with adipocyte hypertrophy [146]. Similarly, adipose tissue depots in rats differ in their method of expansion; subcutaneous adipose tissue and retroperitenal adipose are more likely to increase in adipocyte number whereas mesenteric and epididymal adipose increases cell volume [147]. Interestingly, omental adipose tissue of humans is less responsive to PPARγ agonists in regards to differentiation compared with subcutaneous adipose tissue [148] but the SVF cell number of the subcutaneous depot of obese humans is lower than that of the omental or mesenteric adipose tissue depots [133].

Metabolic function also varies across adipose tissue depots. Adipocytes from visceral depots are documented as having greater lipolytic rate than subcutaneous depots [134-136]. Furthermore, mesenteric adipose tissue is less sensitive to insulin and

lipolytic hormones [147, 149]. Similarly, human omental adipose tissue have a lower efficiency in insulin signaling than visceral adipose tissue as observed by a decrease in GLUT4 and an increased abundance of the lesser efficient insulin receptor splice variant [137].

Dysfunction of Adipose Tissue (causes, consequences, and adiponectin)

Causes of Adipose Tissue Dysfunction

The primary insult responsible for the initiation of inflammation in adipose tissue is still under investigation. Obesity creates a lipid-rich environment in adipose tissue. This condition increases adipose tissue susceptible to the development of inflammation. The inflammatory profile (e.g., cytokines, transcription factor activation) present during adipose tissue dysfunction indicates endoplasmic reticular stress (ER stress) may be involved.

Endoplasmic Reticulum Stress

The ER is an organelle responsible for the processing of cellular proteins. Dysfunction of the ER results in misfolded and non-functional proteins. Accumulation of misfolded proteins in the ER leads to inflammation and apoptosis. The unfolded protein response (UPR) is a key rescue system used by the cell to correct ER stress [150]. The UPR utilizes the following signaling proteins to alleviate stress; PKR-like ER-localized eIF2α kinase PERK, IRE-1, and activating transcription factor 6 (ATF-6). Briefly, all of these proteins are associated with the ER membrane and are activated when misfolded or non-functional proteins accumulate in the ER lumen [150, 151]. PERK and IRE-1 both

autophosphorylate and activate subsequent signaling pathways. ATF-6 is a nuclear transcription factor that must be modified in the golgi prior to translocation to the nucleus [151, 152]. PERK signals the phosphorylation and inhibition of translation factors like eIF2α, IRE-1 activates XBP1, which, in turn, mediates an increase in the transcription of protein chaperones. An increase in the production of protein chaperones enhances the ER secretion of proteins and promotes ER biogenesis [152, 153]. Chaperone proteins are also increased via ATF-6 transcriptional control.

The inflammation present during UPR is mediated through IRE-1 and PERK. IRE-1 activates the transcription factors c-Jun N-terminal kinase (JNK), NFkB pathways, while PERK only activates NFkB. Translocation of the transcription factors JNK and NFkB to the nucleus results in the transcription of pro-inflammatory proteins (IL6, IL8, TNFα, and MCP1) [154-156]. Reactive oxygen species (ROS) generated as byproducts of increased disulfide bond formation during UPR also causes inflammation [154, 156, 157].

Several of the inflammatory proteins that are elevated with ER stress are also present during obesity. These inflammatory proteins include IL6, TNFα and MCP1. In addition, obesity is also associated with elevated ROS. Similarly, elevated PERK and JNK activation have been detected in the adipose tissue of obese mice [158]. The link between ER stress and inflammation is complicated by the observation that the UPR activates inflammation and is induced by inflammation. Xue et al. identified an increase in the UPR in murine fibrosarcoma cells treated with ROS and TNFα [159]. These findings indicate that ER stress may be initiated by inflammation. ER stress-induced

inflammatory cytokines may cause a feed forward response resulting in a cyclical inflammatory cascade.

Unresolved low grade inflammation is a hallmark indicator of increased susceptibility for the progression of insulin resistance in obese animals. Nakatani et al. linked ER stress with a decrease in insulin sensitivity in obese mice [160]. Furthermore, ER stress may be involved in the progression of adipose tissue mass accretion. Scheuner et al. developed a knockout mouse model that allowed for non-interruptible protein synthesis [161]. This model causes a protein overload and subsequent ER stress [161]. When Scheuner et al. exposed these ER stress susceptible mice to a high fat diet, they developed almost twice the adipose tissue mass of the wild type controls [162]. The evidence presented here indicates ER stress is likely a key player in the progression to physiological dysfunction during the obese state.

Consequences of Adipose Tissue Dysfunction

Inflammation

Clinical observations indicate a positive correlation between obesity and systemic inflammation [163, 164]. In the commonly used mouse obesity models, adipose tissue inflammation has been suggested as a contributing factor of systemic inflammation. Furthermore, i*n vitro* studies demonstrate that the 3T3L1 adipocytes secret inflammatory cytokines when subjected to extended lipid filling, saturated fatty acid (SFA), or an inflammatory agent like lipopolysaccharide (LPS) [165-167]. As mentioned previously, the primary pro-inflammatory cytokines of interest in adipose tissue are IL6, TNFα, and

MCP1. These inflammatory cytokines have the distinct ability to modulate metabolism and promote macrophage infiltration into adipose tissue.

The Role of Adipocytes, Pre-adipocytes, and Immune Cells in Adipose Tissue Dysfunction

Fain, Ross and McIntosh [168] propose that cells located in the SVF of adipose tissue are responsible for the majority of the tissue inflammation [69, 169, 170]. Further investigation reveals pre-adipocytes and macrophages are the main cytokine secretors in the SVF [171]. Although adipocytes are not considered the primary cytokine producers, they do have a pivotal role in the initiation of inflammation in the SVF. Adipocyte stress results in TNFα and IL6 secretion that stimulates the pre-adipocyte population and resident macrophages to produce inflammatory cytokines. The compounding inflammation derived from adipocyte and the SVF provides the ideal environment for monocyte accumulation within adipose tissue.

The establishment of an inflammatory macrophage population in adipose tissue leads to a cyclic inflammatory milieu and systemic inflammation. Immunohistochemstry of adipose tissue has demonstrated that macrophages infiltrate and tend to form a "crown" like structure around dying adipocytes that stain negative for perilipin [28]. Furthermore, Weisberg et al. suggests adipose tissue macrophages are the primary source of circulating TNFα in obese animals and are responsible for 50% of the IL6 secreted from adipose tissue [171].

Insulin Resistance as an Outcome of Inflammation

A direct consequence of low-grade inflammation is the peripheral down regulation of insulin signaling. Inflammation-induced insulin resistance is caused by insulin post receptor signaling inhibition [172]. Specifically, excess TNFα results in the increased inhibitory phosphorylation of serine on insulin receptor substrate (IRS) [173-176]. Furthermore, IL6 has similar properties as TNFα in the induction of insulin resistance (reduced IRS and GLUT4 activation) in the adipocyte [177].

The development of insulin resistance in muscle, liver, and adipose tissue is detrimental to systemic homeostasis. Inhibition of insulin receptor secondary signaling in adipose tissue removes the inhibitory stimulus of hormone-sensitive lipase (HSL). Increased fatty acid release by HSL and the increase in TNFα and/or IL6 stimulated lipolysis [178, 179] results in an increase in circulating lipid concentration [180-183]. Obesity-associated inflammation also affects adipose tissue lipid storage via inhibition of adipose tissue growth. TNFα is also shown to reduce the expansion of adipose tissue by inhibiting adipocyte hypertrophy [184]. TNFα also inhibits adipogenic potential through a reduction in PPARγ signaling [185-187].

Insulin resistance in muscle and liver tissue reduces the amount of glucose entering the tissue causing an elevation in serum glucose. An increase in serum glucose and lipid that is accompanied by deceased capacity for adipose tissue storage can lead to lipotoxicity and its associated complications.

Adiponectin and Adipocyte Dysfunction

Adiponectin has Anti-inflammatory Functions

Early research on adiponectin identified a domain that is homologous to complement protein 1 and a domain with structural similarity to TNFα (hence the early name C1q) [98, 99]. Presence of a complement protein homology domain and the similarity to TNFα spurred the investigation of adiponectin as an inflammatory protein. However, clinical observations identified an inverse correlation between adiponectin and mild inflammation, suggesting that adiponectin may repress inflammation [129, 188]. Furthermore, adiponectin is reported as reducing the presentation of TNFα-associated adhesion molecules in endothelial tissue [189]. Adiponectin is also a known inhibitor of NFκB and ROS and promotes the production of the anti-inflammatory cytokine IL10 [190].

Adiponectin Regulates Macrophages

Adiponectin decreases adipose tissue inflammation through the reduction of macrophage infiltration [124, 171, 191]. Adiponectin reduces the adipose tissue macrophage population through multiple mechanisms: inhibition of monocyte adhesion to epithelial membranes, reduction of pro-macrophage cytokines, and retardation of macrophage growth.

The NFkB transcription factor family of cytokines (e.g., MCP1, TNFα, IL6) are known inducers of macrophage infiltration into adipose tissue [192-194]. Adiponectin-documented inhibition of NFkB and its activation of PPARα both reduce the

concentration of MCP1 [165, 195]. Adiponectin also down regulates the expression of the adhesion molecules VCAM-1, ICAM-1, and E-selectin [189]. These findings are corroborated by the observation that immune cell interactions with endothelial cells increase during adiponectin deficiency [196]. Lastly, adiponectin inhibits the growth of monocytic progenitors and macrophages [197]. Collectively, these key reports indicate that adiponectin reduces macrophage infiltration into adipose tissue by inhibiting inflammatory cytokines, decreasing binding proteins, and retarding of macrophage cellular growth.

Adiponectin and Metabolism (Prevention of Lipotoxicity and Insulin Resistance)

Prior to the 2007 publication by Kim et al. [198], the accepted mechanism of adiponectin-mediated correction of metabolic disorders was through insulin sensitization and the resolution of inflammation. Briefly, reduction of inflammatory cytokines (as described in the previous sections) by adiponectin is known to result in favorable insulin signaling. When resolution of inflammation is accompanied by the strong glucose sensitizing and lipid lowering effects of adiponectin in the liver, muscle, and adipose tissue, there is a direct abatement of insulin resistance [199]. However, Kim et al. [198] published a report that suggests adiponectin prevents insulin resistance through promotion of adipose tissue expansion [198]. This study examined the effects of transgenic overexpression of a mutated form of adiponectin in the obesity prone ob/ob mouse model. The combination of the *ob/ob* obesity model and high concentrations of adiponectin produced the fattest obese mouse model to date. Intriguingly, these massively obese mice presented serum metabolic markers within the normal range. Unlike the

transgenic animals, the non-transgenic *ob/ob* mice exhibited severe metabolic disturbances. The authors concluded that massive expansion of subcutaneous adipose tissue allowed for excessive lipid storage, thereby preventing the adverse consequences related to lipotoxicity. Although this study was well executed, it failed to address the physiological ramifications of a lack of leptin. Furthermore, the author's state that the product of the mutated adiponectin transgene never exited the adipocyte. The authors only found native adiponectin in the circulation. The authors disregarded the physiological implications of adiponectin intracellular accumulation. Kim et al. [198] further concluded that the increase in subcutaneous adipose tissue mass was a result of adipogenesis, as based on histological procedures. This controversial study is one the first to suggest adiponectin protects homeostasis through adipose tissue growth. However, the research on the effects of adiponectin on adipose tissue morphology is limited and contradictory [108, 200, 201].

Adiponectin: Proliferation and Differentiation

Evidence from an *in vitro* 3T3L1 cell culture model indicates proliferation and differentiation of preadipocytes is increased with the hyperexpression of adiponectin [200]. Furthermore, adiponectin was also shown by Carmo-Avides and colleagues to increase the differentiation of human pre-adipocytes [202]. Conversely, a recent publication with adiponectin overexpression showed lower adipocyte differentiation specific signals (e.g., CEBPα) and an increase in the presence of preadipocyte markers (e.g., Pref-1) in the inguinal fat of transgenic mice [203]. Additively, activation of the main secondary signaling molecule of the adiponectin receptor, AMPK, has been shown

to reduce the expression of the transcription factors PPARγ and CEBPα in mesenchymal cells and preadipocytes [204-206]. Supporting these data is the observation that AMPK null mice experience increased adipocyte hypertrophy and differentiation [207].

Methods for Influencing Obesity-Associated Complications

The modulation of adipose tissue dysfunction and preservation of metabolic homeostasis in obesity can be influenced by multiple factors. Significant weight loss is a proven method of obesity management and is a successful way to reduce the inflammatory cytokine profile in adipose tissue [208]. Weight loss is attributed to the improvement of inflammation, insulin resistance, and the return of normal adiponectin concentrations. A secondary approach to reducing obesity associated complications is through the consumption or avoidance of specific dietary fatty acids. Fatty acids and their metabolic by-products are known effectors of inflammation. A classic representation of this is the proinflammatory role of arachidonic acid. There has been a recent focus on the biological activity of SFA and the omega 3 (n-3) fatty acids as modulators of obesity associated inflammation and metabolic dysregulation

Dietary Fatty Acids and Obesity

Saturated fatty acids, specifically medium chain fatty acids (palmitate, myristic, laurate) are described by multiple groups as inducers of adipocyte and macrophage inflammation in adipose tissue [73, 166, 209, 210]. Conversely, there is an increasing amount of data indicating n-3 fatty acids and monounsaturated fatty acid (MUFA) are beneficial for the prevention and resolution of obesity-associated inflammation [211,

212]. Consumption of n-3 fatty acids is associated with a reduction in circulating TNFα, C-reactive protein (CRP), and IL6 [212-214]. Likewise, dietary MUFA has been shown to decrease CRP and IL6 [211].

The majority of the current literature studying the anti-inflammatory effects of n-3 fatty acids focuses on eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) dietary supplementation. EPA and DHA are the byproducts of alpha-linolenic acid (αLA) modification by multiple steps: desaturation, elongation, and oxidation. Briefly, the conversion αLA to EPA consists of the desaturation of αLA by Δ6 desaturase, a subsequent elongation to 20 carbons, and desaturation by Δ5 desaturase [215]. The conversion of EPA to DHA requires the elongation to a 24 carbon chain followed by desaturation with Δ5 desaturase and the shortening of the carbon chain to 22 by peroxisomal oxidation [215]. Although humans possess the appropriate enzymatic machinery for the conversion of αLA to EPA and DHA, the conversion rate is estimated to be less than 6% [216]. The limited data on αLA feeding describes an increase in plasma and tissue concentrations of EPA, but not DHA [217].

Interestingly, not all animals rely on hepatic tissue for the *de novo* production of fatty acids. One key tissue that is commonly overlooked as a site for fatty acid production is adipose tissue. Depending on the species, adipose tissue can support up to 90% (porcine) or as low as 5% (humans) of *de novo* lipogenesis [218]. Although not required, the presence of *de novo* lipogenesis and a high concentration of proteins related to lipid metabolism make adipose tissue an ideal target for the investigation of fatty acid conversion. There is currently a paucity of data on the conversion of αLA to EPA or

DHA in adipose tissue. Regardless of the origin of the n-3 fatty acids (*in vivo* conversion or consumed), previous literature indicates n-3 fatty acids are important for prevention and resolution of adipose tissue inflammation.

Effects of FA on Adipose Tissue

n-3 FA and Membrane Enrichment

Consumption of a diet rich in n-3 fatty acids results in the enrichment of cell membranes through the substitution of n-6 fatty acids [219]. Membrane enrichment with n-3 fatty acids has multiple physiological implications, including alteration of the functional capacity of receptors located in lipid rafts [220, 221]. It is therefore likely that n-3 fatty acids are able to modify insulin or inflammation-associated receptor signaling in adipose tissue. Interestingly, the enrichment of n-3 fatty acids does not occur at the same rate in all tissues. Purified EPA supplementation increases plasma phospholipids in humans after six weeks of intervention [222]. Enrichment of DHA in adipose tissue, skeletal muscle, and cardiac muscle have been shown to occur within three to six months in humans supplemented with DHA [217, 223-227].

n-3 FA and Macrophage Recruitment and Inflammation

Macrophage infiltration into adipose tissue is an indicator of tissue inflammation. The macrophage-associated increase in inflammation has been characterized as a contributing factor in the development of insulin resistance [228]. Enrichment of the cellular environment whether *in vivo* or *in vitro* with n-3 fatty acids reduces inflammation and inhibits immune cell interaction with adhesion molecules, preventing monocyte

infiltration [229-232]. Furthermore, n-3 fatty acids are able to decrease the inflammatory contribution of resident tissue macrophages. Novak et al. showed an inhibition of NFκB pathway by n-3 fatty acids in culture using LPS-stimulated macrophages [231]. Likewise, Fujisaka et al. identified a phenotypic switch in resident macrophages from M1 (associated with the proinflammatory cytokines TNFα, IL6, and MCP1) to M2 (produce anti-inflammatory cytokines IL10 and IL1 receptor antagonist) with n-3 supplementation [81, 230]. Similarly, purified EPA supplementation suppresses macrophage inflammation in obese animals and increases the secretion but not the transcription of adiponectin [233]. Conversely, multiple groups have reported that supplementation of n-3 fatty acids increases PPAR nuclear receptor activation and subsequent adiponectin transcript abundance [234-236].

n-3 FA and Peroxisome Proliferator Activated Receptors

The n-3 fatty acids EPA and DHA have been shown to increase the expression of adiponectin through activation of the PPARγ nuclear receptor [237]. Neschen et al. identified an increase in adiponectin in mice fed n-3 fatty acids and was able to abolish this increase with PPARγ inhibitors [238]. Furthermore, Banga and colleagues showed an increase in adiponectin by DHA activation of PPARγ in 3T3L1 adipocytes [239].

Interestingly, n-3 FA also activate the PPARα nuclear receptor [237]. PPARα controls the expression of oxidative enzymes [240]. As described previously, adiponectin is also able to activate PPARα [116]. The theoretical combined action of n-3 fatty acid stimulation of PPARα and the secondary stimulation through adiponectin (increased by PPARγ activation) would present a strong signal for increased fatty acid oxidation.

These effects were described by Flachs et al. in male mice consuming n-3 fatty acids. Flachs et al.[234] identified a threefold increase in mitochondrial biogenesis and a significant increase in the oxidation of palmitate in the n-3 treatment group [234].

n-3 FA: Resolution of Serum Inflammation and an Increase in Glucose Uptake

Supplemental intake of fish oil, which is rich in EPA and DHA, decreases serum TNFα and IL1β by approximately 80% in healthy subjects after eight weeks [229]. Furthermore, dietary supplementation of n-3 fatty acids protected mice against high fat diet-induced adipose tissue inflammation [232]. The reduction of known insulin signal disruptors with the supplementation of n-3 fatty acids is also accompanied by an increase in GLUT4 transcription and subsequent glucose uptake in the adipose tissue of rats [241].

n-3 FA and Adiposity

Previous research indicates n- 3 fatty acids inhibit the growth of adipose tissue through modulation of proliferation and differentiation of preadipocytes. Data by Ruzickova et al. indicates preadipocyte proliferation is inhibited in the adipose tissue of mice consuming n-3 fatty acids [241]. Differentiation of preadipocytes in rats and mice, are inhibited with the reduction in adipocyte derived PGI2 by n-3 fatty acid [219, 242]. Furthermore, n-3 fatty acids reduce differentiation by induction of apoptosis in post confluent preadipocytes, thereby lowering the preadipocyte pool [243].

Along with the alteration of adipose tissue cellularity, n-3 fatty acids also decrease hypertrophy of mature adipocytes. Adipose lipid filling is decreased in mice when 9 % of a high fat diet is replaced with EPA/DHA [234]. Similarly, obese mouse

models exhibit a decrease in adipocyte size with EPA supplementation [233, 241]. A reduction in adipocyte hypertrophy also occurs in the visceral adipose tissue of rats consuming EPA and DHA [244-246].

Saturated Fatty Acid-Induced Inflammation

Saturated fatty acids are capable of inducing inflammation and are the main constituents of the immunoreactive segment LPS called the lipid A component [247, 248]. The SFAs; myristic, palmitic, and stearic acids are highly enriched in the lipid A component of LPS. Multiple groups have observed induction of an inflammatory response in adipose tissue with the individual SFAs of the lipid A tail [165, 249]. Induction of inflammation by SFA in adipose tissue has profound implications for dietary FA and the development of obesity.

Recent research has focused on the role of toll like receptors, specifically TLR4 and TLR2, in inducing inflammation related to SFA [250-252]. Previous literature has narrowed in on TLR4 and TLR2 because of their distinct ability to bind LPS [251]. Data from our laboratory has shown a significant induction above control in NFκB and its gene product IL6 in 3T3L1 adipocytes treated with palmitate or LPS [253]. Furthermore, TLR4 null mice were protected from inflammation and insulin resistance when challenged with a high SFA diet [167, 254].

Saturated Fatty Acid –Associated Inflammation and Insulin Resistance

High SFA content in cells results in high diacylglycerol and/or ceramide concentrations, both of which activate protein kinase C (PKC) [255, 256]. PKC activates

IκB kinase and c-jun-N-terminal kinase, which, in turn, induce serine phosphorylation and inhibition of IRS-1. PKC also induces the production of pro-inflammatory cytokines [209].

Saturated fatty acids increase TNFα, IL6, and MCP1 production through the activation of NFkB and AP1 transcription factors via the TLR4 and TLR2 signaling pathway [252, 257]. As described in a previous section, TNFα and IL6 are both involved in insulin resistance. The over accumulation of SFAs in tissue increases inflammation through the production of ROS and an increase ER stress caused by phosphorylation of eIF2α and JNK [258]. Research also indicates that laurate, palmitate, and myristate increase the production of chemotactic proteins in adipose tissue, increasing the probability of macrophage infiltration [259]. The combined inflammatory induction, immune cell recruitment, and direct inhibition of insulin signaling by SFAs indicate a strong role for SFAs in obesity-related disease.

Summary

In the US, a staggering amount of economic, medical, and intellectual resources are devoted to the treatment of obesity and its co-morbidities. Alleviation of obesity-related lipotoxicity and inflammation would reduce the incidence of the obesity-associated co-morbidities: insulin resistance and CVD. Lipotoxicity is absent under normal metabolic conditions because adipose tissue sequesters excess lipid, preventing ectopic deposition. The plasticity of adipose tissue allows for rapid tissue expansion through hypertrophy and adipogenesis when under an increased demand for lipid storage. However, when the lipid load surpasses the limit of adipose tissue expansion, multiple

stress signals develop. Stressed adipose cells secrete inflammatory cytokines. Paracrine cytokine activation of neighboring cells can lead to a cyclical inflammatory cascade. These pro-inflammatory cytokines cause the disruption of metabolic homeostasis in insulin-sensitive tissues like muscle and liver. Furthermore, inflammation results in increased adipose tissue lipolysis. A lipolysis-mediated increase in circulating lipid can induce lipotoxicity and subsequent cyclic inflammatory, response metabolic dysregulation, and eventual tissue necrosis.

The adipose tissue-derived protein adiponectin has anti-inflammatory, insulin-sensitizing and adipogenic properties that can decrease the negative effects of adipose tissue stress. However, the concentrations of this protein are negatively associated with the inflammatory milieu present in obesity. Previous literature indicates adiponectin reduces obesity-associated stress by promoting adipose tissue expansion. High circulating concentrations of adiponectin in a leptin-deficient model of obesity results in massive adipose tissue accretion. Furthermore, the massive expansion of adipose tissue, mediated by adiponectin, protects against obesity associated inflammation and metabolic complications. The role of adiponectin in promotion of adipose tissue expansion represents a divergence from the previously published body of literature indicating that adiponectin promotes insulin sensitization through an increase in lipid oxidation and a decrease in glucose concentrations. Thus, further examination of the affects adiponectin has on adipose tissue morphology is warranted. There is currently no pharmaceutical treatment for low concentrations of adiponectin. However, dietary fatty acids of the n-3 family have been shown to increase adiponectin concentrations and decrease the

inflammation and metabolic disarray associated with obesity. It is unknown from these data if n-3 fatty acid consumption is required or if the endogenous conversion from precursor fatty acids is sufficient for the prevention of adipose tissue inflammation.

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CHAPTER 3. METABOLIC AND INFLAMMATORY CHANGES IN ADIPOSE TISSUE OF PIGS FED DIETS HIGH IN LARD OR CANOLA OIL

Abstract

Low-grade inflammation associated with obesity is affected by multiple variables including dietary fatty acids. Previous literature has identified saturated fatty acids as an inducer of the pro-inflammatory cytokines TNFα and IL6 and the chemokine MCP1. Conversely, the omega 3 fatty acids, EPA and DHA, are associated with anti-inflammatory effects. There are limited data on the effects of EPAs precursor αLA. The aim of this study was to characterize the inflammatory profile, tissue FA profiles, and metabolic state of swine after 11 weeks of consuming either a 30% canola oil diet, 25% lard diet, or control diet. We also sought to elucidate the role of dietary fatty acids in adiponectin production. Our data indicate no difference in serum inflammatory profile, metabolic profile, or adiponectin in swine after 11 weeks of diet. Furthermore, liver concentrations of EPA and docosapentaenoic acid (DPA) in liver were similar between the canola and control diets regardless of dietary fatty acid content. The pigs fed the canola diet had significantly lower numbers of subcutaneous adipose tissue macrophages compared to animals fed lard or control diets. Overall, the data presented herein indicate dietary enrichment of canola oil reduces the inflammatory potential of the subcutaneous adipose tissue, whereas a diet rich in saturated fatty acids may take longer than 11 weeks for inflammation to occur in the commercial pig.

Introduction

Obesity-associated inflammation and insulin resistance are among the most prevalent detriments to human health and well-being. These chronic disease states impose enormous economic burdens on the U.S. and global economies. Consumption of diets high in saturated fat acids (SFA) are a major determinant of health, particularly with respect to cardiovascular disease, obesity, and insulin resistance (1, 2). The contribution of SFA to the development of disease is due to its biological activity, which extends far beyond that of a simple energy source. The biological activity of SFA is exemplified by palmitate, which induces inflammation in cultured macrophages (3), dendritic cells (4), and 3T3-L1 adipocytes. Conversely, n-3 fatty acids stimulate adiponectin secretion from adipocytes, attenuate adipose expansion, and protect against inflammation in some models (5-14). Whereas n-6 polyunsaturated fatty acids (PUFA) have been reported as pro-inflammatory, the n-3 PUFAs attenuate inflammation through competition with n-6 PUFA as a substrate for cyclooxygenase 2 and also via inhibition of the NFκB transcription factor (3, 15, 16). Consequently, there is considerable interest in enriching tissues with n-3 fatty acids via dietary means to alleviate inflammation.

The anti-inflammatory effects of n-3 PUFA are largely attributed to dietary docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) because the conversion from their precursor alpha-linolenic acid (αLA) is minimal *in vivo* (i.e., 6% or less of circulating DHA is from αLA) under normal dietary conditions (17). Recent evidence indicates that diets rich in αLA do, in fact, alleviate some types of inflammation and reduce the risk for cardiovascular disease (18, 19). Furthermore, Ren et al. (20) reported

that αLA attenuates the induction of NF*k*B and iNOS production in Raw 264.7 cells. It is relatively unknown, however whether αLA directly affects inflammation or if its byproducts EPA and DHA mediate the effects.

Rodent models have been the pillar of obesity and metabolic research, especially when invasive techniques are required. However, there are clear metabolic and physiological differences between humans and rodents, and these differences complicate the translation of biomedical research findings into effective dietary intervention strategies (21). Consequently, alternative model species are being pursued, and the pig is emerging as an attractive biomedical model. Contemporary genetic lines of pigs used in the commercial pork industry have been intensely selected for lean body composition over the past five decades. However, these genotypes are an excellent humanoid model for atherosclerosis when fed substantial quantities of lard and cholesterol, and researchers have quite successfully extended this model to include atherosclerosis accelerated by streptozotocin-induced diabetes (22).

On the other hand, there is a paucity of data addressing obesity-linked inflammation in the adipose tissue of the pig or the impact of specific dietary fats on insulin resistance and inflammation. Our objective was thus two-fold. First, we sought to determine whether inflammation could be induced in adipose tissue of commercial pigs fed a diet high in the saturated fatty acid palmitate from lard, and whether this inflammatory response differed from that induced by a diet high in αLA from canola oil. Secondly, we wanted to determine whether providing a diet high in α-LA resulted in a measurable enrichment of selected tissues with n-3 fatty acids.

Materials and Methods

Animals and diets. All animal procedures where approved by the Iowa State University Animal Care and Use committee. Twenty three, 18-week-old pigs of a Yorkshire cross commercial genotype averaging 63.6 kg were randomly assigned to three dietary treatments and housed in separate pens in an environmentally controlled facility. Water and feed were provided *ad libitum* for the duration of the 11-week study. The dietary treatments consisted of a low fat, corn-based control, a high by saturated fat (Lard) diet and a diet high in canola oil (Table 1). The pigs were brought to a surgical plane of anesthesia with a mixture of ketamine, xylazine, and telazole (2.2mg/kg body weight of each component) following a 12 hour fast. After the animals were anesthetized, they were euthanized by exsanguination. At the time of euthanasia, blood was collected from the jugular vein. Samples of liver, longissimus dorsi (LD) muscle and subcutaneous and mesenteric adipose tissues were removed, frozen in liquid nitrogen, and stored at -80° C until removed for sample analyses.

Fatty acid analysis. All collected tissues were analyzed for fatty acid content by procedures as described previously by Gabler et al [23]. Briefly, lipids were extracted from 0.5 g of tissue using the method of Lepage and Roy. Following extraction, 0.74 µM heptadecanoic acid was added to each sample as a positive control. Samples were then analyzed for fatty acid methyl esters using a gas chromatograph (model 6890; Hewlett-Packard, Palo Alto, CA) with an Omegawax 320 capillary column (Sigma-Aldrich).

RNA extraction and quantitative PCR . Total RNA was recovered from liver, muscle, and adipose tissue using Trizol reagent (Invitrogen, Carlsbad, CA), and residual

DNA was removed by DNase treatment (Turbo DNase, Ambion, Houston, TX, USA). The iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) was used to reverse-transcribe 2 µg of purified RNA. Primer sets were all designed for 60°C annealing temperature, allowing for the use of the same thermal cycling parameters: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Primer sequences for porcine SWC3, acetyl Co-A carboxylase (ACC), delta-6 desaturase, adiponectin, uncoupling protein 3 (UCP3), MCP1, 12S rRNA, and RPL32 genes are shown in Table 2. The identity of the PCR products were confirmed by sequencing, cloned into the pGEMT vector (Promega, Madison, WI), and diluted serially to provide a standard curve for each transcript. Reactions were carried out in an iCycler using the IQ SYBR Green Supermix kit (BioRad, Hercules, CA). Transcript abundance was calculated by regression against the standard curve for each gene, respectively, and expressed as log starting quantity using RPL32 as a co-variant via SAS.

Immunofluorescence. Three-µm sections of subcutaneous adipose tissue adhered to cryo-glass slides were fixed in 100% ethanol, and kept at -80° C until immunofluorescence assays were performed. Mounted tissue samples were hydrated by sequential immersion in 100% EtOH, 95% EtOH, 75% EtOH, and water. Porcine myeloid cells where identified by anti-swine SWC3 antibody (VMRD, Pullman, WA) which was applied to the slides in buffer (pH 7.5) containing 10 mM NaPO4, 0.9% PBS, and 0.1%BSA at a dilution of 1:100 for 1.5 hours. Secondary anti-mouse IgG conjugated to FITC (Abbiotec, San Diego, CA) was applied (1:1000) to the slides for 90 minutes. Slides were fixed in 10% formalin for 5 minutes and then mounted with non-drying

fluorescent compatible Vecta Shield medium (Vector Laboratories, Burlingame, CA) and visualized with a Zeiss light microscope with a Zeiss HRc Axiocam digital camera (Carl Zeiss, Thornwood, NY). Adipocyte area, number, and myeloid cell number were measured at 20X magnification in the subcutaneous adipose tissue. Total SWC3 cells were divided by the total cell count to obtain the percentage of SWC3 positive cells. All microscopy analysis were done using Axio Vision v4.5.0.0 (Carl Zeiss, Germany).

Serum metabolites, cytokines, and hormones. IL6 was quantified via ELISA kit (R&D systems, Minneapolis, MN), Insulin and C-reactive protein were measured by an EIA from ALPCO diagnostics (Salem, NH). All kits were used in accordance with the manufacturer's protocol. Serum cholesterol (Roche, Indianapolis, IN), glucose (Roche, Indianapolis, IN), and NEFA (Randox, Crumlin, Co. Antrim, United Kingdom) were determined using an auto-analyzer (Roche, Indianapolis, IN) in the Department of Veterinary Pathology at Iowa State University. Serum adiponectin was arbitrarily quantified by Western blot. Briefly, the serum was diluted 15-fold and mixed with 2 X Laemmli buffer (Bio Rad, Hercules, CA). Samples were incubated at 100°C for 10 minutes. After incubation samples were loaded onto 12% Mini-PROTEAN TGX gels (Bio Rad, Hercules, CA). Total protein was subjected to electrophoresis with a 25 mM Tris/ 192 mM Glycine/ 0.1% SDS buffer at 150 volts for 45 minutes, then transferred onto nitrocellulose membrane for chemiluminesecent detection using standard western blotting procedures.

Statistical analyses. The mixed-model procedure in SAS (Version 9.1; SAS Institute, Cary, NC) was used to analyze all data. For gene transcription data, RPL32 was

used as a covariate. Data were randomized by pig for tissue analysis. All data were presented as least-squares means ± standard error of the mean. Differences were considered significant at $P \leq 0.05$ and trend towards significance at $P \leq 0.1$.

Results

Diet and tissue fatty acid profiles. The fatty acid profiles of the diets and tissues are summarized in Tables 1 and 3. As regards the saturated fatty acids, the major dietary differences were reflected in the proportion of palmitate (C16:0), which was similar in the control and lard diets, and approximately 50% lower in the canola oil diet. Stearate (C18:0) was approximately 3-4 times higher in the lard diet vs. the control and canola oil diets. Substantial differences were also noted for oleic acid (C18:1) with the canola oil diet containing approximately 2 and 1.5 times that of the control and lard diets, respectively. Linoleic acid (C18:2 n-6) was nearly 50% higher in the control diet vs. the lard and canola diets, and arachidonic acid (C20:4 n-6) was detected only in the control diet. As expected, the canola oil diet contained the highest proportion of αLA (C18:3 n-3), and EPA (C20:5 n-3) and docosapentaenoic acid (DPA, C22:5 n-3) were detected only in the control and canola oil diets, respectively.

Differential effects of diets on tissue fatty acid profiles were also readily apparent. Liver and both adipose depots from pigs fed the canola oil diet had appreciably lower proportions of palmitate vs. pigs fed the control and lard diets. However, palmitate in the LD muscle was not influenced by diet. Despite the dietary stearate being higher in the lard diet, the proportion of this fatty acid was reduced in liver by the canola oil diet. Stearate enrichment in the adipose depots were highest in the control pigs, followed by

the groups fed the lard and canola oil in order. Stearate and oleic acid enrichment in LD muscle were not affected by diet. However, oleic acid was higher in all other tissues of the pigs fed the canola oil diet. Linoleic acid was slightly enriched in the liver of pigs fed the canola oil diet. Arachidonic acid was not detected in either adipose depot, but the lard diet increased the concentration of this fatty acid in the liver by 42% vs. the control diet, whereas the canola oil diet reduced it by 23%. With the exception of the subcutaneous adipose depot, the canola oil diet increased concentrations of αLA substantially across tissues. Neither EPA nor DPA were detected in adipose tissue or LD muscle; in liver, the proportions of both fatty acids were lower in the pigs fed the lard diet than either the control or canola oil diet.

Body weight and subcutaneous adipocyte size. Final body weights and adipocyte size data are presented in Figure 2 A and B. Despite the high fat and metabolizable energy content of the lard and canola diets, pigs fed these diets were not heavier than controls. The p-value for the difference between the canola diet and lard diet adipocyte size was $p = 0.057$, but neither diet resulted in a difference vs. the control diet.

Serum metabolites, insulin and inflammatory markers. As shown in Table 4, total cholesterol was significantly greater ($p< 0.05$) in pigs fed the lard diet relative to the other two groups, and although diet did not influence LDL concentrations, HDL concentrations were increased ($p<0.05$) in pigs fed the lard diet compared to the control group. Serum concentrations of glucose, insulin, NEFA and triglycerides were not influenced by either high fat diet, nor were the inflammatory markers, IL6 or C-reactive protein. Serum adiponectin concentrations were not different between treatments (data not shown).

Adipose myeloid cell infiltration and inflammation markers. The number of cells positive for the porcine myeloid marker, SWC3, in subcutaneous adipose tissue was significantly lower ($p<0.05$) in pigs fed the canola oil diet than in either the control or lard group (Figure 3A). However, this result was not confirmed by SWC3 mRNA expression (Figure 3B). Likewise, MCP1 expression (Figure 2C) was not altered by either high fat diet. Adiponectin transcript abundance was higher in the subcutaneous adipose tissue versus mesenteric adipose tissue irrespective of diet (Figure 4A). Adiponectin transcript was not influenced by diet in either subcutaneous or mesenteric adipose tissue depots.

Metabolic and mitochondrial responses. Irrespective of diet, acetyl Co-A carboxylase (ACC) expression was the lowest in the liver and greatest in the subcutaneous adipose tissue (Figure 5B). Acetyl Co-A carboxylase expression was not influenced by diet in the liver or mesenteric adipose tissue, but there was a trend for a reduction in expression with the canola diet in the subcutaneous depot. Subcutaneous adipose tissue was identified as having the greatest abundance of ACC and the liver as the lowest, mesenteric adipose tissue was excluded in the investigation of desaturase transcript abundance. As shown in Figure 4A, Δ-6 desaturase was expressed similarly in liver and subcutaneous adipose tissue and diet did not alter transcript abundance. The expression of mitochondrial specific 12S rRNA , a proxy measure for mitochondrial density, was not responsive to diet in the liver, LD muscle, or subcutaneous adipose tissue, while the lard diet significantly reduced 12S rRNA in mesenteric adipose vs. the control and canola oil diets (Table 4D). Expression of UCP-3 in LD muscle (Figure 4B), the primary location of this

protein, was increased in pigs fed the lard diet relative to both the control and canola oil diets

Discussion

We report herein a significant enrichment of EPA in the liver of swine fed a diet enriched with canola oil and negligible in concentrations of EPA or DHA. The liver fatty acid profile of the pigs fed the control diet, which had low amounts of EPA and DHA from fish meal, were similar to the canola fed pigs. One of the main enzymes involved in the conversion of αLA to EPA is delta 6 desaturase. The mRNA concentration of delta 6 desaturase was unchanged with diet, but was greater in the liver compared to adipose tissue. We expected to see an increase in the expression of delta 6 desaturase in the tissues of pigs fed the canola diet based on previous data indicating n-3 fatty acids increase PPARα activation (23) and subsequently increase delta 6 desaturase activity (24). Previous literature also indicates n-3 fatty acids mediate a decrease in the transcript abundance of genes involved in *de novo* lipogenesis (25). ACC transcript was observed in the subcutaneous depot of animals fed the canola diet. We observed a significant decrease in ACC transcript abundance in the subcutaneous adipose tissue of animals consuming the canola diet compared to the lard or control fed animals. In line with previous literature we report greater ACC transcript abundance in subcutaneous adipose tissue compared to the liver regardless of diet (26). Based on transcript abundance only, these data indicate SQ adipose tissue is the primary location for *de novo* lipogenesis, while fatty acid desaturation is most likely to occur in the liver of swine.

Previous literature indicates dietary fatty acids can increase electron transport chain uncoupling by increasing the concentration of uncoupling proteins in multiple tissues (27, 28). Uncoupling protein 3 (UCP3) gene transcript was measured to determine if addition of n-3 fatty acids in the diet increased UCP3 abundance. The UCP3 transcript abundance of the longissimus dorsi was significantly greater in the animals consuming the lard diet compared to the other two diets. This was unexpected based on previous data that indicates rats fed diets enriched with 40% fish oil exhibit an almost two-fold increase in UCP3 expression in muscle tissue versus a control diet (29). Furthermore, Bassaganya-Riera et al. (30) indicates dietary supplementation with fish oil in an inflammatory bowel disease pig model significantly increased UCP3 mRNA expression in colonic tissue compared to animals fed a control diet.(30). A third study reported an induction of UCP3 expression with unsaturated fatty acids supplement and to a lesser extent with palmitate supplementation (28). A possible explanation for the lack of n-3 mediated increase in UCP3 is that our n-3 diet consisted of αLA and not EPA and DHA rich fish oil used in previous studies.

Previous work by Flachs et al. indicates mitochondrial density increases in adipose tissue of mice consuming a diet supplemented with n-3 fatty acids (31). We assessed mitochondrial density in the muscle, liver, subcutaneous and mesenteric adipose tissues. Our approach was to examine mitochondrial number through transcript abundance of the mitochondrial specific 12S rRNA. We report a significant reduction of 12S rRNA in the mesenteric adipose tissue of animals fed the lard diet compared to the control or canola diets. When the 12S rRNA data and the UCP3 data are interpreted together it indicates that the lard diet increases energy dissipation as heat and

subsequently decreases mitochondrial density. Further research is needed to identify how the lard diet reduced the mitochondrial density in mesenteric adipose tissue and the possible role of UCP3.

Our laboratory as well as others have previously shown an inflammatory response with high SFA diets in mice (32, 33). In the current study, we did not identify a significant difference in serum concentrations of the pro-inflammatory cytokines IL6, CRP and MCP1 or the anti-inflammatory protein adiponectin with any of the diets. Since previous literature indicates adiponectin expression is up-regulated by n-3 fatty acids, we sought to measure adiponectin transcript abundance in adipose tissue (34). Adiponectin transcript abundance in adipose tissue was not affected by diet, but was significantly greater in the SQ adipose depot compared to the MES adipose depot.

Immunofluorescent visualization of myeloid cell infiltration into SQ adipose tissue identified significantly fewer positive cells in the tissue of canola diet fed animals versus the control or lard fed animals. Given the lack of inflammatory, chemotactic, or anti-inflammatory signals, we and others postulate that the reduction in myeloid cells is a direct outcome of the dietary fatty acids incorporation into this tissue. Previous literature reported that OA and EPA interfered with the interactions between immune cells and the adhesion molecule ICAM-1 (35). Furthermore, αLA supplementation in humans caused a decrease in the soluble adhesion molecules sVCAM-1 and sE-selectin in the absence of a change in serum inflammatory profile (36). Interestingly, the subcutaneous adipose tissue of the canola fed animals had significantly greater enrichment of OA and αLA than the other diets. Based on previous literature, it is inferred that adipose tissue enrichment

of OA and αLA mediated a decrease in immune cell adhesion leading to reduced adipose tissue infiltration.

According to previous literature, adipocyte size is reduced with n-3 fatty acid supplementation (37). Although not significant, our data indicates a trend ($P<0.1$) for a reduction in SQ adipocyte area in animals fed the canola diet versus the lard diet. A reduction of adipocyte size with n-3 fatty acid enrichment has been previously attributed to the induction of oxidation and/or decreased preadipocyte differentiation and increased preadipocyte proliferation (31). Regardless of the mechanism behind the reduction in adipocyte size, smaller adipocytes are not associated with inflammation and thus are more desirable.

In conclusion, we report data herein indicating that αLA is converted to EPA in the liver of pigs fed a diet enriched with canola oil at concentrations equal to a diet containing fish meal. Furthermore, the abundance of ACC and delta 6 desaturase in liver and adipose tissue suggests these tissues are responsible for fatty acid conversion and lipogenesis, respectively. Our identification of no difference in serum inflammation, a lower accumulation of adipose tissue immune cells, and a trend for small SQ adipocytes in pigs fed the canola diet indicates these animals had a low risk of developing lipid induced insulin resistance. Our data also indicates that the lard diet was not sufficient in generating systemic inflammation within the short duration of 11 weeks, but did result in greater UCP3 transcription in muscle. It is possible that this genetic line of pig is predisposed to SFA induced activation of energy dissipation through electron transport uncoupling and not through adipocyte storage, thus protecting it from the adverse effects

of SFA. From our data, it is apparent that a longer feeding duration is needed to fully elucidate the effects of αLA and SFA in the pig.

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Table 1. *Fatty acid profiles of swine diets*

Diet	SFA		MUFA	Omega 6			Omega 3		
	C16:0	**C18:0**	**C18:1 (OA)**	**C18:2 n6 (LA)**	**C18:3n6 (γLA)**	**C20:4n6 (ARA)**	**C18:3n3 (αLA)**	**C20:5n3 (EPA)**	**C22:5n3 (DPA)**
	g/100g								
Control	20.1	4.2	22.5	38.7	n.d.[1]	0.3	2.7	0.9	0.6
Lard	19.4	14.3	32.9	24.8	n.d. [1]	n.d. [1]	2.3	n.d.[1]	n.d.[1]
Canola	9.1	3.3	49.0	26.6	n.d. [1]	n.d.[1]	8.1	n.d.[1]	0.18

Table 2. *Swine PCR primers*

Gene	Forward	Reverse
RPL32	TGGAAGAGACGTTGTGAGCAA	CGGAAGTTTCTGGTACACAATGTAA
ACC	TGTTGTCATGGTCACACCCGAAGA	TTGTTGTTGTTTGGTCCTCCTGGC
SWC3	TCCTTGACAACTCCGACAACTGGA	ACCTTGTAGAAGACGTGGAGCCTT
MCP 1	AGTCACCAGCAGCAAGTGTCCTAA	TCAGGCTTCAAGGCTTCGGAGTTT
Δ6 Desaturase	ACTTAAAGGGTGCCTCTGCCAACT	AAGTGGTGCAGAAGAACCAGCCTT
Adiponectin	CGTGATGGCAGAGATGGCGT	CCACACTGAATGCTGAACGG
UCP 3	ACGATGGATGCCTACAGGAC	TCCGAAGGCAGAGACAAAGT
12S RNA	AAACTCAAAGGACTTGGCGGTGCT	TAGGGTTTGCTGAAGATGGCGGTA

Table 3. *Fatty acid profiles of porcine tissues following 11 weeks of diet*

Tissue	Diet	SFA		MUFA	Omega 6			Omega 3		
		C16:0	**C18:0**	**C18:1 (OA)**	**C18:2 n6 (LA)**	**C18:3n6 (γLA)**	**C20:4n6 (ARA)**	**C18:3n3 (αLA)**	**C20:5n3 (EPA)**	**C22:5n3 (DPA)**
		g/100g								
Liver	Control	16.41±1.52[a]	28.20±0.79[a]	13.27±1.00[a]	14.86±1.67[a]	0.03±0.08[a]	14.15±0.87[a]	0.07±0.18[a]	3.36±0.48[a]	3.08±0.32[a]
Liver	Lard	13.21±1.44[a]	28.72±0.75[a]	15.62±0.93[a]	16.90±1.58[a]	0.18±0.08[ab]	18.57±0.82[b]	0.24+0.17[a]	0.22±0.46[b]	0.94±0.30[b]
Liver	Canola	7.42±1.42[b]	23.75±0.74[b]	23.22±0.92[b]	22.00±1.56[b]	0.30±0.08[b]	10.92±0.81[c]	1.99±0.17[b]	2.74±0.45[a]	2.24±0.30[a]
LD	Control	22.18±0.40[a]	14.17±0.58[a]	31.56±2.32[a]	21.69±1.64[a]	n.d. [1]	7.15±0.81[ab]	n.d. [1]	n.d. [1]	n.d. [1]
LD	Lard	20.64±0.38[a]	14.17±0.54[a]	30.18±2.19[a]	23.58±1.55[ab]	n.d. [1]	9.67±0.76[b]	n.d. [1]	n.d. [1]	n.d. [1]
LD	Canola	16.99±0.37[b]	12.39±0.54[b]	33.99±2.16[a]	27.01±1.53[b]	n.d. [1]	6.05±0.75[a]	1.96±0.37b	n.d. [1]	n.d. [1]
Mes.	Control	26.89±1.83[a]	19.66±0.55[a]	34.34±0.78[a]	12.13±0.94[a]	1.81±0.29[a]	n.d. [1]	0.39±0.12[a]	n.d. [1]	n.d. [1]
Mes.	Lard	23.70±1.73[a]	15.27±0.52[b]	38.14±0.74[a]	13.10±0.89[a]	3.72± 0.27[b]	n.d. [1]	0.57±0.12[a]	n.d. [1]	n.d. [1]
Mes.	Canola	12.53±1.71[b]	10.16±0.52[c]	43.23±0.73[b]	16.11±0.88[b]	4.60±0.27[c]	n.d. [1]	4.38±0.12[b]	n.d. [1]	n.d. [1]
SubQ	Control	20.49±0.95[a]	12.15±0.48[a]	37.05±1.06[a]	18.64±1.15[a]	5.78±0.64[a]	n.d. [1]	0.66±0.42[a]	n.d. [1]	n.d. [1]
SubQ	Lard	17.98±0.90[a]	9.68±0.45[b]	39.98±1.00[a]	20.59±1.08[a]	5.89±0.60[ab]	n.d. [1]	0.65±0.40[a]	n.d. [1]	n.d. [1]
SubQ	Canola	8.89±0.89[b]	5.21±0.45[c]	48.77±0.99[b]	23.74±1.07[b]	6.76±0.59[b]	n.d. [1]	1.38±0.39[a]	n.d. [1]	n.d. [1]

Different letters signify a $P \leq 0.05$

[1]n.d. represents a non-detectable concentration

Table 4. *Serum inflammation and metabolic profile of swine following 11 weeks of diet*

	Control	**Lard**	**Canola Oil**
Total Cholesterol (mg/dl)	97.31 ± 5.3[a]	116.14 ± 5.3[b]	94.61 ± 5.1[a]
LDL (mg/dl)	50.58 ± 9.6	73.56 ± 8.4	56.19 ± 9.2
HDL (mg/dl)	61.53 ± 5.4[a]	82.68 ± 4.7[b]	73.48 ± 5.1[ab]
NEFA (mg/dl)	0.18 ± 0.1	0.26 ± 0.1	0.22 ± 0.1
Triglycerides (mg/dl)	40.43 ± 7.0	33.38 ± 6.1	37.96 ± 6.7
Glucose (mg/dl)	124.8 ± 19.8	121.03 ± 17.3	130.56 ± 18.8
Insulin (ug/ml)	0.09 ± 0.02	0.04 ± 0.02	0.06 ± 0.03
C-Reactive protein (ng/ml)	25.94 ± 3.1	20.0 ± 2.8	24.40 ± 3.9
IL6 (pg/ml)	45.37 ± 7.7	54.86 ± 6.7	46.89 ± 7.3

Different letters signify $P \leq 0.05$

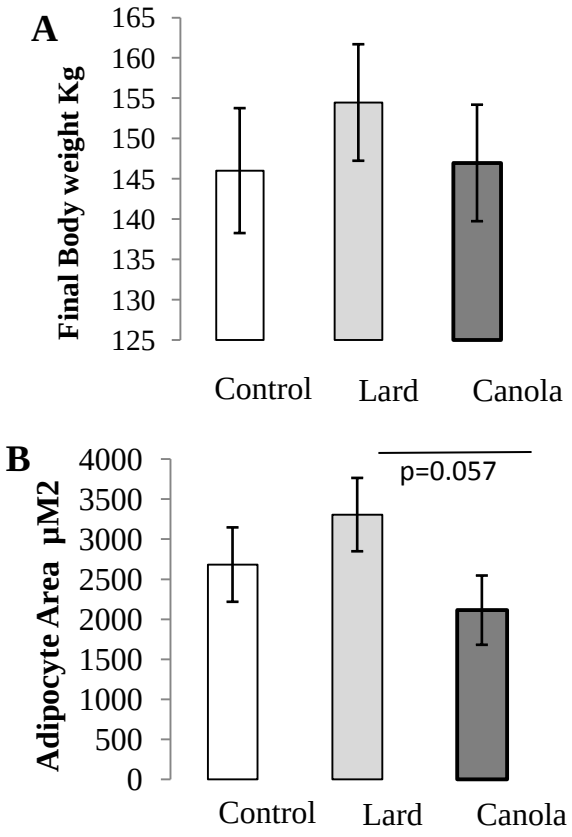

Figure 2. B*ody weight or adipocyte size were not altered by diets*

All measurements were reported after 11 weeks of diet in Control (n=7) pigs, Lard (n=8) pigs, and Canola Oil (n=8) pigs. A) Final body weight of pigs separated by diet B) Average area of subcutaneous adipocytes.

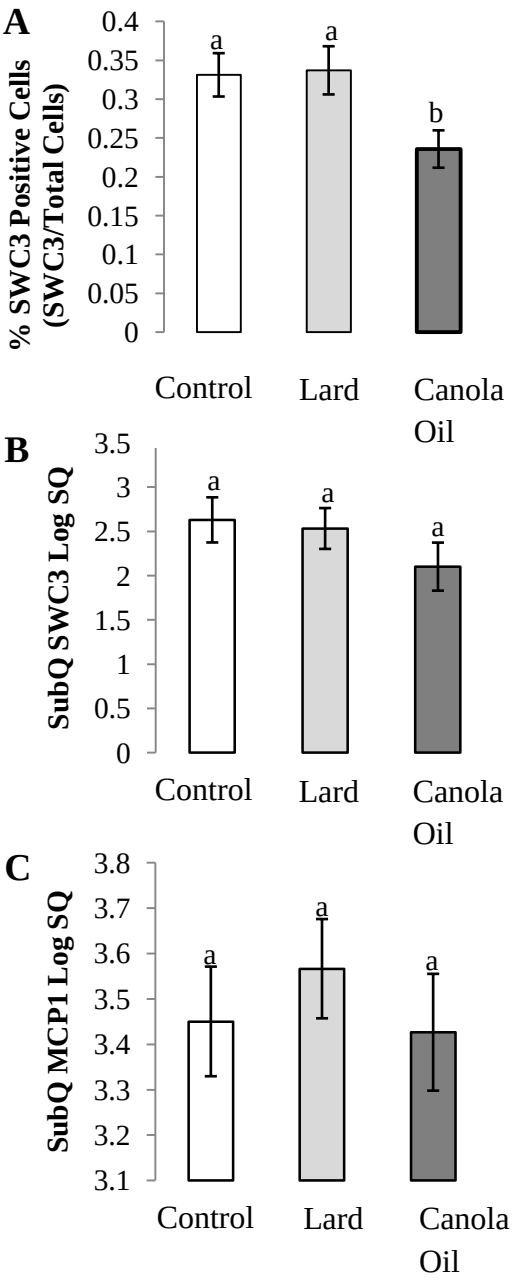

Figure 3. *Infiltration rate of myeloid immune cells and transcript abundance of chemotactic protein in subcutaneous adipose tissue*

Measurements were performed on tissue collected at the completion of the 11-week diet treatment. A) Immunofluorescence of myeloid immune cells visualized at 20 X magnification in subcutaneous adipose tissue B) Transcript abundance of the myeloid specific marker SWC3 in subcutaneous adipose tissue C) Transcript abundance of MCP1 in subcutaneous adipose tissue. Different letters indicate $P \leq 0.05$.

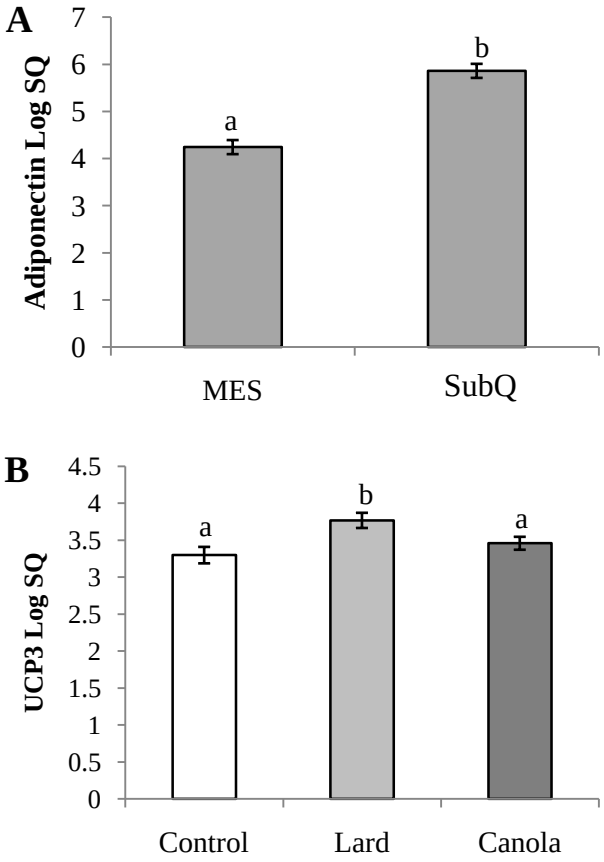

Figure 4. *Adipose tissue expression of adiponectin is depot specific. Transcript abundance of UCP3 in LD muscle was influenced by diet.*

Measurements were obtained from tissue procured after 11 weeks of diet. A) Transcript abundance of adiponectin in the mesenteric and subcutaneous adipose tissue depots of all animals regardless of diet B) UCP3 transcript abundance in the LD muscle separated by diet. Different letters signify $P \leq 0.05$.

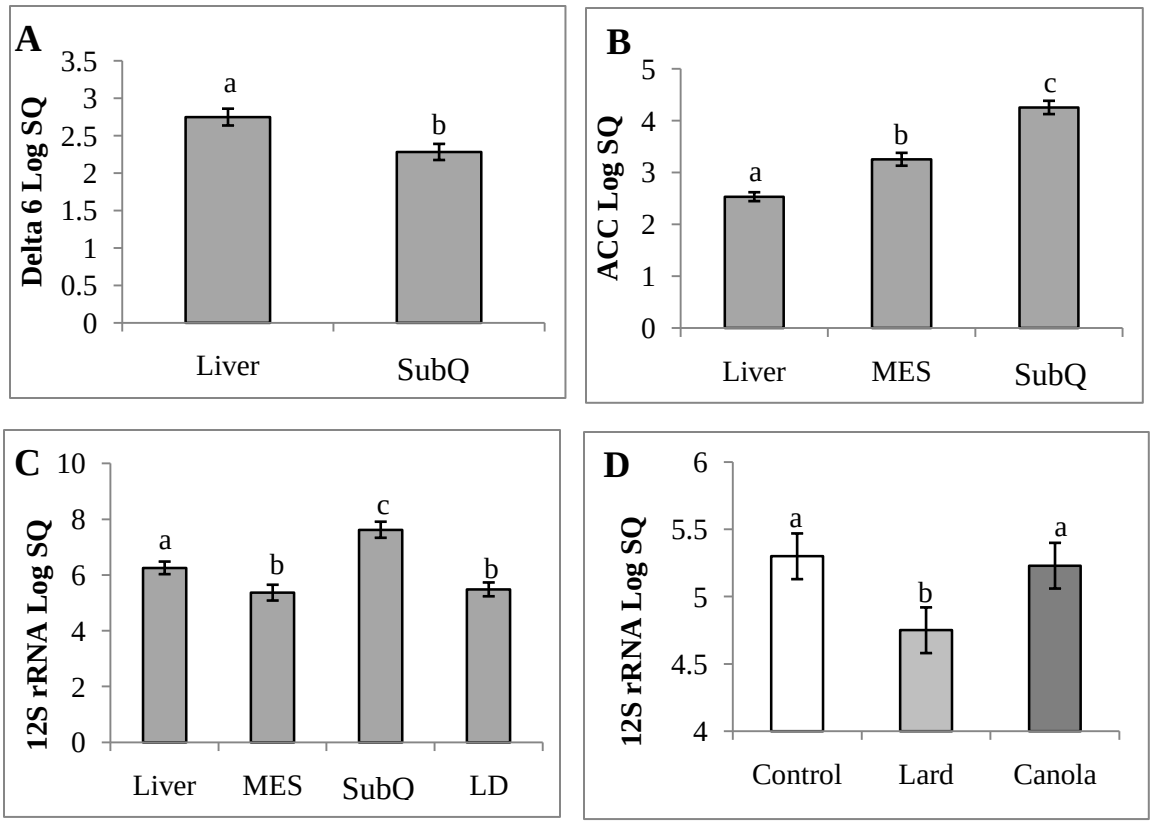

Figure 5. *Acetyl-CoA Carboxylase and delta 6 desaturase enzyme abundance. Tissue and diet-dependent alterations in mitochondrial specific gene transcript.*

All gene expression measurements were obtained from 1 g of tissue harvested from animals at completion of 11-week diet. Tissues consist of: liver, mesenteric adipose tissue (MES), subcutaneous adipose tissue (SubQ), and longissimus dorsi muscle (LD). A) Transcript abundance of delta 6 desaturase in liver or subcutaneous tissues B) Acetyl-CoA carboxylase transcript abundance in lipogenic tissue C) Tissue-specific mitochondrial specific transcript abundance regardless of diet D) Transcript abundance of mitochondrial specific 12S rRNA in subcutaneous adipose tissue by diet. Different letters signify $P \leq 0.05$.

CHAPTER 4: ADIPONECTIN NULL MICE FED A HIGH FAT DIET HAVE UNALTERED BODY COMPOSITION AND METABOLIC PROFILE

Abstract

Obesity is associated with reduced expression and circulating concentrations of adiponectin and low grade inflammation in adipose tissue, which contributes to the onset and progression of insulin resistance. Previous studies using adiponectin-deficient mice have shown conflicting results as regards to the development of insulin resistance and change in body weight in response to high fat diets. The aim of this study was to quantify changes in body fat and protein content in relation to the adiponectin wild type ($ADN^{+/+}$), heterozygous ($ADN^{-/+}$) and knockout ($ADN^{-/-}$) genotypes fed normal low fat vs. high fat diets. We also sought to determine whether inflammation was influenced by adiponectin genotype. Groups of male and female mice from each genotype were fed a high fat or low fat diet for 16 weeks. Our data indicate that adiponectin genotype did not influence total carcass fat and protein content, even in mice fed the high fat diet. However, adipocyte size (area) was increased and adipocyte number decreased in $ADN^{-/-}$ females fed the high fat diet, perhaps indicative of a unique role for adiponectin in high fat diet induced adipogenesis in females. Neither serum concentrations of CRP or macrophage infiltration in adipose tissue were altered by diet, sex, or genotype. Collectively, these results indicate that adiponectin deficiency does not alter body fat or protein accretion, nor does it exacerbate inflammation associated with diet induced obesity.

Introduction

Adiponectin is produced largely by adipocytes and is linked to the regulation of energy metabolism, insulin sensitivity and inflammation (see [1,2] for detailed reviews). Early work with recombinant protein indicated that adiponectin not only promotes

postprandial triglyceride clearance from the blood, but also stimulates fatty acid oxidation in skeletal muscle and cultured myocytes [3,4]. Collectively, these actions seemed to explain in part the suppression of adipose expansion in mice fed a high-fat obesigenic diet and treated with adiponectin [3,5], and to explain adiponectin mediated improvements in insulin function in models of obesity and insulin resistance [6,7]. However, more recent findings have implicated adiponectin as a metabolic signal which stimulates adipogenic pathways fundamental to adipose expansion. Overexpressing the high molecular weight form of adiponectin in mice lacking leptin resulted in marked expansion of the subcutaneous adipose depot and simultaneously corrected the metabolic disturbances associated with the morbid obesity and the accompanying inflammation and insulin resistance [8]. Although several studies [9-12] have evaluated the effect of adiponectin on body weight or the size of specific fat pads, direct determinations of total lipid and protein mass have not been reported in relation to adiponectin status, nor has there been a direct assessment of adipocyte size. The primary objective of the study reported herein was to determine whether adiponectin status influences body composition or markers of inflammation in mice fed a high fat diet.

Materials and Methods

Animals. Adiponectin-null mice were generated by removal of exon 2 of the adiponectin sequence on chromosome 16. Founder adiponectin null (ADN $^{-/-}$)mice were back crossed onto the original C57BL/6J wild type strain, and ADN $^{+/-}$ breeding pairs were established to produce ADN $^{-/-}$, ADN $^{+/-}$, and ADN $^{+/+}$genotypes. Genotypes of offspring were determined using DNA extracted from tail tissue. The PCR was performed using a three primer set (Forward1: $^{5'}$CTTTACGGTATCGCCGCTC$^{3'}$,

Forward2: $^{5'}$CAACTAAGACACTGATGA$^{3'}$, Reverse: $^{5'}$CTTTACGGTATCGCCGCTC$^{3'}$). PCR products were separated by electrophoresis and detected with SYBR Safe reagent (Invitrogen, Carlsbad, CA). Wild type primers produced a product size of 250 kb, whereas the adiponectin product was 550 kb. At six weeks of age (+/- 1 week) mice where placed onto experimental diets in blocks of 8-12 mice with all three genotypes represented. A total of 139 mice (68 females and 71 males) were used. The diets consisted of a low-fat chow control and an obesisgenic diet high in monounsaturated fatty acids (Table3).

Body composition. After 16 weeks on experimental diets, mice were euthanized by CO_2 asphyxiation. Blood was quickly collected via cardiac puncture and serum was stored at -80C prior to analysis. Gonadal fat pads, defined as the fat pad adjacent to uterine horns in females and next to epididymis in males, were excised, weighed, frozen in liquid nitrogen, and stored at -80°C. Whole carcasses (less the blood and fat pad) were autoclaved for 45 min at 250°C in a volume of water equal to one half the carcass weights. After autoclaving, carcasses were homogenized in a blender and the homogenate freeze dried for 48 hours. The dried homogenate was reground in the blender under liquid nitrogen, and sub-sampled for fat and protein determinations via ether extraction and the Kjeldahl procedure, respectively as previously described [13,14]. A subset of gonadal fat pads were homogenized in lysis buffer A (50mM Tris·Hcl pH 7.5, 1mM EDTA, 1mM DTT, 10% glycerol, 1% Triton X-100, 50mM Sodium fluoride, 5mM sodium pyrophosphate, 0.1mM PMSF, 5mg/ml aprotinin, 5mg/ml leupeptin, and 5mg/ml pepstatin A) for protein recovery. The protein was quantified by the BCA assay (Pierce, Rockford, IL). Fat pad protein averaged less than 3% of total fat pad weight.

Consequently, fat pad weights were multiplied by the correction factor and included in the calculation of total fat percentage.

Immunohistochemstry. Sections of gonadal fat pad were fixed overnight in 10% buffered formalin pH 6.8 and imbedded in paraffin. Thereafter, sections of 3 µm thickness were mounted onto microscope slides for morphometry and macrophage determinations. Immunohistochemstry was performed as previously described to identify macrophages in the tissue [15]. Briefly, an anti-F4/80 antibody and ABC Kit (Vector Labs, Burlingame, CA) were used to detect macrophages. Thereafter, the sections were counter-stained in eosin and hematoxylin. Adipocyte size (μm^2), adipocyte number, and macrophage number were acquired by visualization with a Zeiss light microscope at 20X magnification in four different fields and averaged.

Serum analyses. Glucose, total cholesterol and triglycerides were measured using an auto analyzer at the pathology lab at Iowa State University. ELISA or EIA were used to quantify adiponectin and IL6 (R & D Systems, Minneapolis, MN) C-reactive Protein and insulin (Alpco Diagnostics, Salem, NH).

Statistics. The mixed-model procedure in SAS (Version 9.1; SAS Institute, Cary, NC) was used to analyze all data. A blocked study design was used with blocks corresponding to the dates for each group of mice started on dietary treatments, therefore diet, sex, and genotype are treated as fixed variables. All data are presented as least-squares means $\pm$ standard error of the mean. Differences were considered significant at $P \leq 0.05$ and trend towards significance were noted at $P \leq 0.10$. Mean separations were performed by the *pdiff* procedure when a significant interaction of main effects was identified. Serum adiponectin concentrations were analyzed without the $ADN^{-/-}$ given their

lack of measurable concentrations. Thus the genotype variable for the serum adiponectin concentrations consisted of the ADN $^{+/-}$ and the ADN $^{+/+}$ mice only.

Results

Adiponectin concentrations. Final serum adiponectin concentrations are presented in Figure 6. As expected, mice with the heterozygous genotype had adiponectin concentrations at approximately 40% (genotype effect, P<0.0001) of those in the ADN $^{+/+}$ group, and females had higher concentrations (sex effect, P <0.0001) than males. However, the sex by diet and genotype by sex interactions were significant (P = 0.006 and 0.03, respectively). These interactions resulted from the ADN $^{+/+}$ females having significantly higher adiponectin concentrations (P = 0.05) than all other groups, and the high fat diet reduced serum adiponectin only in the ADN $^{+/+}$ females.

Serum inflammation and metabolic parameters. Interleukin 6 (IL6) was largely undetectable in the serum, with no indication of sex, diet or genotype being a contributing factor. Consequently, inflammatory status was assessed by measuring serum C-reactive protein (CRP). Neither dietary treatment, gender nor genotype significantly altered serum CRP concentrations (Table 5), and interactions of main effects were not significant.

The metabolic state of the mice was assessed based on serum concentrations of glucose, insulin, cholesterol, and triglycerides (Table 5). Mice fed the high fat diet had lower glucose concentrations than those fed the control diet (diet effect, P = 0.002), and females had lower concentrations than males (sex effect, P < 0.0002). However, the significant genotype by sex interaction (genotype by sex, P = 0.02) indicated that the higher concentration of glucose in males and lower concentration in females did not

occur in the ADN $^{+/+}$ mice. There was a significant three-way interaction (sex by diet by genotype, P = 0.04) for serum insulin concentrations; although wild-type males and all females were unresponsive to diet, the insulin concentrations increased appreciably (P<0.0001) in ADN $^{+/-}$ males fed the low fat diet, male ADN $^{+/-}$ and ADN $^{-/-}$ both had higher concentrations (male vs. female with in genotype, P<0.0001) than females of the same genotype, irrespective of diet. Cholesterol concentrations were greater in males (sex effect, P = 0.0001). Serum triglyceride concentrations were not significantly influenced by genotype or sex, but mice fed the high fat diet had lower concentrations (diet effect, P = 0.01).

Body composition. Body composition and caloric intake data are presented in Table 6. Final body weights were greater in mice fed the high fat diet (diet effect, P = 0.001). However, the significant sex by diet interaction (sex by diet, P = 0.03) indicates that females were more responsive to the high fat diet than males, with the exception of the female ADN $^{-/-}$ mice (sex by diet by genotype, P = 0.009). In terms of actual weight gain over the experimental period, males gained more weight (sex effect, P = 0.0007). A significant sex by diet interaction (sex by diet, P = 0.04) indicates the females on the low fat diet gained less than the other groups. Body fat (percentage basis and actual mass) was lower in mice fed the high fat diet (diet effect, P =0.02), and this was especially apparent in the males (sex by diet, P = 0.05). Additionally, males had higher percentages of protein and higher actual protein mass (sex effect, $P \leq 0.01$). There was also a trend (sex by diet, P = 0.08) for a sex by diet interaction which reflects the lower protein mass in the females on the high fat diet.

Immunohistochemistry (IHC) and adipocyte number. The gonadal fat pads where excised for IHC and determinations of adipocyte size and number. Female ADN $^{-/-}$ mice responded to the high fat diet by significantly increasing adipocyte area (P=0.02) and decreasing the adipocyte number (P=0.008) (Figure 7). The ADN $^{+/-}$ females did not differ from ADN $^{-/-}$ or ADN $^{+/+}$ female mice. In the males, adipocyte morphology was not affected by diet or genotype. Although macrophages were identified in the adipose tissue of all mice, macrophage infiltration was not influenced by diet, sex or genotype, nor were there significant interactions of main effects (Figure 8).

Discussion

The effects of adiponectin genotype (i.e., ADN $^{-/-}$, ADN $^{+/-}$ and ADN $^{+/+}$) on circulating adiponectin concentrations have been reported by multiple groups [9-12]. We confirmed an absence of serum adiponectin (i.e., undetectable concentrations) in the homozygous knockout mice, whereas the ADN $^{+/-}$ mice had measurable, but substantially lower serum concentrations than the wild type (ADN $^{+/+}$) mice. Consequently, our model allowed us to evaluate the effects of adiponectin status on the response to the high fat diet at distinct concentration differences ranging from undetectable to normal wild type concentrations.

In addition to genotype, serum adiponectin concentrations were also influenced by sex. Females had higher adiponectin concentrations than males, and ADN $^{+/+}$ females fed the high fat diet exhibited a decrease in adiponectin. This sexual dimorphism and the reduction in adiponectin caused by the high fat diet (females only) has also been documented by Gui et al. [16] and Bullen et al. [17], respectively. Furthermore, the lack

of a dietary response in our male mice is consistent with the report by Barnea et al. [18] which indicated no reduction in serum adiponectin concentrations in male mice fed a high fat diet for 20 weeks. Collectively, these findings indicate a unique susceptibility of females vs. males to the suppressive effects of a high fat diet on circulating adiponectin. However, the underlying mechanism for the sexual dimorphic response in serum adiponectin concentrations with high fat diet has not been identified.

The data presented herein support the findings of several groups that adiponectin null mice are not different than wild type mice in terms of body weight [9-12]. Moreover, our results show clearly that the absence of adiponectin does not affect total fat or protein content on an absolute mass or percentage basis. We also report that a high fat diet consistently promotes an increase in final body weight within genotype, and that this response is more pronounced in the females. Although there were not significant genotype differences observed in body composition measures, the data clearly indicate distinct gender differences. Specifically, as compared with males, the female mice were less likely to have a change in percent fat or protein when fed the high fat diet. Interestingly, in the absence of differences in overall body composition, the female $ADN^{-/-}$ mice had larger, but fewer, gonadal adipocytes. Adipose tissue composed of larger, but fewer, adipocytes suggest that adipogenesis may be diminished in adiponectin-deficient females. This finding is intriguing in light of the recent finding that over expressing adiponectin in the leptin deficient obese mouse stimulates expansion of subcutaneous adipose tissue [8]. Surprisingly, the male $ADN^{-/-}$ mice did not show adipocyte hypertrophy. Whether the lack of adipocyte hypertrophy relates directly to male sex hormones remains to be determined.

Given implicated role of adiponectin as a local and systemic anti-inflammatory factor [13,14], it was pivotal to investigate inflammatory status in this model. Clinical investigations have identified an increase in serum IL6 and CRP in patients with the metabolic syndrome, which commonly presents with low adiponectin concentrations [19,20]. Ouchi et al. [21] reported an inverse correlation between serum adiponectin and serum CRP concentrations in humans and an increase in mouse CRP mRNA transcript abundance in adipose tissue of adiponectin knockout animals. Based on these findings, we expected an increase in serum IL6 and CRP in the $ADN^{-/-}$ mice, especially those fed the high fat diet. However, IL6 was largely undetectable in the serum of our mice, and whereas CRP was readily measurable, adiponectin genotype did not influence CRP concentrations, regardless of sex or diet. These data indicate that the absence of adiponectin does not necessarily predispose mice to a chronic inflammatory state, even when fed a high fat diet. The fact that macrophage infiltration was not altered by genotype, irrespective of diet, further substantiates the lack of differences in the inflammatory status of adipose tissue in these mice.

Adiponectin is a known moderator of insulin sensitivity, but reports on insulin sensitivity in adiponectin null mice are conflicting [9-12]. The fasting glucose and insulin concentrations of the mice used in this study seemed high as compared with previous reports. The explanation for this difference is not readily apparent, but it is possible that the difference relates to the shorter duration of fasting that we used vs. that described in other reports (6 hours vs. 12-16 hours) [9-11,18]. However, relative to the present study, the glucose concentrations were influenced by genotype, although a genotype by sex interaction was indicated in that glucose concentrations were higher in wild type females

fed the low fat diet. We also found higher serum insulin concentrations in the ADN $^{+/-}$ male mice on the low fat diet. Perhaps most important, our collective glucose and insulin data to not indicate that the absence of adiponectin, or the low circulating concentrations in the ADN$^{+/-}$ mice, is sufficient to predispose mice to the insulin resistance associated with high fat diets. Albeit, we cannot rule out the possibility that the type of fat fed in the high fat diet (i.e. unsaturated vs. saturated fats) may impact this outcome. Although the overall effect of the high fat diet was not as expected, the fact that male mice exhibited differences in insulin concentrations while females did not, further highlight the importance of sex in the interpretation of results obtained in studies comparing genetic lines and(or) dietary factors.

In summary, our findings indicate that adiponectin deficiency alone does not predispose mice to chronic inflammation associated with a diet high in unsaturated fat, nor does it cause significant changes in the accretion of protein or fat in mice fed normal low fat or obesigenic high fat diets. However, the female-specific adipocyte hypertrophy and corresponding reduction in adipocyte number in ADN$^{-/-}$ mice fed the high fat diet support a possible unique role for adiponectin in adipogenesis in females.

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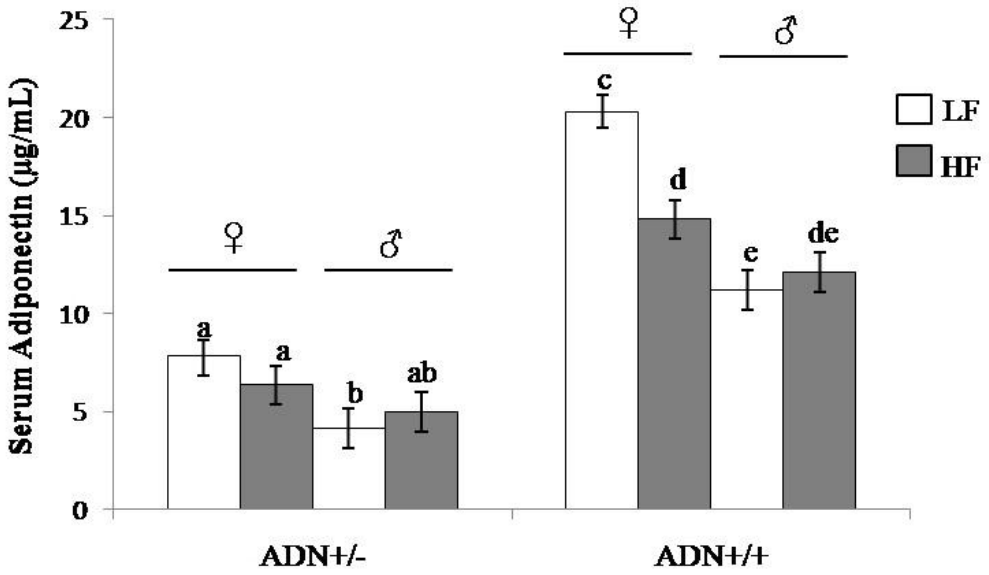

Figure 6. *Serum adiponectin in heterozygote and wild type mice after 16 weeks of diet*. Main effects of sex and genotype were significant (12.3 ± 0.5 vs. 8 ± 0.5 µg/ml for females vs. males, P<0.0001) and (5.8 ± 0.5 vs. 14.6 ± 0.5 µg/ml for $ADN^{+/-}$ vs. $ADN^{+/+}$ P=0.03) as well as the sex*diet and genotype*sex two-way interactions (P=0.006 and P= 0.03 respectively). Main effects of diet exhibited a trend (P=0.08) while genotype*diet two-way interaction and the three-way interaction were not significant (P=0.16). Mean ± SEM. Different letters signify P ≤ 0.05

Table 5. *Serum Metabolic and Inflammatory Markers*

Genotype	Sex	Diet	Glucose (mg/dl)[1]	Insulin (ng/ml)[2]	Total Cholesterol (mg/dl)[3]	Triglycerides (mg/dl)[4]	C-Reactive Protein (ng/ml)
$ADN^{+/-}$	F	HF	206.8 ± 17.5^{ab}	0.71 ± 0.36^{a}	142.6 ± 24.6^{a}	83.7 ± 16.7^{a}	29.6 ± 2.7^{a}
$ADN^{+/-}$	F	LF	233.7 ± 17.5^{abcd}	0.7 1± 0.31^{a}	116.6 ± 20.9^{a}	112.5 ± 10.0^{ab}	29.2 ± 2.5^{a}
$ADN^{-/-}$	F	HF	199.3 ± 17.4^{a}	0.69 ± 0.31^{a}	132.0 ± 25.0^{a}	86.3 ± 13.9^{a}	29.4 ± 2.4^{a}
$ADN^{-/-}$	F	LF	217.5 ± 18.4^{abc}	0.55 ± 0.31^{a}	105.1 ± 21.4^{a}	122.7 ± 11.9^{ab}	28.0 ± 2.5^{a}
$ADN^{+/+}$	F	HF	208.1 ± 17.4^{ab}	0.87 ± 0.31^{a}	133.6 ± 24.6^{ab}	134.7 ± 13.7^{b}	29.7 ± 2.3^{a}
$ADN^{+/+}$	F	LF	285.0 ± 23.2^{e}	0.84 ± 0.36^{a}	139.0 ± 33.2^{ab}	140.0 ± 23.7^{b}	31.3 ± 2.7^{a}
$ADN^{+/-}$	M	HF	278.5 ± 17.4^{de}	1.09 ± 0.31^{a}	224.9 ± 21.4^{c}	112.2 ± 10.7^{ab}	30.4 ± 2.4^{a}
$ADN^{+/-}$	M	LF	284.9 ± 16.5^{e}	3.05 ± 0.33^{c}	203.5 ± 21.5^{bc}	134.9 ± 12.0^{b}	29.6 ± 2.5^{a}
$ADN^{-/-}$	M	HF	249.4± 17.5^{cde}	2.20 ± 0.33^{b}	231.7 ± 21.8^{c}	117.1 ± 10.9^{ab}	33.5 ± 2.7^{a}
$ADN^{-/-}$	M	LF	284.0 ± 17.4^{e}	1.66 ± 0.46^{ab}	188.2 ± 20.3^{bc}	139.4 ± 16.7^{b}	26.5 ± 3.9^{a}
$ADN^{+/+}$	M	HF	224.6 ± 17.4^{abc}	1.01 ± 0.36^{a}	205.0 ± 27.6^{c}	118.3 ± 13.7^{ab}	32.8 ± 2.6^{a}
$ADN^{+/+}$	M	LF	262.7 ± 15.8^{cde}	1.44 ± 0.33^{ab}	185.1 ± 9.3^{bc}	139.0 ± 9.7^{b}	29.1 ± 2.5^{a}

[1]Significant effect of diet (227.8 ± 7.7 vs. 261.3 ± 8.1 for HF vs. LF diet, P=0.002); Significant sex effect (225.1 ± 8.2 vs. 264.0 ± 7.6 for females vs. males, P= 0.0002); Significant genotype*sex interaction, P= 0.02)

[2]Significant effect of sex (0.73 ± 0.25 vs. 1.75 ± 0.18 for females vs. males, P<0.0001); Trend for sex*diet interaction (P=0.08) and significant genotype*sex effect (P=0.057); Significant effect of genotype*diet (P=0.017) and significant genotype*sex*diet effect (P=0.04)

[3]Significant effect of sex (128.1 ± 15.2 vs. 206.3 ± 14.7 for females vs. males, P<0.0001); Trend for effect of diet (178.2 ± 15.4 vs.156.2 ± 14.7 for HF vs. LF diet, P=0.07)

[4]Significant effect of diet (108.7 ± 6.1 vs. 131.4 ± 6.4 for HF vs. LF diet, P= 0.01)

Table 6. *Body Composition, Gain and Energy Intake*

Genotype	Sex	Diet	FBW (g)[1]	Total Fat (%) [2]	Total Fat (g)[3]	Total Protein (%)[4]	Total Protein (g)[5]	Total Gain (g)[6]	Energy Intake (kcal/wk)[7]
ADN+/-	F	HF	39.1 + 1.5^{a}	30.5 + 4.4^{ab}	11.8 + 2.0^{ab}	14.3 + 0.9^{ab}	5.2 + 0.3^{ab}	17.6 + 1.5^{de}	95.7 + 0.4^{c}
ADN+/-	F	LF	34.2 + 1.6^{b}	33.0 + 4.0^{ab}	11.9 + 1.9^{ab}	14.9 + 0.8^{ab}	5.1 + 0.3^{a}	12.7 + 1.6^{ab}	119.2 + 0.3^{e}
ADN$^{-/-}$	F	HF	37.0 + 1.6^{ab}	31.0 + 4.1^{ab}	11.9 + 1.9^{ab}	13.5 + 0.9^{ab}	5.0 + 0.3^{a}	15.1 + 1.5^{abcd}	93.4 + 0.4^{c}
ADN$^{-/-}$	F	LF	34.5 + 1.6^{b}	35.4 + 3.9^{ab}	12.1 + 1.8^{ab}	14.9 + 0.8^{ab}	5.0 + 0.3^{a}	12.9 +1.6^{abc}	115.0 + 0.3^{de}
ADN$^{+/+}$	F	HF	39.5 + 1.6^{a}	39.2 + 3.7^{b}	16.1 + 1.8^{b}	12.9 + 0.8^{a}	5.2 + 0.2^{ab}	18.1 + 1.6^{de}	92.7 + 0.4^{c}
ADN$^{+/+}$	F	LF	33.2 + 1.6^{b}	35.2 + 4.5^{b}	12.7 + 2.1^{ab}	15.2 + 1.0^{ab}	5.2 + 0.3^{ab}	11.5 + 1.6^{a}	112.9 + 0.3^{de}
ADN$^{+/-}$	M	HF	38.2 + 1.5^{ab}	25.4 + 4.1^{a}	10.2 + 1.9^{a}	15.9 + 0.9^{b}	5.9 + 0.3^{b}	18.0 + 1.5^{de}	82.8 + 0.4^{a}
ADN$^{+/-}$	M	LF	36.1 + 1.6^{ab}	38.5 + 4.3^{b}	15.2 + 2.0^{ab}	16.5 + 0.9^{b}	6.0 + 0.3^{b}	16.2 + 1.5^{bcd}	100.0 + 0.3^{d}
ADN$^{-/-}$	M	HF	39.7 + 1.6^{a}	30.5 + 4.2^{ab}	12.1 + 2.0^{ab}	15.7 + 0.9^{b}	5.9 + 0.3^{b}	20.1 + 1.5^{e}	90.2 + 0.4^{a}
ADN$^{-/-}$	M	LF	36.9 + 1.6^{ab}	35.2 + 4.5^{b}	13.7 + 2.1^{ab}	16.1 + 1.0^{b}	5.9 + 0.3^{b}	16.8 + 1.6^{cde}	91.5 + 0.3^{b}
ADN$^{+/+}$	M	HF	36.1 + 1.5^{ab}	26.4 + 4.4^{a}	9.6 + 2.0^{a}	17.1 + 1.0^{b}	6.0 + 0.3^{b}	16.1 + 1.5^{bcd}	82.6 + 0.4^{a}
ADN$^{+/+}$	M	LF	38.2 $\pm$ 1.5^{a}	35.8 $\pm$ 3.7^{b}	13.9 $\pm$ 1.8^{ab}	15.6 $\pm$ 0.8^{b}	6.0 $\pm$ 0.2^{b}	17.5 $\pm$ 1.5^{de}	102.7 $\pm$ 0.3^{d}

[1]Significant effect of diet (38.3 $\pm$ 0.8 vs. 35.5 $\pm$ 0.8 for HF vs. LF diet, P=0.001); Significant sex*diet interaction (P=0.03); Trend for genotype*sex*diet interaction (*P*=0.09)

[2]Significant effect of diet (30.5 $\pm$ 2.2 vs. 35.51 $\pm$ 2.3 for HF vs. LF diet, P=0.02); Significant sex*diet interaction (P=0.05)

[3]Significant sex*diet interaction (P=0.01)

[4]Significant effect of sex (14.3 $\pm$ 0.4 vs. 16.1 $\pm$ 0.4 for females vs. males, P=0.01); Trend for sex*diet interaction (P=0.08)

[5]Significant effect of sex (5.1 $\pm$ 0.2 vs. 5.9 $\pm$ 0.2 for females vs. males, P=0.0002)

[6]Significant effect of sex (14.7 $\pm$ 0.9 vs. 17.5 $\pm$ 0.9 for females vs. males, *P*=0.0007); Trend for sex *diet interaction (P=0.08)

[7]Significant effect of sex (2.56 $\pm$ 0.02 vs. 2.50 $\pm$ 0.02 for females vs. males, P<0.0001); Significant diet effect (2.42 $\pm$ 0.02 vs. 2.64 + 0.02 for HF vs. LF diet, P<0.0001)

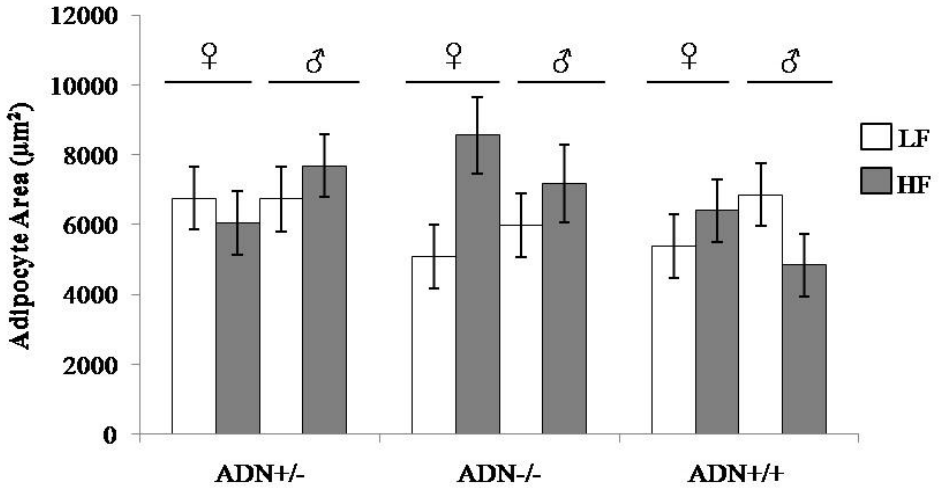

Figure 7. *Mean adipocyte area from gonadal fat pads after mice were fed experimental diet for 16 weeks.* Main effects of sex, diet, genotype were not significant (P= 0.20), nor were two-way or three-way interactions significant (P= 0.17). Mean ± SEM.

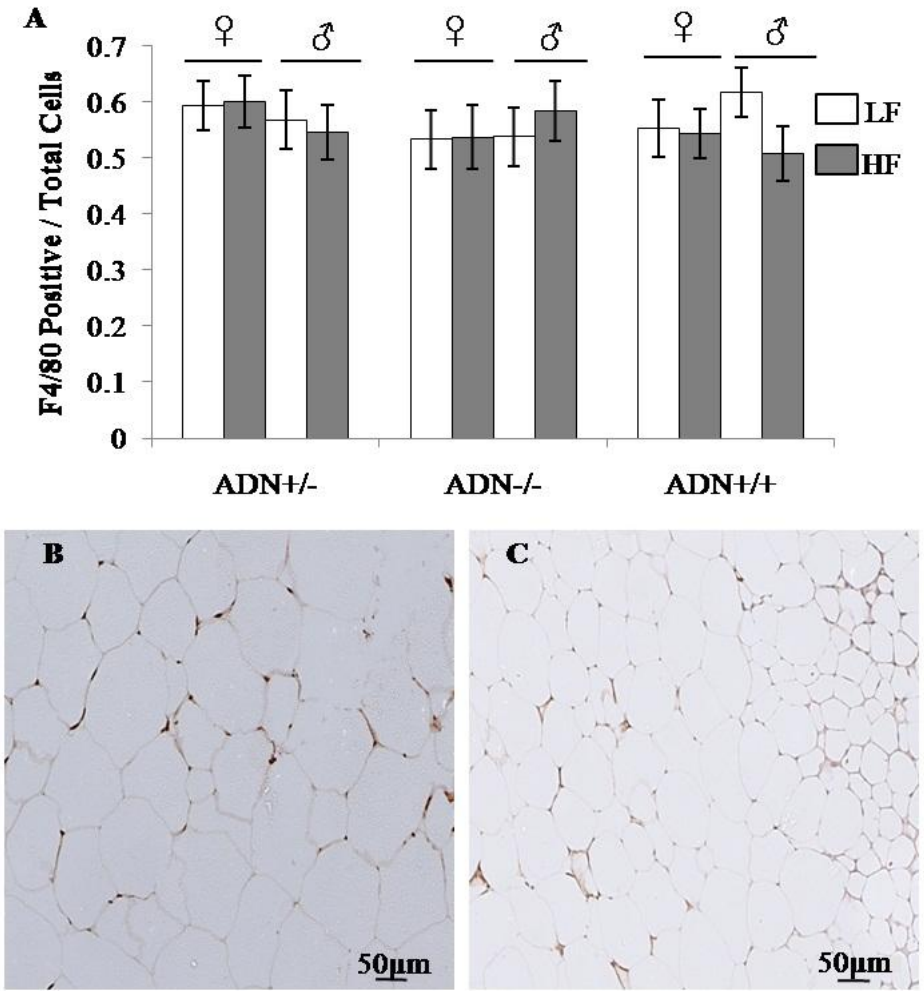

Figure 8. *Immunohistochemstry of gonadal fat pad excised after 16 weeks of diet*. A) Percentage of F4/80 positive cells in fat pad. Mean ± SEM. B) Representative picture of an $ADN^{-/-}$ female on a high fat diet. C) Representative picture of an $ADN^{-/-}$ female on low fat diet

CHAPTER 5. ADIPONECTIN SUPPRESSES THE PROLIFERATION AND DIFFERENTIATION OF STROMAL VASCULAR CELLS DERIVED FROM *ob/ob* MICE.

Abstract

Adiponectin has been suggested to protect against metabolic dysfunction in leptin-deficient (*ob/ob*) obese mice by increasing adipose tissue expansion. The goal of our work was to investigate the role of adiponectin in proliferation and differentiation of stromal vascular cells (SVC) recovered from subcutaneous adipose tissue of *ob/ob* and wild type (WT) mice in an *in vitro* system. Cells were grown until 5 days post differentiation. The cell culture media were supplemented with or without adiponectin and leptin. Proliferation of SVC was examined after 18 hr of culture. We identified a greater basal rates of proliferation in and lower rates of differentiation in WT cells compared to the *ob/ob* cells. The WT cells were more proliferative whereas the *ob/ob* cells had greater differentiation rates. Adiponectin decreased the transcript abundance of the differentiation-associated transcription factors CEBPα and PPARγ in the *ob/ob* SVC by 1 and 2 fold respectively. Furthermore, adiponectin decreased the proliferation of ob/ob cells compared with media control. Overall, we report different growth patterns for WT and *ob/ob* SVC cells and that adiponectin decreases the differentiation and proliferation of *ob/ob* SVC cells.

Introduction

Excessive expansion of adipose tissue during obesity increases proinflammatory cytokine secretion and suppresses the anti-inflammatory, insulin-sensitizing hormone

adiponectin [1-3]. Previous literature indicates that inflammation and insulin resistance can be suppressed when adiponectin concentrations are restored to normal [4, 5]. Suppression of inflammation by adiponectin occurs through inhibition of the nuclear factor kappa B transcription factor (NFκB) or through direct inhibition of TNFα signaling [6, 7]. Reduction of insulin resistance by adiponectin is the result of increased β-oxidation and/or the direct interaction of AMP-activated protein kinase (AMPK) with insulin receptor agonists [8]. These physiological effects of adiponectin were the accepted dogma until 2007.

In 2007, Kim et al. published a manuscript suggesting adiponectin overexpression in the leptin-deficient *ob/ob* obese mouse model protects against obesity-related insulin resistance and inflammation through expansion of subcutaneous adipose tissue [9]. An increase in circulating native adiponectin concentrations comparable to PPARγ agonist treatment was achieved by transgenic overexpression of a mutated form of adiponectin. This mutated adiponectin was not detected in the serum, indicating it was retained by adipocytes while promoting the secretion of native adiponectin. It is unknown if this mutated adiponectin has a role in the regulation of adipose tissue growth. Furthermore, the physiological implications of leptin deficiency were ignored in this study [9].

To adequately examine the effects of high adiponectin concentrations on preadipocytes, we investigated the role of exogenous adiponectin in the absence of intracellular adiponectin retention. My study was designed to identify the role of adiponectin in the proliferation and differentiation of stromal vascular cells isolated from the subcutaneous adipose tissue of *ob/ob* mice. Based on the work of Kim et al. [9], we

hypothesized that adiponectin treatment would increase proliferation and differentiation of preadipocytes in the SVC fraction of *ob/ob* adipose tissue.

Materials and Methods

Stromal vascular cell isolation. Six week old *ob/ob* and C57BLK/6J mice were procured from Jackson Laboratories (JAX). Six *ob/ob* mice and ten C57BLK/6J mice were euthanized via CO_2 asphyxiation and inguinal subcutaneous adipose tissues were collected in 37°C PBS with 3% penicillin/streptomycin (SP) mix. All of the tissue within each genotype was pooled together to create one cell lot. Adipose tissue was digested for 40 minutes at 37°C, mixed at 150 oscillations per minute (opm) in low glucose DMEM containing 1mg/ml collagenase (Worthington Biochemical, Lakewood, NJ) and 1% SP. After digestion, samples were centrifuged at 1000 X g for 8 minutes. After centrifugation the supernatant and adipocytes were decanted. The SVC pellet was resuspended in 5 ml of red blood cell lysis buffer (0.154 M NH_4Cl, 10mM $KHCO_3$,0.1mM EDTA, with a pH of 7.3) for 10 minutes and then spun at 1000 X g for 8 minutes. Supernatant was decanted and the pellet was resuspended in low glucose DMEM containing 10% fetal bovine serum (FBS) and 2% SP. Following re-suspension the cells were incubated in 12 well plates and 75 cm^2 flasks. Cells were grown in 5% CO_2 at 37°C for all experiments. Media were changed at 2 hours post plate and again at 24 hours post-plating. At 48 hours post plating, the cells were washed with PBS and trypsinized to break up densely populated clusters. After trypsinization, the cells were thoroughly mixed by gentle pipetting and diluted 1:1 in low glucose media containing 10% FBS and 1% SP and allowed to re-adhere to culture plate.

Differentiation cell culture. Three days after the cells were re-plated, confluence was 85% as based on visual identification via microscopy. Media were changed to differentiation media consisting of low glucose (1,000 mg/L) DMEM (Sigma Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) 1% penicillin/streptomycin (SP) mix, 1.7 µM insulin, 1.0 µM dexamethasone, 1.5 mM isobutylmethylxanthine, and 0.4 mM biotin. The differentiation media were then treated with or without either 20 µg/ml recombinant mouse adiponectin (BioVendor, Candler, NC, USA), 10 ng/ml recombinant mouse leptin (R and D Systems, Minneapolis, MN, USA), or the combination of adiponectin and leptin at respective concentrations. Cells were incubated for 72 hours at which time the media were changed to low glucose DMEM containing 10% FBS, 1% SP, insulin, and adiponectin or leptin. The treatment was ended 48 hours after the second media change, 5 day post differentiation, at which time media and cells harvested and mRNA and protein were collected for G3PDH or PCR assays. All treatments were replicated 9 times for analysis of PPARγ and C/EBPα transcript abundance, and 3 times for G3PDH.

Proliferation cell culture. Cells were plated at 2,000 cells per well in a 96-well culture plates and incubated overnight. The following day the media were changed to basal media containing BrdU with either 20 ug/ml adiponectin or 10 ng/ml leptin treatments. Cells were incubated for 18 hours and at completion were processed according to the ELISA manufacturers protocol (Millipore, Billerica, MA, USA). Each treatment was repeated 5 times across 3 plates.

G3PDH assay. At 5 days, post differentiation media was removed and the cells were washed with an isotonic phosphate buffer. Lysis buffer (0.25 M sucrose, 1mM Na_2EDTAH_2O, 5 mM tris base, 1 m M dithiothreitol, pH to 7.4) was added to each well and cells were scraped and transferred to tubes on ice. Cells were mechanically lysed by repeated passage through a needle and syringe. Lysate was spun at 12,500 X g for 5 minutes at 4°C. Supernatant was removed to new tubes on ice and 0.15 ml of supernatant was then transferred to a cuvette. Addition of 0.1 ml substrate buffer (0.71 mg/ml dihydroxyacetone phosphate lithium in H_2O) and 0.8 ml of assay buffer (200 mM triethanolamine, 5 mM Na_2EDTAH_20, 1/10 final volume β-mercaptoethanol, 20 µM NADH) were added immediately prior to measurement of absorbance. Absorbance was measured every 2 seconds for 3 minutes at 340nm. The total protein concentrations of the supernatants were identified using the protocol for a BCA reagent kit (Pierce, Rockford, IL, USA). The change in optical density was used to calculate the activity of G3PDH per minute and was corrected for total protein.

mRNA Extraction and Quantitative Real Time PCR. Recovery of total RNA was performed as described by the Trizol reagent (Invitrogen, Carlsbad, CA, USA) protocol. Residual DNA was removed with DNase treatment according to manufactures protocol (Turbo DNase, Ambion, Houston, TX, USA). Reverse transcription of 2 µg of purified RNA was performed using a iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). All primers were designed for use with the following thermal cycler parameters: 95°C for 3 min, followed by 40 cycles of 95 °C for 15s, 60°C for 30s, and 72°C for 30s. The primer sequences for GAPDH, PPARγ and CEBPα are reported in Table 7. Serial

dilutions of sequence confirmed gene target clones (pGEMT vector, Promega, Madison, WI, USA) were used to generate a standard curve for each transcript. Quantitative real time PCR reactions were performed on an iCycler using IQ SYBR Green Supermix (BioRad, Hercules, CA, USA). Regression analysis of the standard curve was used to calculate the transcript abundance, expressed as log starting quantity. GAPDH was used as the housekeeper control gene.

Statistical Analysis. All data were analyzed using the mixed-model procedure in SAS (Version 9.0; SAS Institute, Cary, NC, USA). GAPDH transcript abundance was used as a covariate for the gene transcription data. Treatments were randomly assigned and represented once per cell culture plate. The fixed effects consisted of plate, treatment, and genotype. All data are presented as least-square means $\pm$ standard error of the mean. Differences were considered significant at $P \leq 0.05$ and trends toward significance were reported at $P \leq 0.1$. Differences among means were determined by the pdiff procedure when a significant interaction was identified for treatment, genotype, or treatment by genotype.

Results

Media adiponectin and leptin. The concentrations of adiponectin and leptin in media are reported in Figures 9 and 10. Concentrations of both proteins were dependent on the supplementation status of each treatment. Media from leptin treatments had concentrations of approximately 10 ng/ml while non-supplemented media had less than 2 ng/ml. Interestingly, the media from the WT cells receiving leptin supplementation had greater concentrations of leptin compared with all other treatments. Adiponectin

concentrations in the media were also treatment dependent with values of 10 µg/ml and 1 µg/ml for adiponectin supplementation and control media respectively.

Proliferation. Cellular proliferation is reported in Figure 11 as concentration of cellular incorporation of BrdU reagent. The WT cells receiving the control, adiponectin, or leptin media had significantly greater concentrations of BrdU compared to the combined adiponectin/leptin treatment or any of the o*b/ob* cells. BrdU concentrations of the o*b/ob* cells were unchanged with leptin or adiponectin/leptin supplementation. However, treatment of *ob/ob* cells with adiponectin alone resulted in significantly lower ($p< 0.05$) BrdU incorporation compared to the other treatments.

G3PDH enzyme activity. The enzyme activity of G3PDH is reported in Figure 12 and was significantly greater in the cells from o*b/ob* animals. Treatment of *ob/ob* cells with leptin, whether alone or in conjunction with adiponectin, significantly reduced G3PDH activity. In the WT cells, G3PDH enzymatic activity was not significantly different between treatments.

Gene expression markers of differentiation. PPARγ and CEBPα transcript abundance are summarized in Figures 13 and 14 respectively. PPARγ transcript abundance was greater ($p= 0.0001$) in all of the *ob/ob* treatments compared to WT cells. The PPARγ transcript of o*b/ob* cells treated with the combined adiponectin/leptin treatment were lower than the control or leptin treatment ($p=0.007$ and $p= 0.036$ respectively). Likewise, the adiponectin treatment in *ob/ob* cells resulted in a trend for reduced ($p=0.067$) PPARγ transcript compared to the control. CEBPα abundance was greater ($p=0.0001$) in *ob/ob* cells compared to WT cells regardless of treatment.

Adiponectin treatment reduced CEBPα transcript 1.7 fold compared to control in the o*b/ob* cells. The WT cells treated with adiponectin, leptin or the combination of both exhibited a 2 fold decrease in CEBPα compared to the control.

Discussion

We report herein significant phenotypic differences in differentiation and proliferation of subcutaneous SVCs obtained from leptin deficient *ob/ob* and wild type mice. Cells from WT animals were highly proliferative while *ob/ob* cells were prone to differentiation. Furthermore, our data indicates a significant difference in the responsiveness of *ob/ob* SVC cells and WT cells to adiponectin and leptin treatment.

Treatment of *ob/ob* SVCs with leptin, whether alone or with adiponectin, reduced differentiation compared to control as measured by G3PDH. This finding is supported by Thomas et al. who showed a significant decrease in adipocyte differentiation of human stromal bone marrow treated with leptin [10]. It appears from our data that adiponectin does not influence adipocyte differentiation as assessed by G3PDH assay. Contrary to these findings, previous literature indicates adiponectin increases the differentiation of human preadipocytes as confirmed by microscopy [11].

It is possible that genetic markers of differentiation are present prior to protein expression of G3PDH. The gene expression profile of adipocyte differentiation is well characterized and consists of two distinct phases of transcription factor abundance [12-18]. Early in the differentiation process there is an increase in the transcription factors CEBPβ and CEBPδ [17, 19]. These two transcription factors mediate the production of

CEBPα, which accompanies PPARγ as a late phase transcription factor [14]. Previous literature indicates that the early phase differentiation signals CEBPβ and CEBPδ are increased by STAT3. STAT3 is a transcription factor that associates with the JAK/STAT family of receptors, which includes the leptin receptor [20]. Leptin receptor signaling can also increase the activation of two inhibitors of adipocyte differentiation, ERK and AMPK [21]. Our data showed no change in CEBPα or PPARγ with leptin treatment in either cell type. However, the co-treatment of leptin and adiponectin decreased PPARγ transcript abundance in the *ob/ob* cell line compared to media control. The treatment of cells with adiponectin alone reduced the transcript abundance of PPARγ and CEBPα in *ob/ob* SVCs compared to vehicle control. Based on these data it appears that adiponectin is the driving force in the reduction of PPARγ observed in our co-treatment experiment.

Multiple signaling proteins associated with adiponectin are involved in the regulation of CEBPα/β/δ and PPARγ. For instance, adiponectin has been shown to inhibit STAT3 via activation of JNK in hepatocytes [22]; if this occurs in preadipocytes, the inhibition of early phase transcription factors could occur. Furthermore, activation of AMPK by adiponectin decreases the expression of PPARγ and CEBPα in mesenchymal cells [23]. Previous reports using AICAR to activate AMPK show a significant reduction in PPARγ and CEBPα in preadipocytes [24, 25]. Likewise, the absence of AMPK in mice results in adipose tissue expansion, characterized by hypertrophy and differentiation [26]. Interestingly, the *ob/ob* SVCs in our study present with a similar phenotype as the adipose tissue of AMPK KO mice. These data indicate that the greater differentiation rate observed in our *ob/ob* SVCs may be mediated by lower basal AMPK concentrations.

Given that adiponectin is a strong inducer of AMPK [8, 27] and that AMPK inhibits differentiation transcription factors, it is likely that the inhibition of adipocyte differentiation by adiponectin in our study is mediated through AMPK.

Previous literature indicates leptin suppresses proliferation of 3T3L1 preadipocytes [28, 29]. Likewise, adiponectin has been implicated as an antagonist of key growth factors and thus inhibiting proliferation of multiple cell types [30]. We report a lower basal proliferentiation rate of *ob/ob* subcutaneous SVC cells compared to wild type control cells. Our data indicates leptin does not affect SVC proliferation. Furthermore, we observed a significant decrease in the proliferation of *ob/ob* cells with adiponectin treatment. The proliferation of WT cells remained unchanged with leptin and adiponectin treatments. However, WT proliferation was significantly decreased from control with the co-treatment of adiponectin and leptin. This data suggests the combined activation of the adiponectin and leptin pathways, most likely AMPK, results in a decrease in WT cell proliferation. These data contribute to our developing hypothesis that ob/ob cells have lower basal AMPK activity and therefore are more sensitive to its affects. If our hypothesis is correct, greater AMPK activation would be required to suppress WT cell proliferation as compared to the *ob/ob* cells.

In summary, we report a decrease in *ob/ob* SVC differentiation associated transcription factors and a reduction in proliferation with recombinant adiponectin treatment. Examination of our data has lead to the formation of a new hypothesis that *ob/ob* cells are physiologically more sensitive to the effects of active AMPK than WT mice. This hypersensitivity to AMPK explains, at least in part, the phenotypic differences

in differentiation and proliferentiation observed between the WT and *ob/ob* cells in this study. Based on our findings, it is evident that further investigations into the role of AMPK concentrations in *ob/ob* subcutaneous SVCs are warranted.

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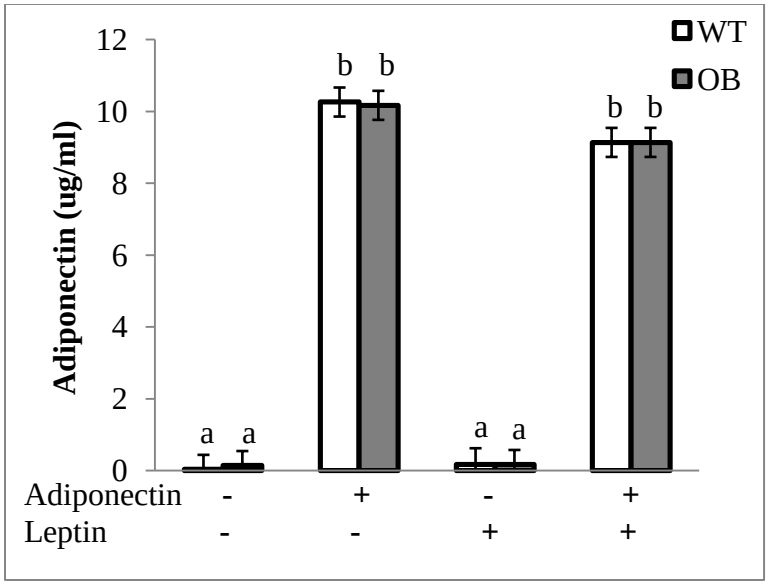

Figure 9. *Adiponectin concentration in the media at 5 days post differentiation*. SVC derived from ob/ob or WT mouse subcutaneous adipose tissue were cultured with or without 20 µg/ml adiponectin and/or 10 ng/ml leptin in standard differentiation media for 5 days. Different letters identify $P \leq 0.05$. Mean $\pm$ SEM

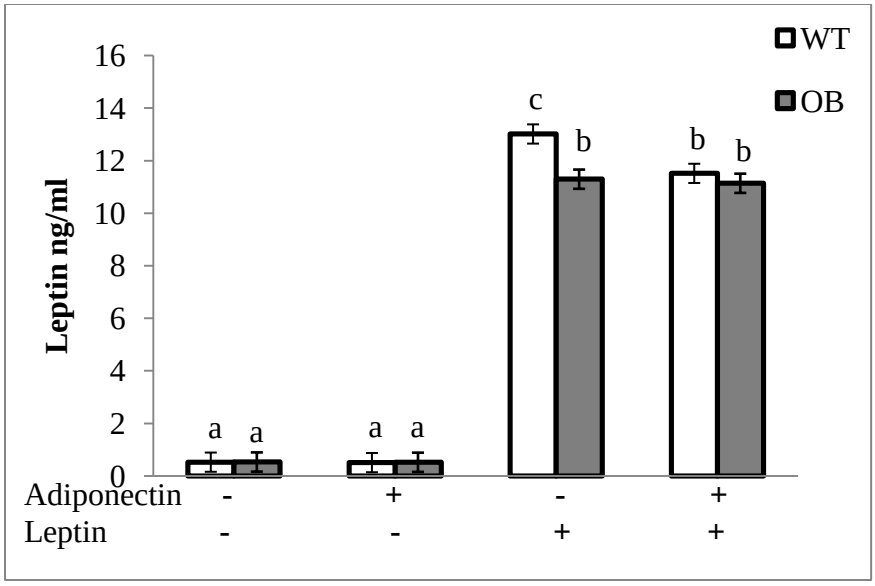

Figure 10. *Leptin concentrations in the media at 5 days post differentiation*. SVC derived from ob/ob or WT mouse subcutaneous adipose tissue were cultured with or without 20 µg/ml adiponectin and/or 10 ng/ml leptin in standard differentiation media for 5 days. Different letters identify $P \leq 0.05$. Mean $\pm$ SEM

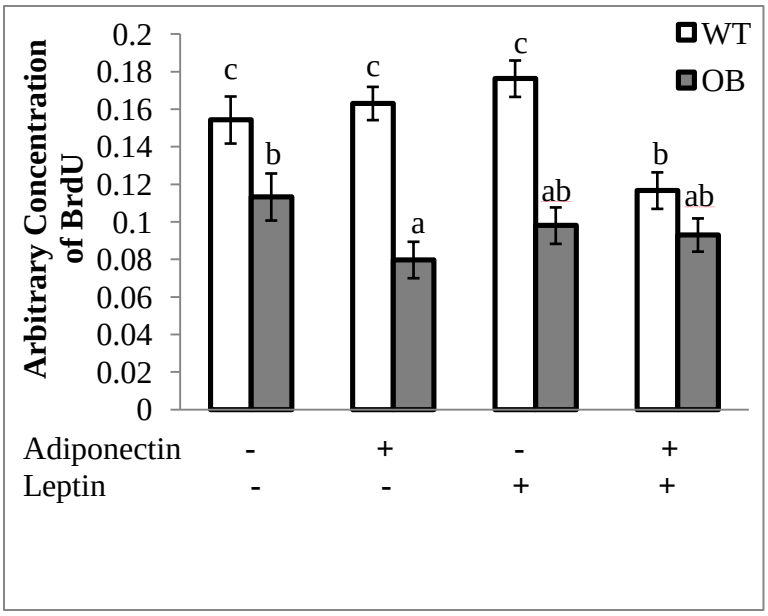

Figure 11. *Differences in proliferation of stromal vascular cells.* Incorporation of BrdU into newly formed cells was assessed in SVC derived from ob/ob or WT mice. Cells were cultured for18 hours in media treated with or without 20 µg/ml adiponectin and/or 10 ng/ml leptin. Different letters identify $P \leq 0.05$. Mean $\pm$ SEM.

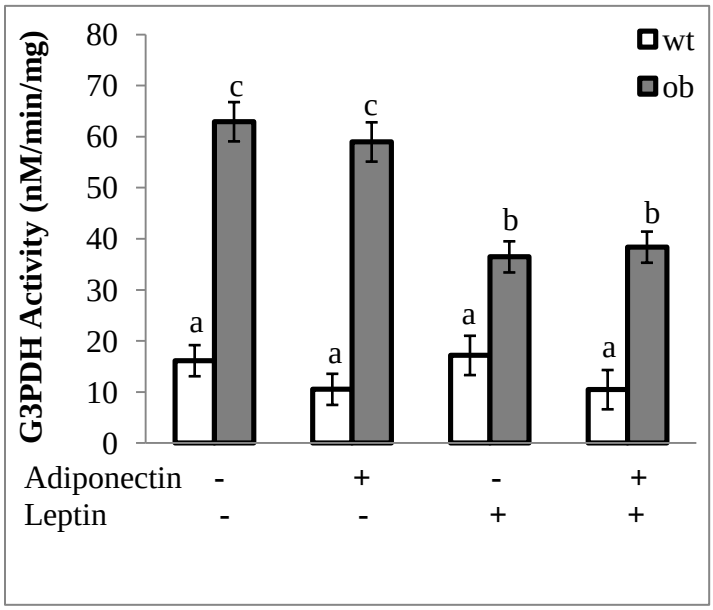

Figure 12. *Differences in differentiation assayed by G3PDH activity.* G3PDH activity in stromal vascular cells obtained from wild type or ob/ob mice and cultured for 5 days post differentiation. Culture media were treated with or without 20 µg/ml adiponectin and/or 10 ng/ml leptin. Different letters identify $P \leq 0.05$. Mean $\pm$ SEM.

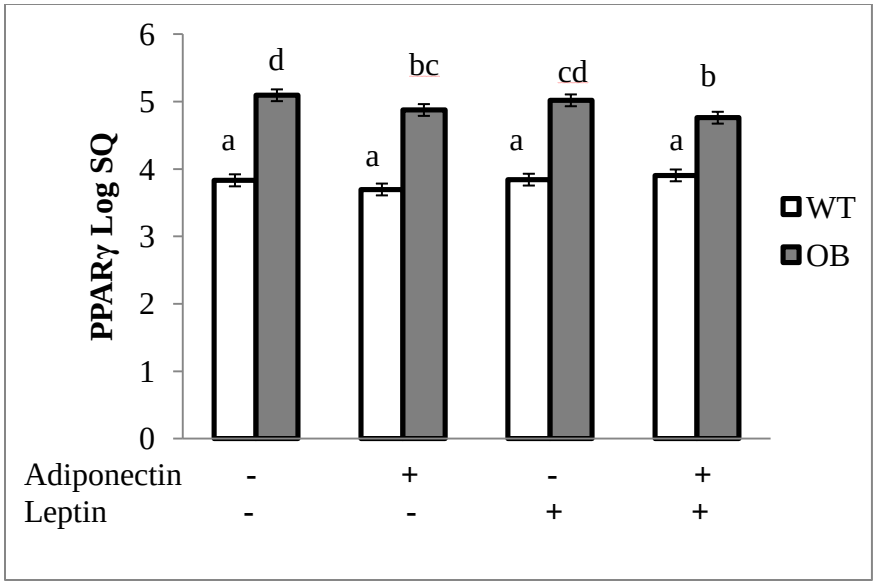

Figure 13. *Transcript abundance of the late phase differentiation specific gene PPARγ.* Transcript abundance of PPARγ from stromal vascular cells derived from *ob/ob* or wild type mice at 5 days post differentiation. Cells were treated with or without 20 µg/ml adiponectin and/or 10 ng/ml leptin. Different letters indicate $P \leq 0.05$. Mean ± SEM

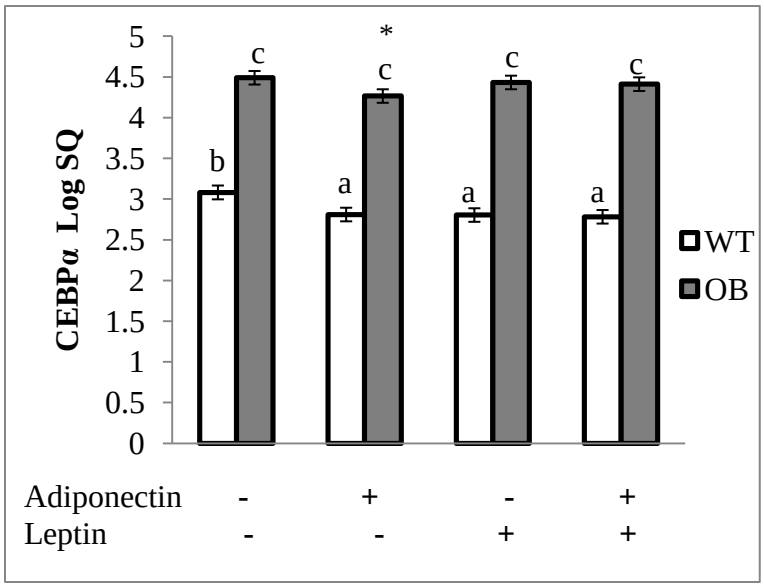

Figure 14. *Differences in mRNA markers of differentiation.* Transcript abundance of CEBPα from stromal vascular cells at 5 days post differentiation cultured in media treated with or without 20µg/ml adiponectin and/or 10 ng/ml leptin. Different letters signify $P \leq 0.05$. *P = 0.067. Mean ± SEM.

Table 7. *Realtime PCR Primer Sequences*

Gene	Forward	Reverse
Pref-1	AGA AAG GCC AGT ACG AAT GCT CCT	TTG CGG CTA CGA TCT CAC AGA AGT
CEBPα	AGA AGT CGG TGG ACA AGA ACA GCA	GCG TTG TTT GGC TTT ATC TCG GCT
PPARγ	ACA TAA AGT CCT TCC CGC TGA CCA	AAA TTC GGA TGG CCA CCT CTT TGC

CHAPTER 6. GENERAL CONCLUSIONS

We demonstrated that adiponectin transcript abundance and serum concentrations in swine are not altered with the consumption of large amounts of αLA or SFA. However, this study led to the identification of significant liver enrichment with EPA and DPA, indicating *in vivo* conversion of αLA in swine fed canola oil. Interestingly, liver tissue had greater transcript abundance of delta 6 desaturase compared to adipose tissue, which had higher ACC transcript. These findings support previous literature that has identified adipose tissue of swine as the main location of *de novo* lipogenesis [1]. However, the identification of a higher potential for desaturation in the liver compared to adipose tissue is not represented in the literature. Given the similar concentrations of EPA and DPA in only the liver of the control and canola diets, it is difficult to form a conclusion on physiological relevance. It appears that the fatty acid enrichment in the liver of these diets is basally regulated, given the similar fatty acid profiles even though the canola diet had an overabundance of substrate. We also identified significantly less myeloid cell infiltration in the subcutaneous adipose tissue of pigs fed the canola diet compared with control, which was accompanied by a trend for smaller adipocytes in this group. It is not completely apparent whether the reduction of myeloid cell number is a result of αLA supported mechanisms or through oleic acid mediated reduction in adhesion molecules as seen in previous literature [2]. Together, these results indicate that a diet high in canola oil reduces the inflammatory potential of adipose tissue by reducing macrophage infiltration and increasing adipocyte number. The use of a production pig line that has been selected for leanness over many generations and a dietary period that was not long enough to induce obesity may have precluded the observation of a dietary alteration in

adiponectin or inflammation. Therefore, it would be of interest to examine the effect of these diets in an obesity-prone pig line like the Ossabaw pig. An obese pig model would have a more pronounced difference in inflammation and adiponectin concentrations allowing for a better examination of dietary effects.

To further elucidate the effects of high fat diets on adiponectin function, specifically its role in body composition, we utilized an adiponectin null murine model was used. The mice had varying serum adiponectin ranging from non-detectable to wild type concentrations, based on their genotype: adiponectin null homozygotes, adiponectin null heterozygotes, and adiponectin wild type mice. Adiponectin null mouse models have been utilized for the examination of inflammation and metabolism in the absence of adiponectin. A controversial finding in adiponectin null mice is the development of insulin resistance with a high fat diet but no change in body weight from wild type control animals [3-6]. Therefore, we sought to investigate body composition differences between the adiponectin-null mice and wild type animals. To determine the effect of a lack of adiponectin on body composition, we fed adiponectin null and wild type mice high fat diets. We reported no significant difference between wild type or adiponectin null mice for body composition, metabolic profile, or inflammatory status. Interestingly, we identified significantly greater adipocyte size and lower total adipocyte numbers in adiponectin null female mice. Conversely, previous literature has reported a significant increase in adipocyte number in the adipose tissue of adiponectin-transgenic mice with high serum adiponectin concentrations [7]. These findings imply adiponectin has a direct influence on adipocyte size and number.

To determine whether adiponectin regulates preadipocyte differentiation and proliferation, we treated mouse stromal vascular cells with recombinant adiponectin. In order to align our work with previous reports, we chose to use SVC from obese *ob/ob* leptin-deficient and non-obese wild type mice. To account for the leptin deficiency in *ob/ob* cells, we added a positive control treatment that consisted of leptin-supplemented media. We identified a significant reduction in proliferation and differentiation of adiponectin treated *ob/ob* cells. Adiponectin treatment of wild type cells did not alter proliferation or differentiation, but the combined leptin adiponectin treatment significantly lowered proliferation. Furthermore, we identified a significant difference between wild type and *ob/ob* cells in basal proliferation and differentiation rates. Cells from wild type mice were more proliferative while *ob/ob* cells were more adept at differentiating. These data indicate that in the leptin-deficient animal high concentrations of adiponectin inhibit the expansion of subcutaneous adipose tissue.

It is unknown what molecular mechanisms are controlling inhibition of proliferation and differentiation by adiponectin and why only in leptin-deficient cells. Given the identification of a suppression in wild type SVC proliferation with the combined leptin and adiponectin treatment, the first logical step in this investigation of molecular mechanisms would be AMPK. Previous literature indicates AMPK activation in preadipocytes reduces the transcript abundance of the late phase differentiation factors PPARγ and C/EBPα [8, 9]. Likewise, the lack of leptin- mediated AMPK activation in *ob/ob* cells may predispose them to a higher sensitivity for AMPK activation. The use of the AMPK agonist 5-Aminoimidazole-4-carboxyamide ribonucleoside (AICAR) in *ob/ob*

cell culture would further elucidate the role of AMPK in the inhibition of proliferation and differentiation in this cell line. Furthermore, to fully investigate the role of adiponectin in adipose tissue expansion, it is essential to determine its relationship with leptin. Investigation of adiponectin in a leptin-resistant model such as the *db/db* mouse would further elucidate this relationship and directly relate to the leptin-resistance seen in obese humans. Moreover, further work is needed to determine if high concentrations of adiponectin are needed to mediate the desired adipose tissue effects or if restoration of normal concentrations in obese animals is sufficient.

Collectively, our data provides evidence that high concentrations of adiponectin influence the adipocyte population of adipose tissue. However, it seems that the potency of adiponectin as a determinant of preadipocytes proliferation or differentiation is dependent on the genetic background of the cells. Furthermore, we show no change in body composition in the absence of adiponectin, indicating the modulation of adipocyte growth by adiponectin reflects a pharmacological, rather than physiological, mechanism. In the absence of a dietary change in adiponectin in the pig, we identified tissue enrichment of essential fatty acids and a significant reduction in myeloid cells. A decrease in myeloid cell accumulation in adipose tissue suggests that dietary fatty acids are a valid option for prevention of obesity associated inflammation in a lean line of pigs, regardless of its effects on adiponectin. With further investigation, it appears that alteration of adiponectin concentrations through dietary or pharmaceutical means may be a promising treatment for prevention of obesity-related complications.

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