The role of adipocyte-specific Interferon Regulatory Factor 8 in diet-induced obesity and metabolic complications

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ABSTRACT

Adipose tissue is traditionally considered a reservoir of energy but has recently emerged as an important endocrine and immune organ in the regulation of systemic metabolism. In combination with genetic and environmental factors, excessive energy consumption is known to cause dysfunctional adipocyte metabolism. Obesity-associated adipocyte dysfunction is considered a primary causal factor in the development of metabolic complications, such as insulin resistance, inflammation, and non-alcoholic fatty liver disease (NALFD). Chronic overnutrition overwhelms the capacity of adipocytes to store lipids, leading to accumulation of free fatty acids (FFA), intracellular stress, and the release of pro-inflammatory and pro-apoptotic signaling molecules. As a result, inflammation and insulin resistance occur in other tissues, including the liver, brain, and skeletal muscle.

The present study investigates the mechanisms underlying obesity-associated metabolic complications. Our laboratory previously observed a 5.6-fold increase in interferon regulatory factor 8 (IRF8) mRNA expression in the adipocytes of mice fed a high fat diet (HFD) relative to mice fed a low fat diet (LFD). IRF8 belongs to a transcription factor family of nine proteins known as the interferon regulatory factors (IRFs), which function as stress sensors in macrophages important for the immune response. However, recent data has demonstrated their presence and significance in additional tissues, including adipocytes. The role of IRF8 in adipocytes has not been fully defined. Thus, the specific aims of this study are (1) to determine the role of lipopolysaccharide (LPS) in regulating IRF8 mRNA and protein expression in mouse derived cultured 3T3-L1 cells, and the role of IRF8 expression in adipocyte function and expression of downstream genes including FasR, adiponectin, and GLUT4, and (2) to investigate the effect of adipocyte-specific IRF8 expression and protein levels on systemic insulin resistance and associated metabolic complications during diet induced obesity (DIO) in mice.

To investigate the role of IRF8 in obesity associated with adipocyte dysfunction, we used an IRF8 adipocyte-specific knock out mouse model ($IRF8^{ad-/-}$) fed an HFD and measured serum glucose and insulin levels and tolerance via glucose and insulin tolerance tests (GTT and ITT, respectively), and liver triglyceride (TG) accumulation. Gene and protein expression levels of IRF8 and downstream targets were measured in the harvested adipose and liver tissues. Additionally, relevant genes and proteins were analyzed in vitro in 3T3-L1 cells to better understand signaling pathways in adipocytes during obesity.

In vitro data showed that IRF8 is regulated post-translationally by LPS. It was also shown that IRF8 regulates the expression of two important proteins relevant to hepatic lipid metabolism, FasR and adiponectin. When male $IRF8^{ad-/-}$ mice were fed a HFD, protection against the development of NAFLD and changes in gene expression of CD36, an important hepatic fatty acid transporter, were observed.

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CHAPTER I: REVIEW OF THE LITERATURE

Introduction

Obesity has become a global public health crisis in recent decades.[1] Data from 2017-2018 showed that 42.4% of adults in the United States are obese.[2] Obesity is associated with comorbidities that reduce life expectancy, including, but not limited to, type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), and cancer.[1,3] These consequences of obesity not only reduce the quality of life of affected individuals, but also place a significant economic burden on the health care system.[4] The etiology and pathophysiology of obesity is highly complex. The molecular mechanisms underlying obesity-associated metabolic diseases are not completely understood and are complicated by lifestyle, genetic, and environmental factors. Importantly, it has been shown that obesity causes adipocyte dysfunction and promotes the development of obesity-associated metabolic diseases.[5,6,7] Characterized by inflammation and cell death, obesity-associated adipocyte dysfunction has revealed a significant link between immunology and nutrient metabolism.[8,9] Signaling pathways such as toll-like receptor 4 (TLR4) are affected and downstream gene expression, protein levels and function are altered.[10,6]

Ultimately, the root cause of adipocyte dysfunction is a surplus of energy.[11,12] Persistent overnutrition exhausts the storage capacity of adipocytes, rendering them incapable of metabolic homeostasis.[13] The adipose tissue is the body’s largest energy reserve and has been widely studied for its role in obesity and associated metabolic disorders. As such, adipocyte dysfunction has become a significant focus of research in recent decades in an effort to understand the myriad of pathologies associated with obesity.

Adipose tissue

Adipose tissue is classically recognized for the unique ability to remodel its cellular dimensions in order to adapt to the metabolic milieu. Adipocytes represent most of the volume of the adipose tissue, but only accounts for 50% of all types of cells. In addition to adipocytes, the adipose tissues contains macrophages, eosinophils, mast cells, fibroblasts, lymphocytes, vascular cells, adipocyte precursor cells, and multipotent mesenchymal stem-like cells.[14] The adipocytes in adipose tissue present in different forms with unique metabolic functions.[15,14] White adipocytes typically contain a single, large lipid droplet, have a high storage capacity for energy as TG, and are collectively referred to as white adipose tissue (WAT).[16] Brown adipocytes, localized in brown adipose tissue (BAT), have a higher concentration of mitochondria and are involved in adaptive thermogenesis. Beige adipocytes have also been observed and have been researched for their ability to dissipate energy when needed.[15] Distinct depots of adipose tissue are found throughout the body: the subcutaneous region and the visceral region. Subcutaneous white adipose tissue (ScWAT) is the largest storage site for excess energy and is considered the most plastic of the metabolically relevant tissues, enabling the body to adapt to the given energy status. Visceral fat is located in the thorax and abdomen and in general is more likely to undergo detrimental changes with obesity, such as insulin resistance, as compared to ScWAT depots6

Lipolysis and lipogenesis are two classic functions of adipocytes. As a fuel reservoir, the adipose tissue stores excess energy as neutral TGs, and provides energy during periods of undernutrition. Lipolysis occurs when the body is in need of energy. Stored TGs are broken down into free fatty acids (FFA) and glycerol and used for energy in other tissues. In addition to being a storage organ, the adipose tissue has important secretory functions. Advances in adipocyte biology research have highlighted the endocrine nature of adipose tissue and is considered to be a “master regulator” of systemic energy homeostasis The dynamic functions of the adipose tissue shows the significant influence on whole body metabolism.[12]

Role of adipose tissue in inter-organ crosstalk

The concept of inter-organ crosstalk was first described in 2002 whereby Dietze et al. found that myocytes cocultured with adipocytes inhibited IRS-1 phosphorylation, suggesting that adipocyte factors induced insulin resistance in myocytes.[10] The adipose tissue has since been found to be a major source of communication between tissues, as illustrated in Figure 1. Crucial to this regulation are bioactive factors referred to as adipokines, which consists of growth factors, complement factors, hormones, and cytokines are secreted from adipose tissue and influence processes such as energy expenditure, food intake, metabolism, blood pressure, and the immune response.[17]. Adipokines, such as adiponectin and leptin, act locally and in peripheral organs to maintain metabolic homeostasis. This is accomplished by complex autocrine, paracrine, and endocrine networks between tissues, including the adipose tissue, brain, liver, muscle, heart, and pancreas that enable coordinated responses to a given metabolic environment.[18]

Obesity-associated adipocyte dysfunction

Central to the detrimental health effects associated with obesity is the development of obesity-associated adipocyte dysfunction. Among the most widely studied complications include inflammation, insulin insensitivity, and hepatic steatosis.[19] During obesity, adipocytes become hypertrophic as the demand for energy storage increases. Changes in adipocyte function and an infiltration of macrophages within the adipose tissue lead to hypersecretion of pro-inflammatory and pro-diabetic cytokines and a reduced secretion of adiponectin is observed. Chronic activation of the immune system has been widely recognized as an important factor in obesity-associated metabolic disorders.[20,21] Paracrine interactions between adipocytes and macrophages are known to initiate and maintain adipocyte dysfunction.[22] It has been shown that during obesity, macrophages are more abundant in the adipose tissue of obese subjects when compared to lean subjects.[23,24] These changes in the adipose tissue environment disrupt signaling pathways important for crosstalk with other metabolic organs that is crucial for maintaining metabolic homeostasis, ultimately leading to metabolic diseases.

Metabolic diseases associated with obesity

A wealth of data has highlighted the important role of adipose tissue in inter-organ cross talk and has led to a deeper understanding of how obesity causes stress within adipose tissue, as well as in distal organs.[25] Well-known comorbidities associated with obesity include T2DM and NAFLD, but recent evidence suggests that adipocyte metabolism also affects tumor formation.[26] Here, T2DM, NAFLD, cardiovascular disease, and breast cancer are discussed.

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a complex disorder that accounts for 90-95% of all diagnosed cases of diabetes.[27] It is a progressive metabolic disease characterized by dysregulated nutrient metabolism and is caused by insulin resistance or impaired insulin secretion. High blood glucose concentrations result from obesity-associated insulin resistance in the liver, adipose tissue, and muscle, along with reduced insulin secretion by the pancreas. Early stages of T2DM show β-cell adaptation, whereby β-cell mass and insulin secretion is increased to compensate for excess nutrients. When unresolved, the β-cells become exhausted and β-cell failure is observed, leading to hyperglycemia. While the precise mechanisms have not been completely defined, it has been observed that circulating levels of FFAs are elevated during obesity in animals and humans and are thought to cause inflammation and insulin resistance in peripheral tissues, which contributes to the development of T2DM.[9,28]

Non-Alcoholic Fatty Liver Disease

NAFLD represents the most common form of liver disease worldwide, affecting over one billion individuals.[1] Thirty percent of the United States population had NAFLD in 2016.[30] Stages of NAFLD includes fat accumulation and can progress to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma.[30] The disease is

characterized by hepatic accumulation of TG (ectopic storage of 5.56% of liver volume) followed by lipid droplets in the cytoplasm.[31] The underlying mechanism causing NAFLD remains unknown, but studies have shown that chronic overnutrition places a burden on the liver by increased handling of protein, fatty acids, and carbohydrates for storage. Obesity and insulin resistance are known to cause increased lipolysis in adipose tissue, providing FFA in circulation that can be taken up by the liver. Insulin has important actions in the liver: promotion of glycogenesis and *de novo* lipogenesis, and inhibition of gluconeogenesis.[30] During obesity and insulin resistant states, there are higher levels of lipolysis in adipocytes and thus levels of circulating FFAs to be taken up by the liver, reduced glycogenesis, and uninhibited gluconeogenesis.[31] Consequently, lipids accumulate in the liver and is clinically considered steatosis.[31] Additionally, increased secretion of pro-inflammatory cytokines by adipocytes, insulin resistance, and a pro-oxidative environment are observed during obesity and are thought to contribute to hepatic damage during NAFLD.[32]

Cardiovascular Disease

Along with obesity, rates of atherosclerosis and cardiovascular events are increasing rapidly.[33] It has been proposed that increased adiposity is associated with the development of atherosclerosis. A study of over a million subjects that were observed for 14 years showed a strong association with obesity and cardiovascular mortality.[34] Obesity may increase the risk of CVD through several mechanisms, including systemic inflammation and factors (metabolites, cytokines, and hormones) produced by the adipose tissue that damage the vessel wall.[35] These factors have been shown to alter the function of endothelial cells, macrophages, and arterial smooth muscle cells through changes in gene expression and lead to the production and release of FFA, TNF-a, fibrinogen, IL-6, and PAI, that effect vessel wall health.[36] The visceral adipose tissue is known to present a more significant influence on the development of atherosclerosis due to its proximity to the portal circulation, therefore also damaging the liver and pancreas.[37]

Breast Cancer

Obesity is associated with increased risk of breast cancer development and poor prognoses once diagnosed.[38,39,40] It is hypothesized that abnormal levels of estrogen, insulin, and/or adipokines during obesity may promote breast cancer. Adipocytes represent a prominent cell type in breast tissue.[3] The literature suggests that endocrine and paracrine signaling by cancer cells promote the production of metabolic substrates in adipocytes that in turn promotes cancer cell growth and invasion. This creates a detrimental relationship that has proven difficult to treat and begins to explain why obesity is associated with breast cancer risk and prognosis.[41] Obesity-associated adipocyte dysfunction in particular causes inflammation and altered metabolism that is thought to promote tumor growth.[39]

Adipose tissue regulation

Interferon Regulatory Factors

Complex regulatory signaling pathways enable the unique ability of adipose tissue to adapt to the present nutrient status. It is not clear how the adipose tissue senses overnutrition, but research has shown that pathogen-associated molecules elicit a response from pattern recognition receptors (PRRs) located on adipocyte membranes during obesity. Levels of fatty acids, LPS, double-stranded RNA, and unmethylated DNA are known to be elevated during obesity and to activate the Toll-like receptor (TLR) signaling pathway in adipocytes (Figure 2).[42] Upon binding, a signal transduction cascade is initiated leading to the transcription of pro-inflammatory cytokines and chemokines and is thought to promote insulin resistance.[43] Several well-known transcription factors are targeted in the signaling cascade, including NF-κB and HIF-1α.[44] Recently, another family of transcription factors have been implicated in adipose tissue inflammation referred to as interferon regulatory factors (IRFs).[45] Consisting of nine members, the IRF transcription factors (IRF 1-9) were originally identified for their role in type 1 interferon gene expression in response to pathogen invasion and tumor

suppression.[46] They are predominantly described in the literature for the initiation of the immune response in macrophages. IRFs are described as stress sensors and are activated through signaling receptors. Binding of inflammatory moieties to cell membrane receptors, most notably, the toll like receptor 4 (TLR4), initiates a signaling cascade that results in the phosphorylation and activation of IRFs, enabling their translocation to the nucleus, DNA binding, and ultimately the upregulation of inflammatory compounds such as interferons, chemokines, and genes involved in T helper cell differentiation.[45,47]

Recently, studies have shown that in addition to immunity, IRFs play an important role in metabolism and have been detected in the brain, kidney, liver, and adipose tissue.[48,49,50] LPS is an inflammatory compound that is significantly increased during obesity and is known to bind to TLR4 and increase the expression and activation of IRFs.[45] Several of the IRF proteins have shown to play important roles in adipocytes. Rosen et al showed that in the adipocytes of obese mice, IRF3 protein levels were increased relative to lean mice. [49] Deficiency of IRF3 in obese mice increased ScWAT browning and protected against insulin resistance and adipose inflammation.[51] Repression of adipocyte differentiation by IRF proteins has also been observed *in vitro*, indicating that they are important regulators of adipogenesis.[52] Additionally, IRF4 has been found to be required for the thermogenesis of brown adipocytes and beige adipocyte development via induction of PGC1-α and an important regulator of adipocyte lipid metabolism including lipogenesis, adiposity, and lipolysis.[51]

Interferon Regulatory Factor 8

In preliminary studies, we have found that in the adipocytes of mice fed a HFD, the expression of the IRF8 is increased by 5.6 fold relative to mice fed a LFD (Figure 3). Consistent with this observation, a recent study demonstrated that IRF8 expression is increased in the adipose tissue of genetically obese (*ob/ob*) mice.[53] IRF8 belongs to the IRF family of transcription factors and is traditionally known for its role in promoting the type 1 immune response.[45] In macrophages, IRF8 is important for the transcription of

genes important for differentiation of myeloid progenitor cells into mature macrophages and for the specificity of the inflammatory response.[54] In the intestine, IRF8 is necessary for the function of dendritic cells and thus intestinal T cell maintenance.[55] Here, we aim to elucidate the function of adipocyte-specific IRF8 on whole body energy homeostasis during obesity.

Gap in knowledge

The underlying mechanisms by which obesity associated adipocyte dysfunction leads to associated metabolic diseases are not fully understood. The current literature has identified several pathways that are altered during obesity. However, specific proteins, mechanisms, and metabolic regulation have yet to be fully elucidated. This study aims to add data and insight to the existing body of knowledge regarding metabolic processes during obesity, specifically the role of IRF8 in obesity associated adipocyte dysfunction. The regulation, effects, and signaling pathways of IRF8 have not yet been clearly defined and are investigated in the present research.

Figures

Figure 1. Effect of adipose tissue signaling on distal tissues.

Bioactive factors secreted from adipose tissue communicate metabolic status to the brain, liver, pancreas, immune system, and skeletal muscle influence satiety, glucose and lipid metabolism, insulin sensitivity, β-cell function, immune system activity.

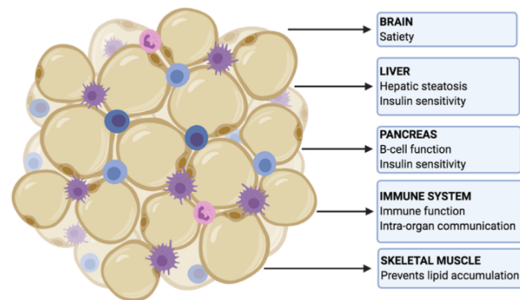


Figure 2. Obesity-associated changes in signaling pathways during obesity. Interactions between macrophages and adipocytes in adipose tissue cause changes in important signaling pathways during obesity due to the presence of ligands such as LPS, dsRNA, and unmethylated DNA. Binding of ligands to TLRs initiates a signaling cascade among HIF-1a, NF-kB, and the IRF family of transcription factors, promoting the transcription of inflammatory proteins.

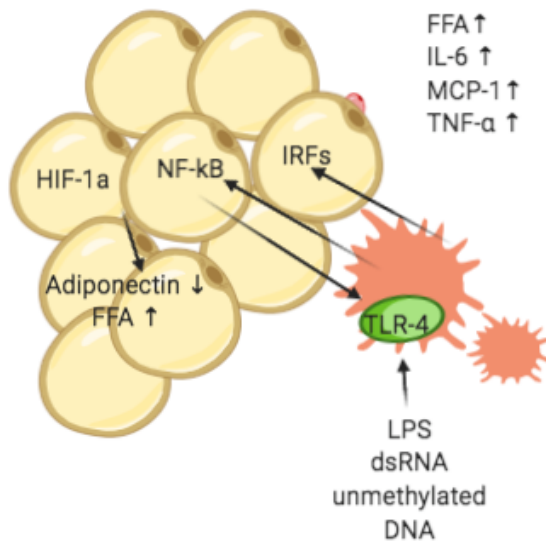
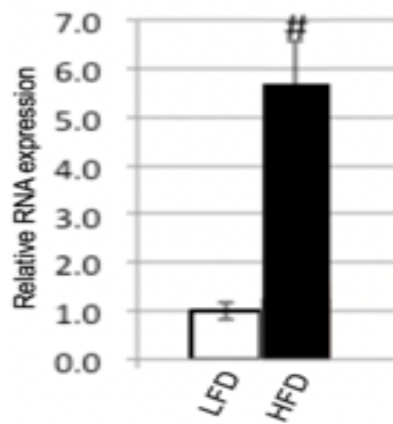


Figure 3. IRF8 expression in wildtype mice on a HFD relative to a LFD.
Previous studies in our laboratory demonstrated that in gWAT adipocytes of obese wildtype mice, the mRNA expression of IRF8 was increased 5.6 fold in control mice fed a HFD when compared to lean mice fed a LFD.



CHAPTER II: STATEMENT OF HYPOTHESIS AND RESEARCH OBJECTIVES

The objective of this thesis is to investigate the role and regulation of IRF8 in adipocytes and how it pertains to obesity-associated adipocyte dysfunction and the development of metabolic diseases.

Specific Aims

Specific Aim 1: To determine the role of LPS in temporally regulating IRF8 mRNA and protein expression in cultured 3T3-L1 adipocytes and the role of IRF8 expression in adipocyte function and expression of downstream genes, including the Fas Death Receptor (FasR), GLUT4, and adiponectin.

Hypothesis: LPS directly increases IRF8 protein expression, but not mRNA levels in the mouse derived adipocytes and will affect downstream signaling pathways.

Specific Aim 2: To investigate the effect of adipocyte-specific IRF8 expression and protein levels on systemic insulin resistance and associated metabolic complications during DIO in mice with an adipocyte-specific deletion of IRF8 ($IRF8^{ad-/-}$) as compared to wildtype ($IRF8^{fl/fl}$) mice.

Hypothesis: Ablation of IRF8 in adipocytes of obese mice mitigates the metabolic complications associated with DIO in mice.

Significance of the study

Obesity associated adipocyte dysfunction is a complex pathology which is not completely understood but is known to promote the development of additional metabolic complications. A wide body of evidence indicates that IRFs have a greater role in

metabolism and have effects in more tissues than is currently defined.[52] The proposed study will contribute to our understanding of adipocyte metabolism during DIO and the underlying mechanisms by which obesity leads to additional metabolic diseases. This contribution is significant because it will further define adipocyte metabolism and lead to potential therapeutic targets for obesity and associated metabolic diseases. By protecting against the development of adipocyte dysfunction during DIO, downstream metabolic complications could be prevented and improve overall health. IRF8 has not been extensively studied in adipocytes; therefore, the present work will add to the body of knowledge regarding IRF8 and its role in obesity associated adipocyte dysfunction and alterations in systemic metabolism.

CHAPTER III: IN VITRO ANALYSIS OF THE REGULATION AND EXPRESSION OF IRF8 AND DOWNSTREAM GENES

Introduction

Knowledge of the genes regulated by a transcription factor is essential to understanding how cells respond to the given metabolic environment. Increasing evidence shows the importance of the IRF family of transcription factors as regulators of adipocyte biology.[49,56,50] In vitro assays were performed to gain a more comprehensive understanding of the IRF8 signaling pathway in adipocytes and how that effects adipocyte metabolism. Analysis of cells during differentiation was assessed to establish gene expression patterns during adipogenesis and to generate a reference before experimental treatments. We used an adenovirus expressing mouse IRF8 to infect and overexpress IRF8 to determine the effects on downstream genes in adipocytes. Proinflammatory signaling was examined through activation of TLR signaling by incubation with LPS. Short and long term incubation periods were used to evaluate the regulation of IRF8 gene expression and protein levels.

Methods

Cell treatment

3T3-L1 preadipocytes were cultured in 10 cm plates at 5 % CO_2 in Dulbecco's modified eagle's medium (DMEM) that included 10% bovine calf serum (BCS), high glucose (4,500 mg/L), L-glutamine (584 mg/L), and HEPES (5958 mg/L). [57] Fresh media was supplied every 2 days. Differentiation of preadipocytes into mature adipocytes was stimulated at 2 days post-confluence (day 0) by the addition of media containing 10% fetal bovine serum (FBS), 10 μg/ml insulin, 1 μM dexamethasone, 500 mM isolutyl-1-methylxanthine, and 1 μM rosiglitazone (Thermofisher) for 2 days. Media was replaced

on day 2 of differentiation with DMEM containing 10% FBS and 10 μg/ml insulin for 2 subsequent days. Ten percent FBS in DMEM was supplied until the experiments conducted at days 7-9 post-differentiation. Cells were serum starved overnight with 0.2% BSA prior to experiments to ensure similar metabolic states between the cells. Upon overnight starvation, the cells were treated with the DMEM with a 2% FBS concentration and experimental treatments.

Differentiation

3T3-L1 preadipocytes were maintained and differentiated at 2 days post-confluence as described above. To determine the gene and protein expression of IRF8 during the process of differentiation, cells were harvested on days 0, 2, 4, 6, 8, and 10. These data were determined by PCR and Western blot using methods described below.

Overexpression

The adenovirus expressing Mouse IRF8 used for infection was purchased from Vector Biolabs. 3T3-L1 adipocytes were grown and maintained as previously described. They were serum starved on day 5 of differentiation and infected with the IRF8 adenovirus (1:10) on day 6 of differentiation. The cells were incubated at 37° C for 2 days and harvested on day 8 for gene and protein analysis using RT-PCR and Western blot, respectively.

LPS treatment

It is known that TLR signaling in adipocytes promotes inflammation and has been implicated in the development of obesity-associated metabolic disorders.[58,59] Considering LPS triggers a TLR signaling cascade, we examined the effects of LPS incubation on gene expression in adipocytes. Cells were serum starved overnight on day 7 of differentiation and treated with LPS (100 ηg/ml) on day 8. Cells were harvested at several different time points according to the protocol provided by the manufacturer

(RNeasy, Qiagen) to look at gene expression; RIPA buffer was used to harvest cells for protein analysis. First, we looked at shorter regulation, the cells were harvested at 0, 5, 15, 30, 60, and 180 minutes. Gene expression was also measured at hours 4 and 24 hours and 5 days.

Gene expression analysis

RNA was extracted from the samples harvested described above using the RNeasy Mini columns (Qiagen). Samples were analyzed for concentration and purity by the Nanodrop 100 spectrophotometer. Two μg RNA per sample was used to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using the Applied Biosystems 7300 RT-PCR System. Data represents the relative gene expression quantified using the $2^{-\Delta\Delta Ct}$ method

Western blot

Protein expression was analyzed as previously described.[60] Cells were harvested in RIPA Buffer (50 mM Tris-HCl, pH 7.4, 150 nM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and then incubated at 37° C for one hour with 15 second vortexing every 20 minutes. Cells were then centrifuged at 14,000g for 10 minutes at room temperature, and the infranatant between the fat and the pellet was taken and frozen at -80C for western blot analysis. Protein amounts per sample used were normalized using the BCA assay (Thermo Scientific). The protein lysates were solubilized and denatured. Equal amounts of protein were fractionated by electrophoresis using a precast 7.5% SDS polyacrylamide gel (Biorad) and the proteins were separated by SDS-PAGE at 75 V for 90 minutes. Proteins were transferred to a nitrocellulose membrane (45μm) in 4° C for 1 hour at 35° C. Anti-IRF8 and anti-GAPDH were purchased from Santa Cruz Biotechnology. The membrane was blocked in 5% milk and incubated with the primary antibody (1:100) overnight. Following overnight incubation, membranes were washed (0.1% Tween-20 PBS) and incubated with 5% milk containing the secondary antibody (1:5000) for 1 hour and washed again. The

membrane was then treated with ECL Reagent (Thermofisher) and proteins were visualized on photo paper using autoradiography.

Results

Overexpression of IRF8 increases the expression of FasR in 3T3-L1 adipocytes

Cells were harvested on days 0, 2, 4, 6, 8, and 10 of differentiation of adipocytes, and it was determined that the highest level of expression of IRF8 is on day 8 (Figure 1), though there was variability in the results. In general, it appears that the level of IRF8 mRNA with differentiation is relatively consistent. Therefore, samples were harvested day 8 for the following experiments in 3T3-L1 cells.

In order to determine the effects of IRF8 to regulate expression of specific genes in adipocytes, we transduced differentiated 3T3-L1 adipocytes with an adenovirus that contained a CMV promoter to overexpress IRF8 mouse cDNA. At various time points (listed in the figures) of transduction, cells were harvested, mRNA was isolated and Real Time PCR (RT-PCR) was used to measure mRNA expression of several genes (Figure 2). Based upon these studies, we concluded that IRF8 overexpression specifically increased the mRNA expression of the Fas receptor (FasR), which has been implicated in obesity-associated adipocyte dysfunction and systemic insulin resistance.[61,62] Interestingly, IRF8 overexpression also significantly downregulated the expression of the circulating adipokine, adiponectin measured using RT-PCR. This is significant because during obesity, adiponectin adipocyte expression and circulating levels are reduced.[63] Circulating levels of adiponectin have been shown to reduce serum free fatty acid levels and improve systemic insulin sensitivity.[63,64]

IRF8 protein levels are increased in 3T3-L1 cells after treatment with LPS, but mRNA expression remains the same

It has been previously reported in macrophages that protein levels of IRF8 were increased within 15 minutes of incubation with LPS (100 ηg/ml) in the absence of changes in mRNA expression levels, indicating that LPS regulates IRF8 post-translationally.[27] To investigate the time course of LPS effects on IRF8 expression within adipocytes, we incubated differentiated 3T3-L1 adipocytes with LPS and harvested the cells at varying time points (0, 5, 15, 30, 60, and 180 minutes) (Figure 3). We observed that incubation with LPS did not alter IRF8 mRNA levels, but protein levels of IRF8 were increased as early as 15 minutes of incubation. As observed in the previously mentioned study, our study indicates that LPS regulates IRF8 protein expression post-translationally. Additionally, when we overexpressed IRF8 in 3T3-L1 adipocytes, there was an increase in the expression of the mRNA for the death receptor, FasR. Furthermore, incubating 3T3-L1 differentiated adipocytes with LPS resulted in increased IRF8 protein expression, and significantly increased mRNA expression of FasR after 5, 10, 60, and 180 minutes (100 ηg/ml) (Figure 3). These data indicate that in response to LPS incubation, IRF8 protein expression is increased secondary to post-translational mechanism.

The time course of IRF8 regulation in adipocytes was also evaluated by measuring the expression of these genes (IRF8 and FasR) after incubation with LPS after 4 hours, 24 hours, and 5 days (Figures 4 and 5). Again, we observed increased level of FasR, but as predicted, it was due to the post-translational regulation of IRF8 not IRF8.

Figures

Figure 1. IRF8 mRNA expression during 3T3-L1 cell differentiation.

The expression of IRF8 was measured on day 0, 2, 4, 6, 8, and 10 of differentiation. IRF8 expression was slightly, but not significantly reduced on day 6 (p = 0.055). The highest level of expression was found on day 8 (p = 0.46).

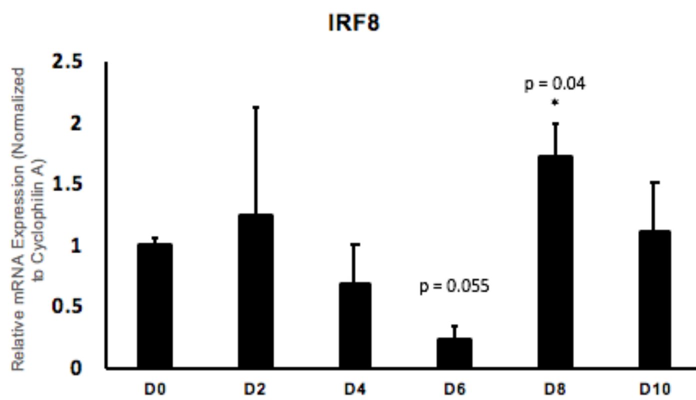


Figure 2. mRNA expression levels in 3T3-L1 cells overexpressing IRF8. Overexpression of IRF8 in 3T3-L1 cells was achieved by incubation of 3T3-L1 cells with an adenovirus expressing IRF8 at a dilution of 1:10. The expression of IRF8 was increased 100 fold ($p = 0.00$). Increased expression was also seen with FasR by about 12,000 fold ($p = 0.00$). No changes were seen in GLUT4 expression, whereas a decrease in expression in adiponectin was observed ($p = 0.00$).

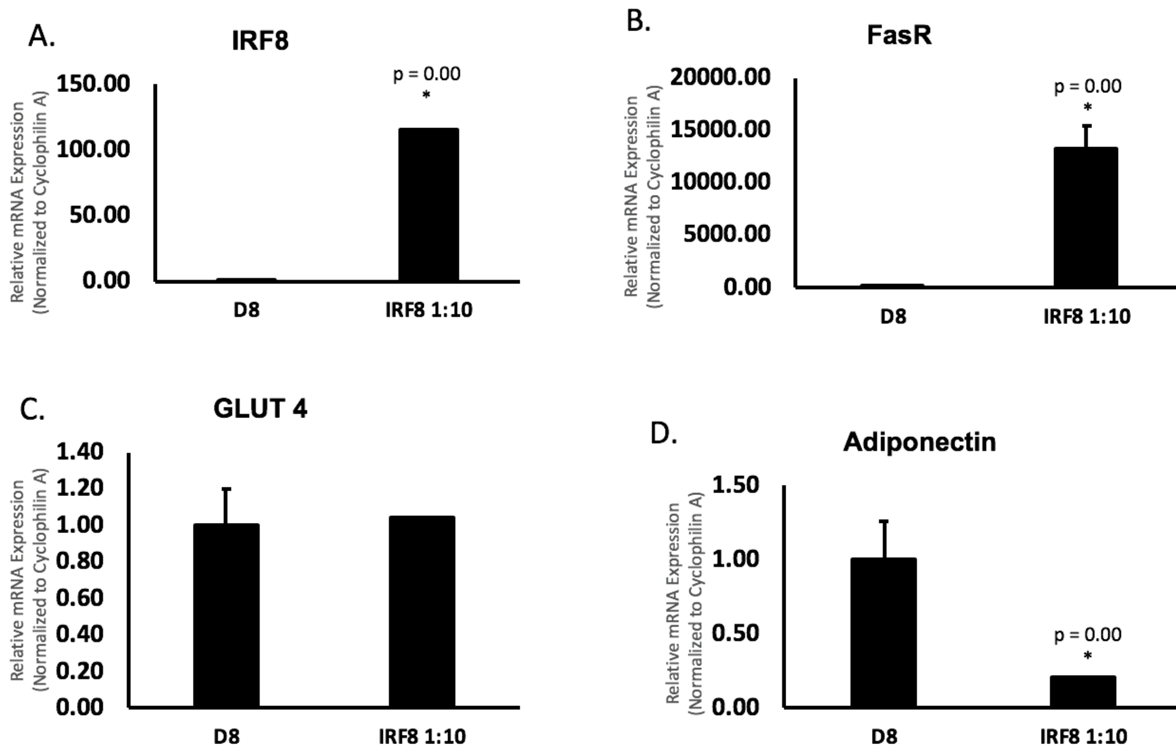


Figure 3. Short term LPS treatment in 3T3-L1 adipocytes.

LPS (100 ng/ml) was used to treat 3T3-L1 cells. Cells were harvested at minutes 0, 5, 15, 30, 60, and 180 minutes. Western blot analysis demonstrated that protein levels of IRF8 (A) were increased by 15 minutes and declined at 180 minutes. No changes were seen in mRNA expression (B). FasR expression was also measured (C) and increases were found at 5 minutes (p = 0.032). 60 minutes (p=0.032), and at 180 minutes (p = 0.026). N = 4 wells of a 12 well plate per treatment. Each experiment was done two times.

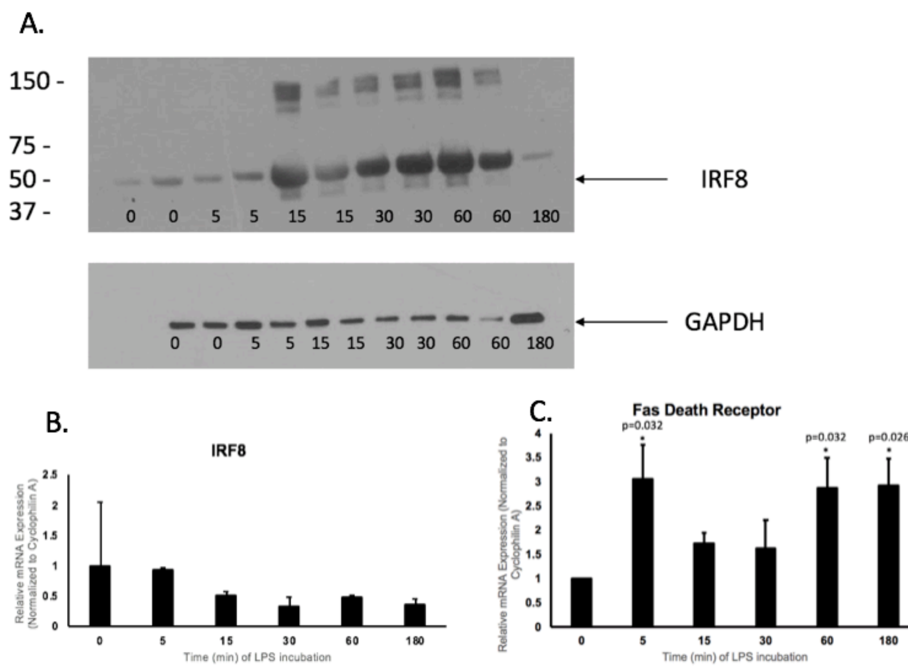


Figure 4. LPS treatment for 4 and 24 hours in 3T3-L1 adipocytes.

To further determine the time course of IRF8 regulation, mRNA expression was measured in 3T3-L1 cells after 4 and 24 hours. No change was seen in IRF8 mRNA expression levels, whereas there was an increase in FasR mRNA expression after LPS incubation at hours 4 ($p = 0.003$) and 24 hours ($p = 0.015$).

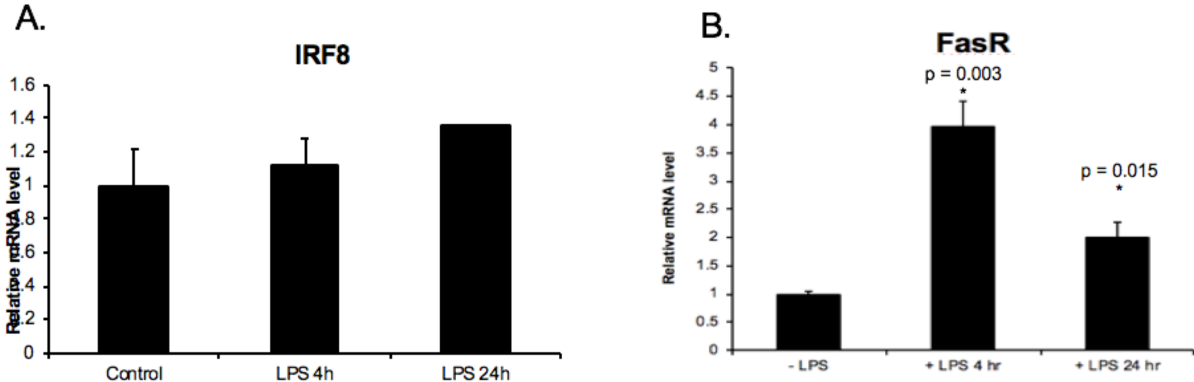
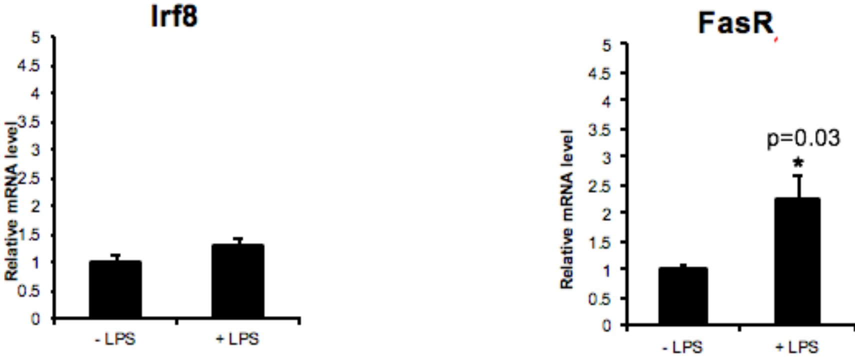


Figure 5. LPS treatment for 5 days in 3T3-L1 cells.

Gene expression was measured in 3T3-L1 cells after incubation with LPS for 5 days. No changes were seen in IRF8 mRNA expression. FasR mRNA expression levels were increased 2 fold (p = 0.03).



CHAPTER IV: EFFECT OF ADIPOCYTE-SPECIFIC ABLATION OF IRF8 ON OBESITY-ASSOCIATED METABOLIC COMPLICATIONS IN VIVO

Introduction

Intricate cell signaling pathways enable a prompt and precise response to tumors and invading pathogens. Increasing evidence shows that transcription factors important for the initiation of the immune response within the context of cancer and pathogen invasion are also important for adipocyte metabolism, including IRF8.[49] Under conditions of metabolic stress, particular proinflammatory proteins in adipocytes such as IRF8 have shown to play an important role in the development of adipocyte dysfunction during obesity or caloric stress.[66] Studies investigating the role of inflammatory transcription factors in adipocytes have shown that insulin resistance and liver fat accumulation caused by obesity are attenuated through adipocyte-specific knockouts of particular genes related to inflammation and adipogenesis.[67,62,8,68] We have previously shown that IRF8 is increased in the adipocytes of mice after DIO when compared to lean mice. Current literature details the role of IRF8 in macrophages and the immune response, but research is limited regarding signaling pathways in adipocytes.

To provide a more comprehensive understanding of IRF8's role in obesity-associated metabolic disorders, we generated a mouse model that specifically deletes IRF8 within adipocytes using the LoxP-Cre system. Mice were fed a 60% high fat diet *ad libitum* to model diet-induced obesity. Body composition and metabolic measurements were examined. Tissue samples were taken, and the analysis included quantification of tissue weights, fat content, and gene expression levels of IRF8 and downstream genes.

Methods

Generation of mice with adipocyte-specific IRF8 deletion

The LoxP-Cre system is a powerful genetic tool that enables tissue-specific knockout models. To delete IRF8 in adipocytes, mice harbored the Adipoq-Cre BAC transgene which enables the expression of Cre recombinase specifically under the control of adiponectin promoter regions in the Adipoq-Cre BAC transgene (JAX stock #010803).[49] Importantly, the Adiponectin Cre-recombinase is only expressed in the white and brown adipose tissue, but not in resident macrophages. These mice are mated to homozygous *loxP* (floxed), in which the IRF8 gene is flanked with *loxP* sites. Mice lacking Adipoq-Cre do not express Cre-recombinase and therefore will not delete the IRF8 gene and served as controls ($IRF8^{fl/fl}$). In the white and brown adipose tissue of mice expressing Cre recombinase, IRF8 was deleted and were used as the experimental mice ($IRF8^{ad-/-}$).

Animal care

Animals were kept at the Jean Mayer U.S. Department of Agriculture Human Nutrition Research Center on Aging at Tufts University in a viral and pathogen-free facility. Animal care and experiments were conducted according to guidelines of the Institutional Animal Care and Use Committee of Tufts University. At 8 weeks of age, $IRF8^{ad-/-}$ and $IRF8^{fl/fl}$ mice were individually caged and were either shifted from a standard chow diet (10% LFD, (Table 1) (Teklad 2016S) to a 60% HFD as previously described, with both diets matched for sucrose. The experiments included several cohorts, with both male and female mice. Details regarding the sex, duration on the HFD, and number of mice per cohort are found in Table 2. Prior to sacrifice, the experimental mice were fasted for 6 hours or 12 hours. Overnight fasting (12 hours) was employed in Cohort 3 to allow mice to acclimate and reduce possible stress prior to FBG measurements. Mice were euthanized via exsanguination and cervical dislocation under isoflurane anesthesia. Fasting blood glucose was measured from the tail vein by glucometer and the liver, gonadal white adipose tissue (gWAT), and subcutaneous white adipose tissue (ScWAT) were harvested, weighed and flash frozen to be prepared for further analyses.

Glucose and insulin tolerance tests

Insulin and glucose tolerance tests were performed following a 6 hour fast or an overnight fast and on non-consecutive weeks. An intraperitoneal injection of glucose (.3 g/kg body weight) or insulin (.75 U/kg) was administered and blood samples were tested at 0, 15, 30, 60, 90, and 120 minutes or until blood glucose levels returned to baseline. Blood glucose from the tail vein was quantified using a portable glucometer.

Real time quantitative PCR analysis of tissues

RNA was isolated from gWAT, ScWAT and liver of the mice and RT-PCR analysis was used to determine the expression of genes related to glucose metabolism, lipid metabolism, and apoptosis. Tissues were mechanically homogenized in TRIzol (Invitrogen) and the RNeasy Mini columns (Qiagen) were used to extract RNA according to the manufacture's protocol. The Nanodrop spectrophotometer (Nanodrop 1000, Wilmington, DE) was used to quantify RNA and check for purity. Equal amounts of RNA were used to make cDNA from the samples and the expression of target genes were analyzed using RT-PCR using SYBR Green (Applied Biosystems 7300, Carlsbad, CA). Relative expression of target genes were quantified as $2^{-\Delta\Delta Ct}$, with Cyclophilin B used as the endogenous control.[69]

Liver triglyceride and enzyme assessment

The frozen livers (30-40 mg/ sample) were pulverized using a mortar and pestle over dry ice and transferred to liquid nitrogen in Eppendorf tubes. The lipid fraction was dried and used to analyze lipid content. Following the drying of the sample, a solution of 1.5% Triton X (Thermofisher) and phosphate-buffered saline (PBS) (Thermofisher) were heated to 65°C and used to dissolve the sample. Triacylglycerides using the Liver TG kit, respectively per the manufacture's protocol (Cayman Chemical).

Statistical analysis

Multiple comparisons were analyzed by a two-way ANOVA followed by a Turkey's HSD when appropriate. A Student's t-test was used to analyze gene expression.

<u>Results</u>

IRF8 expression is reduced in the gWAT of IRF8$^{ad-/-}$ following a HFD.

Progeny from IRF8$^{ad-/-}$ and IRF8$^{fl/fl}$ mice were genotyped prior to the study and separated based on the presence of adiponectin-Cre. After 16 weeks on the 60% HFD, IRF8$^{ad-/-}$ and IRF8$^{fl/fl}$ mice were sacrificed and the gWAT was analyzed for the presence of IRF8. Results showed significantly reduced IRF8 mRNA expression in IRF8$^{ad-/-}$ mice ($p = 0.044$) (Figure 1). Complete ablation of IRF8 mRNA was not expected due to the presence of resident macrophages in adipose tissue that do not contain adiponectin-Cre. Adipocytes were isolated in later studies, but were not suffiecient for gene expression analyses.

Knockout of adipocyte-specific IRF8 shows significantly reduced liver weight after HFD for 13 weeks, but not at 16 weeks.

The present study consisted of three cohorts of male mice with or without adiponectin-Cre (Table 2). Immediately after the experimental diet timeline, mice were scarified for further analysis. Fasting blood glucose, body weight, and weights of the gWAT, ScWAT, and liver of each cohort were recorded (Figure 2). Blood and tissue were flash frozen and saved. Cohort 1 and Cohort 3 IRF8$^{ad-/-}$ and IRF8$^{fl/fl}$ mice were fed a 60% HFD for 16 weeks and did not show differences in body weight, gWAT, ScWAT, or liver tissue. Cohort 2 was sacrificed after 13 weeks on a 60% HFD. Differences were seen liver tissue weight (1.35 g and 1.09 g for IRF8$^{fl/fl}$ and IRF8$^{ad-/-}$ mice, respectively, $p = 0.02$). No differences were seen in gWAT ($p=0.09$) or ScWAT($p=0.08$) in Cohort 2.

IRF8$^{ad-/-}$ mice show reduced expression of CD36 and lower levels of TG in the liver after 13 weeks on a HFD.

Liver tissue from mice used in Cohort 2 were harvested and used for further analysis. Here, we found that TG concentrations in the liver were significantly lower in when IRF8 is knocked out in adipocytes (p=0.0004) (Figure 3). In these samples, we looked at gene expression of enzymes commonly dysregulated in obesity. (Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are involved in gluconeogenesis and were not significantly changed. The expression of CD36, an important fatty acid transport important in facilitating uptake of serum fatty acids into the liver that can be converted into TG, showed significantly lower expression levels in IRF8$^{ad-/-}$ mice (p=0.001) (Figure 3).[70]

Insulin sensitivity is not significantly changed in IRF8$^{ad-/-}$ mice fed a HFD.

To determine if adipocyte IRF8 affects insulin signaling during obesity, measures of insulin sensitivity were taken throughout each study. Insulin tolerances tests were performed at week 4 (Cohort 1), 11 (Cohort 2), and 13 (Cohort 3). After 11 weeks on a HFD, mice showed a more robust response to insulin; however, the results were inconsistent. No differences were found in glucose tolerance tests at weeks 10 (Cohort 1) or 11 (Cohort 3). Figure 4 shows FBG measured throughout the studies for each cohort after either a 6 hour fast or an overnight fast (OVN) (Cohort 3). Overnight fasting was employed to allow the mice to acclimate and reduce stress levels in the mice before measuring glucose levels; however, no differences in variability or glucose levels were observed. At 16 weeks of HFD feeding in Cohort 1, FBG was reduced in IRF8$^{ad-/-}$ mice compared to IRF8$^{fl/fl}$ mice, but this effect was not replicated.

Tables

Table 1. Nutrient composition of the experimental diets.

Nutrient	Source	HFD (g)	LFD (g)
Protein	Casein, Lacric, 30 Mesh	200.00	200.00
	Cystine, L	3.00	3.00
Carbohydrate	Starch, Corn	-	506.20
	Lodex 10	125.00	125.00
	Sucrose, Fine Granulated	72.80	72.80
Fiber	Solka Floc, FCC200	50.00	50.00
Fat	Lard	245.00	25.00
	Soybean Oil, USP	25.00	20.00
Mineral	S10026B	50.00	50.00
Vitamin	Choline Bitartrate	2.00	2.00
	V10001C	1.00	1.00
Dye	Dye, Blye FD&C #1, Alum. Lake 35-42%	0.05	0.04
	Bye, Blue FD&C #1, Alum. Lake 35-42%	-	0.01
	Total (g)	**773.85**	**1055.05**

Table 2. Experimental schedule for male and female IRF8$^{fl/fl}$ and IRF8$^{ad-/-}$ mice fed a HFD.

Cohort	Sex	Weeks on HFD diet	Number
1	Male	16	IRF8$^{fl/fl}$ =9; IRF8$^{ad-/-}$=12
2	Male	13	IRF8$^{fl/fl}$ =10; IRF8$^{ad-/-}$=10
3	Male	16	IRF8$^{fl/fl}$ =6; IRF8$^{ad-/-}$=5

Figures

Figure 1. IRF8 expression in gWAT of IRF8$^{fl/fl}$ and IRF8$^{ad-/-}$ mice.

Gene expression analysis showed reduced expression levels of IRF8 in gWAT following a 60% HFD for 16 weeks (p=0.044).

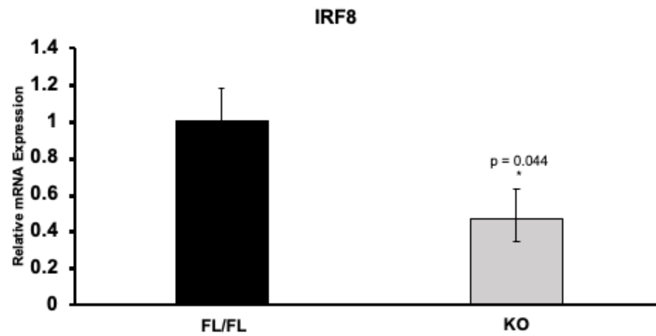


Figure 2. Measurements of body and tissue weights in three cohorts of IRF8$^{fl/fl}$ and IRF8$^{ad-/-}$ following a 60% HFD.

Body weights were taken before sacrifice and tissues were weight immediately after harvest. (A) Body weights and (B) tissue weights from Cohort 1 following a 60% HFD for 16 weeks; (C) body weights and (D) tissue weights from Cohort 2 following a 60% HFD for 13 weeks; and (E) body weights and (F) tissue weights from Cohort 3 following a 60% HFD for 16 weeks. After 13 weeks on HFD, IRF8$^{ad-/-}$ had significantly lower liver weights compared to IRF8$^{fl/fl}$ mice (p=0.02).

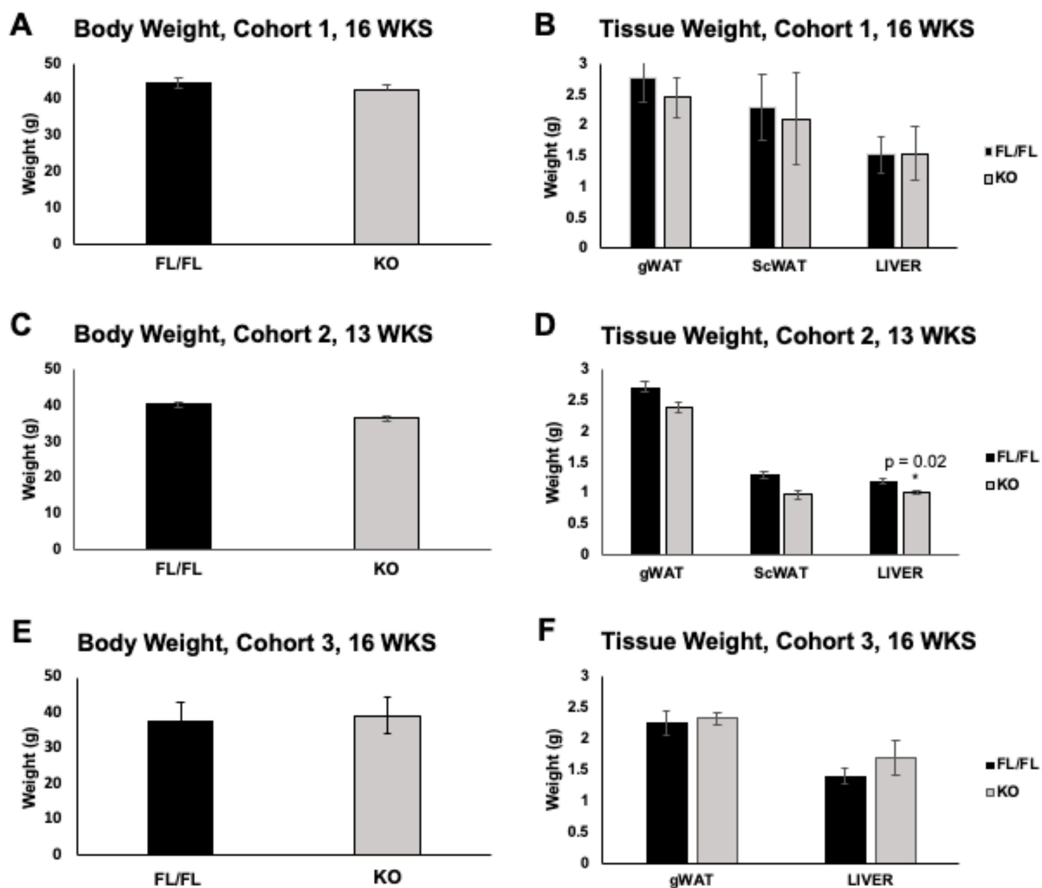


Figure 3. Quantification of hepatic TG and gene expression in $IRF8^{fl/fl}$ and $IRF8^{ad-/-}$ mice.

After 13 weeks on HFD, $IRF8^{ad-/-}$ had significantly lower levels of liver TG compared to $IRF8^{fl/fl}$ mice (4.360 μg/mg and 8.180 μg/mg, respectively) (p=0.0004) (n=10 per group). PCR analysis of the liver showed a significant reduction in CD36 expression (p=0.001) and no changes in PEPCK or G6DPase. While I made this observation here, I was not able to reproduce these data in other studies, and further studies are needed to understand any issues associated with the approach and to confirm whether these data are reproducible.

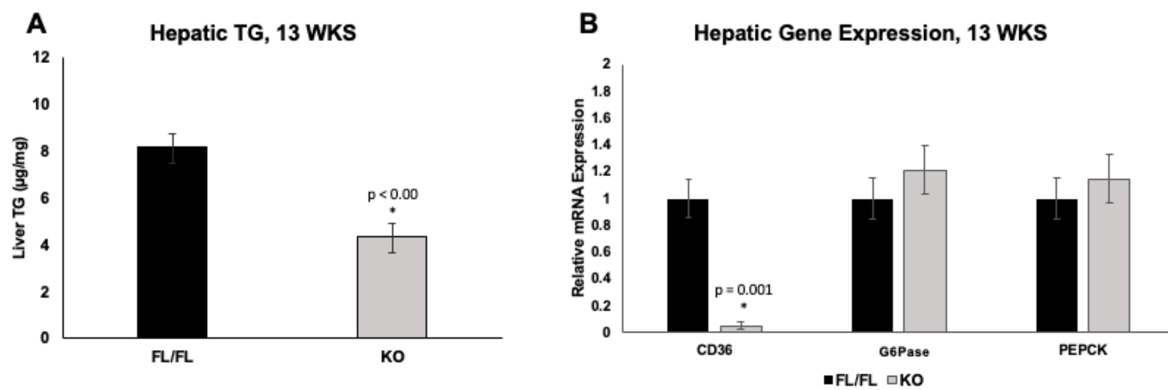
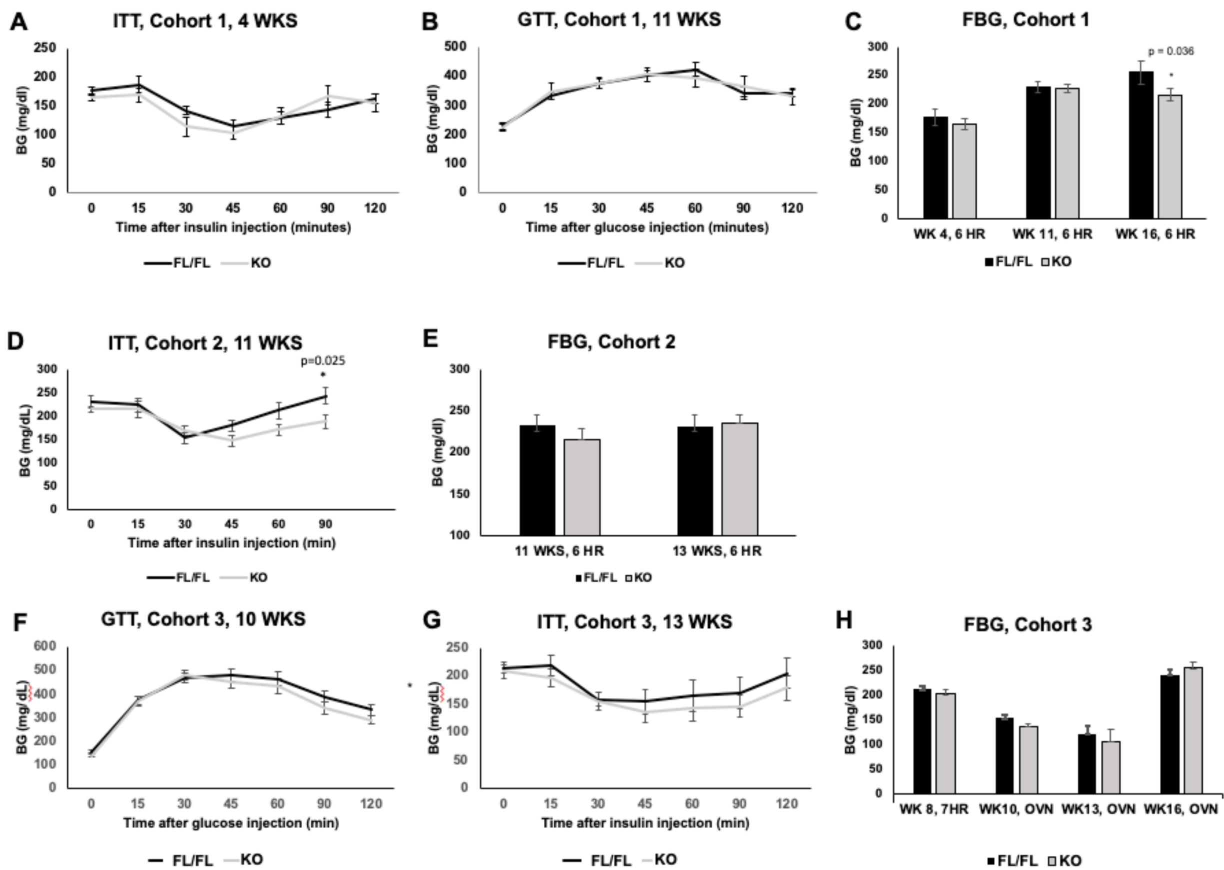


Figure 4. Measures of insulin sensitivity in Cohorts 1-3 of male IRF8$^{fl/fl}$ and IRF8$^{ad-/-}$ on a 60% HFD.

Insulin tolerance, glucose tolerance, and fasting blood glucose measurements throughout experimental studies in three cohorts of IRF8$^{ad-/-}$ and IRF8$^{fl/fl}$ mice. Overall, knockout of IRF8 in adipocytes did not significantly improve insulin sensitivity in mice on a HFD over time.



CHAPTER V: SUMMARY

Discussion

Current literature shows that the expression of the IRF family of transcription factors is altered in adipocytes during caloric overload and contributes to the metabolic dysfunction associated with obesity. Previous studies in our laboratory showed that IRF8 is highly upregulated in the adipocytes of mice fed a HFD when compared to those fed a LFD.[60] Here, we investigate the molecular mechanisms underlying the functions of adipocyte-specific IRF8 in 3T3-L1 cultured adipocytes and measured protein levels and gene expression IRF8 overexpression using a virus and after inducing stress via LPS incubation. Further, the LoxP-Cre system was used to specifically delete IRF8 in adipocytes to evaluate the possible effects of the transcription factor on adipocyte dysfunction and metabolic parameters in an experimental model of obesity in mice. IRF8 floxed and IRF8 knockout ($IRF8^{fl/fl}$ and $IRF8^{ad-/-}$) mice were fed a 60% HFD to induce obesity and caloric stress, and relevant metabolic measures were evaluated.

To understand the IRF8 signaling pathway in adipocytes, we investigated the effect of IRF8 on the expression of FasR in cultured 3T3-L1 adipocytes. FasR is a death receptor involved in the regulation of apoptosis and is a downstream effector of IRF8.[71] In addition to apoptosis, FasR is involved in pro-inflammatory signaling and obesity-associated liver dysfunction. When bound to FasL, FasR has shown to increase the production of IL-1α, IL-β, IL-β, and MCP-1 in cultured 3T3-L1 adipocytes.[72] Deficiency of FasR in the adipose tissue of mice reduces weight gain, adipocyte inflammation, hepatic steatosis, and insulin resistance.[62] We hypothesized that changes in FasR expression is important in the development of obesity-associated adipocyte dysfunction and affected by levels of IRF8. The present study shows that when IRF8 is overexpressed in cultured adipocytes, FasR is upregulated. To further evaluate this pathway, we incubated 3T3-L1 cells with LPS, a known activator of IRF signaling. We found that the expression of FasR was increased following LPS incubation; however, only protein levels of IRF8 were increased, suggesting post-translational regulation of IRF8 by LPS. Taken together, adipocyte-IRF8 regulation of FasR may contribute to the

crosstalk between the adipose tissue and liver and development of NAFLD associated with obesity. Interestingly, these effects are dependent on IRF8 protein levels rather than gene expression.

The liver and adipose tissue are key organs that enable metabolic homeostasis under normal physiological conditions. It is well known that proper crosstalk between the adipose tissue and the liver through adipokines and growth factors plays a crucial role in maintaining homeostasis.[25,70,73] Changes in this communication occur during obesity, as adipocyte metabolism becomes dysfunctional due to caloric overload. Hypertrophy and hyperplasia occur in adipose tissue to compensate for chronic energy surpluses in obesity and cause oxidative stress, cell death, and inflammation.[11]These processes disrupt the secretion patterns of adipocytes and influence the lipid profile in adipose tissue, liver, and other metabolic organs. When unresolved, NAFLD occurs when adipocytes release higher levels of FFAs and adipokines that are delivered to the liver, promote hepatic lipid accumulation, and cause lipotoxicity, characterized by inflammation, apoptosis, and fibrogenesis.[74,74,75] Here, we have investigated the role of adipocyte-specific IFR8 in the development of metabolic complications in the liver during obesity. We hypothesized that the altered regulation of IRF8 in adipocytes observed during obesity affects hepatic metabolism and may contribute to the crosstalk between the adipose tissue and liver.

When challenged with a HFD for 13 weeks, $IRF8^{ad-/-}$ mice had significantly lower liver weights, while ScWAT and gWAT samples did not differ significantly from $IRF8^{fl/fl}$ mice. Additionally, lower levels of TGs were observed in the livers of $IRF8^{ad-/-}$ mice. Evaluation of gene expression in the livers of obese $IRF8^{fl/fl}$ mice showed that the fatty acid transporter, CD36, was significantly downregulated. CD36 is a is a transmembrane glycoprotein found on the surface of adipocytes, macrophages, myocytes, enterocytes, and hepatocytes.[76] A primary function of CD36 is the intracellular uptake, trafficking, and esterification of FFAs. Other functions include regulation of FFA oxidation, VLDL secretion, inflammation, lipogenesis, and autophagy.[77] Importantly, CD36 is known to play a significant role in the progression of NAFLD.[76,78] Under normal physiological conditions, CD36 is not highly expressed, but is induced under conditions of high lipid levels. It is known that CD36 helps maintain lipid homeostasis in many cell types,

including the liver, but less is known about the role of CD36 in conditions of chronic overnutrition and lipotoxicity.[7979,80] A recent study found that ligation of CD36 was associated with inflammation and lipid accumulation in the liver.[79] It is not yet clear if CD36 ligation is a key event in the progression of NAFLD.[79] While CD36 is found to be upregulated under insulin resistant conditions, we did not find differences in insulin sensitivity between IRF8$^{ad-/-}$ and IRF8$^{fl/fl}$ mice; thus, these findings suggest that adipocyte IRF8 effects hepatic liver metabolism and CD36 gene expression through other regulatory pathways such as TLR4 signaling.[78,81]

While T2DM is associated with both obesity and NAFLD, deletion of IRF8 in adipocytes did not protect against HFD-induced insulin resistance. Interestingly, when IRF8 was overexpressed in cultured 3T3-L1 adipocytes reduced the expression of adiponectin, an adipokine that is expressed in the adipose tissue and is positively associated with insulin sensitivity, lipid storage in adipocytes, and lipid oxidation in the liver and muscle.[73] Yet, metabolic measurements in IRF8$^{ad-/-}$ obese mice showed that this effect was not sufficient to see differences in insulin sensitivity compared to IRF8$^{fl/fl}$ mice.[63,82,64] Adiponectin levels are known to be lower in obesity, so the lack of protection against insulin resistance in IRF8$^{ad-/-}$ mice indicates other pathways contribute to the observed reduction in adiponectin during obesity. Previous studies investigating the IRF family of transcription factors in adipocytes have shown protection against insulin resistance, therefore it is possible that this observation may be due to the experimental protocol or specific differences in the isoform.[45,50,83] More studies are needed to determine the role of adipocyte-specific IRF8 in the development of obesity-associated insulin résistance and T2DM.

In conclusion, through *in vitro* and *in vivo* investigations, we show that IRF8 regulation is altered post-translationally during obesity, and can protect against NAFLD by modulating the expression of downstream effectors including hepatocyte CD36 and adipocyte FasR. Additionally, since we did not see differences in insulin signaling or insulin resistance, it is suggested that the observed protection against NAFLD when IRF8 is deleted in adipocytes is due to modulations in inflammatory signaling pathways that affect FasR and CD36. Here, we show that activation of the TLR4 signaling pathway by the presence of LPS is sufficient to increase the gene expression of FasR.

Further studies are needed to determine the full and precise molecular mechanisms underlying the effect of adipocyte-specific IRF8 on the crosstalk between the adipose and liver during obesity.

Limitations and Future Directions

The present study suggests that IRF8 plays a role in adipocyte metabolism and the crosstalk between the adipose tissue and liver during obesity-induced stress; however, several limitations should be considered. Studies investigating other members of the IRF family of transcription factors in adipocytes showed significant impact on insulin sensitivity. Protein and expression levels of other IRFs were not measured, and therefore compensation by similar proteins may have blunted effects we would have expected to see by knocking out adipocyte-specific IRF8. Additionally, metabolic differences between male and female mice warrant separate and equally important analyses. Here, the observations reported are from male mice, leaving a gap in knowledge on the effects of adipocyte-specific IRF8 knockout on insulin signaling and liver triglycerides in female mice. Adipocyte isolation from gWAT of mice used in the experiment did not produce sufficient sample to incubate with LPS and replicate observations made in 3T3-L1 cells. These data would have further defined the underlying mechanisms and regulation of IRF8.

To gain a better understanding of the role of IRF8 in inter-organ cross talk, a liver-specific IRF8 knockout LoxP-Cre model could be used in the same experimental set up. The data would shed light on the significance of the location of IRF8 on function, regulation, and contribution to metabolic dysfunction. IRF8 is also known to regulate acid ceramidase, an enzyme that degrades ceramides. As ceramides regulate the expression of CD36 and influence the development of NAFLD, it would be beneficial to understand the role of IRF8 in ceramide metabolism.

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APPENDIX A. IRF8 regulates extracellular matrix proteins of adipose tissue in obesity

In collaboration with Dr. Madeline Oudin of Tufts University and her laboratory, we incubated subcutaneous adipose tissue (the mammary adipose tissue in mice) with SDS and harvested the remaining extracellular matrix (ECM) for proteomic analysis. Results revealed that the HFD resulted in a dramatic increase in ECM proteins, including upregulation of collagen VI expression.[84] A previous study found that when mice with collagen VI deficiency were fed a HFD, the mice had significantly reduced adiposity in the absence of changes in energy expenditure. Interestingly, proteomic analysis of ECM from subcutaneous adipose of female mice with deficiency of adipocyte-specific IRF8 fed a 60 % HFD for 18 weeks ($IRF8^{fl/fl}$ = 8, $IRF8^{ad-/-}$ = 5) had a dramatic reduction in the numbers of ECM proteins associated with breast cancer (Figure 1), which suggests protection against DIO. Preliminary data suggests that the reduced adiposity in mice with adipocyte IRF8 deficiency have reduced food intake, which protects the mice from DIO.

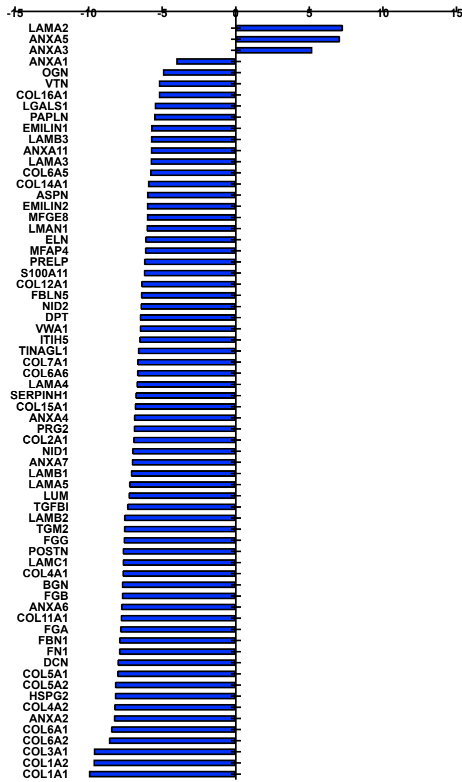

Figure 1. Proteomic analysis of ECM proteins in the subcutaneous fat pads obtained from female IRF8$^{ad-/-}$ mice on a HFD. Relative to IRF8$^{fl/fl}$ mice, IRF8$^{ad-/-}$ female mice show significantly reduced levels of ECM proteins relative to IRF8$^{fl/fl}$ mice, indicating an important role of IRF8 in the regulation of ECM protein secretion from adipocytes.