DUSP5 Regulation of Inflammation and Insulin Signaling

A thesis submitted in partial fulfillment of the requirements for the degree of
Bachelor of Science in Nutrition and the Honors Program

by

Mitra Jefic

Dr. Bradley Ferguson, Thesis Advisor

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We recommend that the thesis prepared under our supervision by

MITRA JEFIC

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DUSP5 Regulation of Inflammation and Insulin Signaling

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Bradley Ferguson, PhD. Thesis Advisor

Tamara Valentine, Ph. D., Director, Honors Program

May 2017
ABSTRACT

Adipose tissue inflammation links obesity to insulin resistance and type 2 diabetes (T2D). Chronic inflammation of adipose tissue is characterized by increased expression of tumor necrosis factor α (TNFα). TNFα stimulates signaling of mitogen-activated protein kinases (MAPKs) within adipocytes (i.e. fat cells) and muscle cells that drive systemic inflammation and insulin resistance, leading to T2D. Until recently, most studies focused on the role of upstream kinases that link TNFα to MAPK activation (i.e. phosphorylation); few studies have examined the role for downstream phosphatases in the deactivation (i.e. dephosphorylation) of this pathway. Therefore, I examined a role for the MAPK-specific dual-specificity phosphatases (DUSPs) in the deactivation of MAPK signaling in response to TNFα-mediated inflammation. We report that four of the ten DUSPs were induced during early stages of obesity development (Stage I), whereas gene expression increased for three out of ten DUSPs during late stage obesity (Stage II). Of the three DUSPs (DUSP1, 5, and 9), we show that DUSP5 regulates phosphorylation of a classical MAP kinase known as extracellular signal-regulated kinase 1/2 (ERK1/2); loss of DUSP5 function led to exacerbated ERK1/2 phosphorylation. These findings suggest that DUSP5 acts as an endogenous regulator of ERK1/2 signaling in response to obesity-mediated inflammation.
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CHAPTER 1: BACKGROUND

INTRODUCTION

Obesity is a growing epidemic in Westernized nations. In the United States, for example, obesity prevalence rates have increased from 20.9% of the adult population in 2001 (Greenberg and Obin, 2006) to over 35% in 2014 (Ogden, Carroll, Fryar, and Flegal, 2015). Obesity increases a person’s risk for multiple chronic diseases such as type 2 diabetes, hypertension, cancer, gallbladder disease, and atherosclerosis (Gregoire, Smas, and Sul, 1998). These obesity-mediated morbidities result in an estimated 300,000 deaths in the U.S. each year (Mokdad et al., 2003).

Organisms tend to evolutionarily adapt to situations that best fit survival, however, as time goes on, what is considered to be optimal tends to change. Although selective pressures do not favor intake of excess energy, they do guard against situations of starvation (Hotamisligil, 2017). Methods for taking in excess nutrients may have been beneficial in times when food was scarce, but this is no longer the case in today’s society. Today, excess energy intake correlates with obesity and the development of metabolic disorders that arise from prolonged excessive caloric intake. This metabolic dysfunction drives inflammatory mechanisms in the cells of the body, resulting in chronic low-grade inflammation. Metabolically driven inflammation was first discovered in adipocytes, although tissues such as the hypothalamus, liver, muscle, pancreatic islet cells, and the gut also show pro-inflammatory mechanisms (Hotamisligil, 2017).

Current evidence demonstrates that adipose tissue dysfunction plays a central role in the sequelae of whole-body metabolic changes that result in the loss of insulin sensitivity and ultimately type 2 diabetes. Central to this argument, obesity-induced chronic, low-grade
inflammation imparts adipose tissue insulin resistance and increased levels of circulating pro-inflammatory cytokines and free fatty acids that drive peripheral insulin resistance in liver and skeletal muscle. Tumor necrosis factor- alpha (TNF-α) is a pro-inflammatory cytokine that plays a causal role in insulin resistance within both adipose tissue and skeletal muscle cells (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Greenberg and Obin, 2006; Hotamisligil, 2017). TNFα has been shown to suppress insulin signaling, alter glucose homeostasis, promote lipolysis, and inhibit lipogenesis in cultured adipocytes (Gregor and Hotamisligil, 2011; Hotamisligil, 2017). In addition, it has been reported that TNF-α is elevated in adipose tissue of obese subjects and that genetic ablation of TNF-α can restore insulin sensitivity in vitro and in vivo (Gregor and Hotamisligil, 2011; Uysal, Wiesbrock, Marino, and Hotamisligil, 1997). Metabolic actions of TNF-α are predominantly mediated through mitogen-activated protein kinase (MAPK) signaling pathways involving extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Greenberg and Obin, 2006). The MAPKs have been shown to be activated in adipose tissue in response to TNF-α and serve as mediators of adipocyte and skeletal muscle insulin resistance through mechanisms involving gene expression as well as the expression and secretion of pro-inflammatory molecules (Guilherme, Virbasius, Puri, and Czech, 2008; Yang et al., 2017). Lastly, MAPK activity has also been linked to cytosolic suppression of insulin signaling through phosphorylation of specific serine residues of insulin receptor substrate-1 (IRS-1) (Guilherme, Virbasius, Puri, and Czech, 2008; Hotamisligil, 2017).

Upstream MAPK kinases have historically been considered the principal regulators of MAPK activity. These kinases serve as activators to add phosphate groups to molecules to initiate a cascade. MAPK kinases activate substrate cascades through various mechanisms,
including by phosphorylation of serine and threonine residues that are made available by substrates in adipocytes and skeletal muscle cells (Wellen and Hotamisligil, 2005; Steinberg et al., 2006). However, recent literature has pointed to phosphatases as powerful and even dominant controllers of many MAPK-dependent biological processes (Jeffrey et al. 2007). Dual-specificity phosphatases (DUSPs) serve to inhibit MAPK activity via dephosphorylation of threonine and tyrosine residues (Kondoh and Nishida, 2007), yet few scholars have studied the role for DUSPs in the regulation of inflammation and insulin resistance in adipocyte and skeletal muscle cell lines. As such, I will use the 3T3-L1 adipocyte cell line and the C2C12 skeletal muscle cell line to delineate the role of DUSPs on MAPK signaling, inflammation, and insulin resistance.

Here, I will elucidate the role of DUSP5 in insulin resistance by examining its role in dephosphorylating ERK 1/2, thereby decreasing the rate of inflammation in adipocytes. Using molecular tools such as immunoblotting and qPCR, I will examine DUSPs expression in an animal model of diet-induced obesity as well as in adipocytes and skeletal muscle myoblasts stimulated with TNF-α. A causal role for the impact of DUSPs on TNF-α-induced MAPK signaling and inflammation in adipocytes and skeletal muscle will further be elucidated using genetic tools to overexpress inducible DUSPs identified in our in vitro and in vivo screens. Markers of inflammation, including interleukin 6, interleukin 1, and TNF-α, have been used as a means of clinical treatment for inflammatory diseases such as rheumatoid arthritis and Crohn’s disease (Hotamisligil, 2017). If these inflammatory diseases have found emerging data to support therapeutic measures for treatment through targeting the inflammatory immune mediated response, the same may be possible for the inflammatory condition of obesity. Combined, these findings have the potential to elucidate novel therapeutic targets for the treatment of obesity-mediated diabetes.
Literature Review

Worldwide obesity rates are increasing, and the World Health Organization estimates that there are over 1 billion overweight adults, 300 million of which are classified as obese, in the world today (Greenberg and Obin, 2006). The presence of excess body mass is classified as the sixth most influential risk factor for the overall affliction of disease globally (Gil, Aguilera, Gil-Campos, and Cañete, 2007) and is the seventh leading cause of death worldwide (Kurek et al., 2016). Obesity is characterized by the presence of chronic, low-grade inflammation in the body, particularly in adipocytes. Although adipose tissue serves as an important endocrine organ with a vital role in overall health, these tissues can wreak havoc on the body when homeostasis is not met. This lack of homeostasis in the body manifests in various disease states. As a result, obese patients are at an increased risk for chronic diseases such as type 2 diabetes, hypercholesterolemia, cardiovascular disease, hypertension, stroke, hypertriglyceridemia, arthritis, asthma, and certain cancers (Greenberg and Obin, 2006). These chronic disease states pose a problem not only medically for those afflicted with the disease, but also economically for the general American population due to the high annual cost associated with management of chronic disease (Kurek et al., 2016). Type 2 diabetes in particular has become increasingly prevalent in modern society.

Type 2 Diabetes

Type 2 diabetes is characterized by impaired glucose tolerance and insulin resistance in the body (Gil, Aguilera, Gil-Campos, and Cañete, 2007). Obesity plays a causal role in type 2 diabetes development due to loss of insulin sensitivity in adipose tissue, liver, and skeletal muscle (Lee and Lee, 2014; Gregor and Hotamisligil, 2011). Adipose tissue functions as an
endocrine organ and has the ability to release adipokines in an autocrine, paracrine, and endocrine manner (Greenberg and Obin, 2006). For example, adipose tissue expresses the hormone adiponectin, which increases insulin sensitivity in muscle and liver (Greenberg and Obin, 2006). The concentration of adiponectin, however, drops significantly as obesity increases, leading to increased insulin resistance (Greenberg and Obin, 2006). Other tissues are also impacted by insulin resistance, including skeletal muscle, in which insulin desensitization impairs GLUT-4 receptor translocation to the plasma membrane (Kurek et al., 2016). In optimal conditions, GLUT-4 translocation to the outer cellular membrane allows for glucose uptake into skeletal muscle; this serves as a source of energy to carry out metabolic processes vital to cell survival. When glucose is not adequately taken up by cells due to insulin resistance, type 2 diabetes is manifested.

Loss of insulin sensitivity in the muscle is multifactorial and can include numerous factors including excessive nutrient intake leading to the accumulation of lipid (i.e. dyslipidemia) metabolites (Kurek et al., 2016; Steinberg et al., 2006) as well as lack of activity. In studies of hind limb immobilization for example, insulin stimulated 2-deoxyglucose uptake was decreased after only 6 hours of immobilization (Kawamoto et al., 2016). In this study, insulin stimulated 2-deoxyglucose was reduced by up to 62%, delineating the fact that higher activity and body movement are positively correlated with increased insulin sensitivity (Kawamoto et al., 2016). This could potentially parallel the relative inactivity common in the obese population, further exacerbating situations of insulin resistance in that subpopulation.
**Insulin Signaling and Development**

Insulin resistance is predominantly characterized by loss of insulin signaling within insulin responsive tissues such as adipose, liver, and skeletal muscle. Insulin signaling has been shown to play a critical role in organelle function and development within cells. Growing evidence has linked obesity to dysfunction of the mitochondria in both adipose tissue and skeletal muscle (Hotamisligil, 2017). The mitochondrion serves as the main power plant and energy source within a cell’s machinery. Dysfunction of this vital organelle can lead to cellular dysfunction. Likewise, the mitochondria in cells of patients with type 2 diabetes show a decrease in mitochondrial activity in both adipose tissue and skeletal muscle (Hotamisligil, 2017). When cells do not function optimally, the organism as a whole ultimately suffers the consequences, for example reduced heart contraction or impaired respiration (i.e. breathing). Insulin signaling is more than a stimulus for adequate glucose uptake however, as this insulin peptide is important for cellular growth and development of many critical tissues. Indeed, studies have shown that insulin-mediated stimulation of insulin-like growth factor I receptor (IGFIR) is pivotal for the development and differentiation of skeletal muscle cells (Yang et al., 2017). Thus, proper response to insulin signaling is not only vital for function in mature, established cells but also in newly differentiating cell types.

**Chronic Inflammation and Insulin Resistance**

The body’s resistance to insulin is mediated by proteins related to the immune system (Gregoire, Smas, and Sul, 1998). The process of the immune response to a viral infection or pathogen mirrors the inflammatory response observed with obesity. In cases of viral infection, the cell’s endoplasmic reticulum becomes overloaded and, in turn, upregulates pathways related
to inflammation (Hotamisligil, 2017; Wellen and Hotamisligil, 2005). When chronic inflammation is present in the system secondary to obesity, the equilibrium of the endoplasmic reticulum is further disrupted (Hotamisligil, 2017). This disruption additionally exacerbates the inflammatory response, making it increasingly difficult for the endoplasmic reticulum to perform its duties within the cell.

As a part of this obesity-mediated inflammatory response, macrophages, phagocytic immune cells, infiltrate adipose tissue and express and secrete pro-inflammatory cytokines (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Wellen and Hotamisligil, 2005). This in turn stimulates adipocytes to express and secrete pro-inflammatory molecules, thus exacerbating adipose tissue inflammation (Hotamisligil, 2017). This inflammatory environment alters molecular signaling events within adipocytes that promote adipocyte insulin signaling resistance (Lee and Lee, 2014).

**Tumor Necrosis Factor-α (TNF-α)**

Infiltrating macrophages drive inflammation with the production of cytokines such as tumor necrosis factor- alpha (TNF-α) (Lee and Lee, 2014; Gil, Aguilera, Gil-Campos, and Cañete, 2007; Hotamisligil, Shargill, Spiegelman, 1993; Xu, Uysal, Becherer, Arner, Hotamisligil, 2002; Wellen and Hotamisligil, 2005). TNF-α is capable of suppressing insulin signaling, altering glucose homeostasis, promoting lipolysis, and inhibiting lipogenesis, which therefore alters the homeostatic state of the system (Hotamisligil, 2017). TNF-α inhibits insulin signaling by suppressing activity of the insulin receptor and insulin receptor substrate proteins that drive GLUT4 translocation to the outer membrane; GLUT4 translocation is necessary for glucose uptake (Gil, Aguilera, Gil-Campos, and Cañete, 2007). In addition, TNF-α signaling
inhibits GLUT4 gene expression while exacerbating inflammatory gene expression within the nucleus of the adipocyte (Kurek et al., 2016; Gil, Aguilera, Gil-Campos, and Cañete, 2007).

Historically, it has been demonstrated that TNF-α is elevated in the blood and adipose tissue in both animals and humans with obesity (Uysal, Wiesbrock, Marino, and Hotamisligil, 1997). Mouse models have been used to analyze insulin resistance in response to TNF-α. One study in particular demonstrated that obese TNF-α +/+ mice, meaning those with excess TNF-α, had increased insulin resistance, whereas TNF-α -/- mice, those lacking the TNF-α gene, did not develop insulin resistance (Uysal, Wiesbrock, Marino, and Hotamisligil, 1997). These findings clearly demonstrate the importance of TNF-α as a pivotal regulator of insulin signaling, glucose uptake and the development of type 2 diabetes.

**Mitogen-Activated Protein Kinases (MAPKs)**

Cytosolic and nuclear actions for TNF-α signaling in adipocytes are regulated via activation (i.e. phosphorylation) of mitogen-activated protein kinases (MAPKs). Phosphorylation of MAPKs on threonine and tyrosine residues via sequential signaling cascades is sufficient and essential for increased MAPK activity. In general terms, MAP kinase kinase kinases (MAP3Ks) phosphorylate MAP kinase kinases (MAP2Ks), which in turn phosphorylate MAP kinases (MAPKs) (Fig. 1.1A) (Bost, Aouadi, Caron, and Binétruy, 2005). In turn, MAPKs activate downstream targets by phosphorylating serine and threonine residues; therefore, they are referred to as serine/threonine kinases (Wellen and Hotamisligil, 2005; Uysal, Wiesbrock, Marino, and Hotamisligil, 1997). Proper activation of this pathway through phosphorylation leads to impairments in insulin signaling, inhibition of glucose uptake and exacerbation of inflammatory gene expression through cytosolic regulation of insulin signaling and nuclear regulation of gene
transcription. This occurs through translocation of the MAPK marker into the nucleus of the cell, thereby allowing it to phosphorylate transcription factors (e.g. c-Jun) that regulate gene expression. Not surprisingly, TNF-α-mediated MAPK activation has been reported to increase TNF-α gene expression in adipocytes, further exacerbating adipose tissue inflammation and decreasing insulin sensitivity.

Figure 1.1 MAPK Signaling Cascade
MAPKs represent a diverse family of proteins that have the capability of transducing multiple extracellular stimuli to activate target-specific responses (Kondoh and Nishida, 2007). It is now understood that MAPK signaling duration, magnitude and localization are critical for the diverse cellular actions ascribed to the MAPK family including cellular proliferation, differentiation, apoptosis, survival and inflammation (Fig. 1.1B) (Kondoh and Nishida, 2007). Several members of the MAPK family link TNF-α to the development of insulin resistance, including extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 (Bost, Aouadi, Caron, and Binétruy, 2005; Seger and Krebs, 1995; Nishimoto and Nishida, 2006; Hirosumi et al, 2002; Pearson et al, 2001; Zhang and Dong, 2007).

Phosphorylation of ERK, JNK, and p38 via upstream MAPK2Ks promotes activation of these MAPKs in response to TNF-α, and these mechanisms have been extensively studied. The JNK kinase activity was first observed in Drosophila (Hotamisligil, 2017). The role of JNK as an upstream inflammatory mechanism in metabolism has also been shown to be conserved in both human and mammalian cell models as well, further delineating the importance of these MAPK signal pathways (Hotomisligil, 2017). Since this discovery, JNK has been viewed as a pivotal benchmark for metabolism with a disadvantageous affect on glucose uptake in cells (Hotomisligil, 2017). The activation of p38 transcripts is highly expressed in differentiation of skeletal muscle cells and cells from the C2C12 muscle cell line (Keren, Tamir, and Bengal, 2006). In addition, myogenesis, or the creation of muscle tissue, has been shown to be regulated by insulin-like growth factors through extracellular cues (Keren, Tamir, and Bengal, 2006). This was first shown through pro-inflammatory cytokines acting on the p38 pathway (Keren, Tamir, and Bengal, 2006). While it is generally concluded that ERK signaling is associated with cell survival and proliferation, non-canonical and even opposing actions of ERK have been reported
(Jain, Phelps, and Pekala, 1999; Keshet and Segar, 2010). For instance, mice deficient in ERK1 were protected against diet-induced obesity and insulin resistance by mechanisms involving decreased adipogenesis and increased energy expenditure (Bost, Aouadi, Caron, and Binetruy, 2005). In contrast, mice deficient in signaling adapter p62, and ERK inhibitor, developed mature-onset obesity and insulin resistance that in part was mediated by increased ERK activity (Rodriguez et al., 2006). Collectively these reports implicate ERK as an important signaling molecule in obesity-induced diabetes.

**Dual-Specificity Phosphatases (DUSPs)**

MAPKs are deactivated by dephosphorylation of threonine and tyrosine residues, which can be completed by serine/threonine phosphatases, tyrosine phosphatases, or dual-specificity phosphatases (DUSPs) (Kondoh and Nishida, 2007; Jeffrey, Camps, Rommel, Mackay, 2007). This serves as a means to remove the phosphate groups from substrates that have been added by kinases, as discussed previously. DUSPs are MAPK-specific phosphatases that have been classified into three groups based on cell localization, substrate specificity, and protein structure (Kondoh and Nishida, 2007). Group I DUSPs localize to the nuclear compartment, and include DUSP1, DUSP2, DUSP3, DUSP4, and DUSP5, Group II DUSPs localize to the cytoplasm and contain nuclear export signals, while Group III DUSPs can localize to the cytoplasm or nucleus (Fig.1.2). Each DUSP has a specific domain and is able to exhibit substrate specificity.
DUSPs, MAPKs and Adipocyte Inflammation

While activation and biological outcome of MAPKs were historically studied via regulation by upstream kinases, phosphatases have recently been shown to play critical roles in the regulation and control of MAPK-dependent biological fate (Jeffrey et al., 2007). For studies outlined in this thesis, we propose the following working model, in which TNF-α-mediated signaling activation increases “inducible” DUSPs in a manner that promotes feedback inhibition of MAPKs and attenuates inflammatory gene expression (Fig. 1.3). While much of this thesis will examine DUSPs in relation to adipose tissue inflammation, it should be noted that this thesis will highlight DUSP5 as it has been demonstrated that this molecule can regulate inflammation in other cell types (Jeffrey et al. 2007). DUSP1 and DUSP9, unlike DUSP5, have been studied
extensively for their role in obesity and adipocyte insulin resistance (Jeffrey et al., 2007; Wu et al., 2006; Zhang et al., 2003; Xu et al., 2003; Emanuelli et al., 2008).

Summary

Inflammatory processes involved in obesity drive localized insulin resistance in body tissues, particularly adipose tissue. Indeed, chronic inflammation of adipose tissue links obesity to the development of insulin resistance (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Gregor and Hotamisligil, 2011; Guilherme, Virbasius, Puri, and Czech, 2008). I have developed the central hypothesis that MAPK-dependent dual-specificity phosphatases (DUSPs) regulate TNFα-mediated MAPK signaling and inflammation in adipocytes and skeletal muscle. We will focus
our efforts on the function of DUSP5 in the regulation of adipocyte and skeletal muscle inflammation and insulin resistance, as it relates to obesity.

CHAPTER 2: EXPERIMENTAL RESEARCH

INTRODUCTION

Obesity is a public health problem in Westernized nations today, with over 35% of the adult population of the United States considered obese (Ogden et al., 2015). Obesity is particularly concerning as it is considered a major risk factor for type 2 diabetes (T2D), hypertension, certain cancers, gallbladder disease, and atherosclerosis (Gergoire, Smas, and Sul, 1998).

Affliction with T2D is common in the obese adult population. T2D is characterized by decreased insulin sensitivity of skeletal muscle and adipose tissue that results from chronic, low grade inflammation in adipose tissue (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Gregor and Hotamisligil, 2011; Guilherme, Virbasius, Puri, and Czech, 2008). Tumor necrosis factor α (TNFα) is an inflammatory cytokine that leads to insulin resistance within adipocytes (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Greenberg and Obin, 2006). Concentrations of TNFα are increased in adipose tissue of obese individuals, and pharmacological or genetic inhibition of TNFα improves insulin sensitivity (Gregor and Hotamisligil, 2011). The mitogen-activated protein kinase (MAPK) signal pathway links extracellular TNFα to intracellular actions that inhibit insulin signaling in the cytosol and activates inflammatory gene expression in the nucleus (Guilherme et al., 2008). The classical MAPKs that have been shown to regulate insulin resistance and inflammatory gene expression; these include extracellular signal-regulated protein
kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Greenberg and Obin, 2006).

Previous research has delineated the role of upstream MAPK kinases on regulation (i.e. phosphorylation) of MAPKs. Recent literature, however, suggests that phosphatases play an equally important role in the regulation of MAPK activity and biological function (Jeffery et al., 2007). A family of ten dual-specificity phosphatases (DUSPs) dephosphorylate the MAPKs and thus inhibit MAPK activity. DUSPs recognize specific MAPK targets where for instance DUSP5 specifically targets ERK 1/2 for dephosphorylation (Kondoh and Nishida, 2007). As few studies have examined a role for DUSPs on MAPK signaling, inflammation and insulin resistance in adipocytes, we used the 3T3-L1 adipocyte cell line stimulated with TNFα as a model of inflammation to elucidate DUSP function on inflammation-induced activation of MAPK signaling. We report that DUSP 1, 5 and 9 expression was increased in white adipose tissue (WAT) in response to diet-induced obesity. Of these, DUSP 1 and 5 expression was increased in adipocytes stimulated with TNFα. Lastly we report that loss of DUSP5 function exacerbated TNFα-induced ERK 1/2 phosphorylation.

MATERIALS AND METHODS

Cell Culture

3T3-L1 preadipocytes were propagated in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) until density-induced growth arrest. At 2 days post-confluence, growth medium was replaced with DMEM supplemented with 10% FBS, 0.5mM 1-methyl-3-isobutylxanthine, 1 µM dexamethasone, and 1.7 µM insulin (MDI).
Experiments described herein were conducted in mature adipocytes (d8) stimulated with 100 pM TNFα. All experiments were repeated at least 3 times to validate results and ensure reliability.

**Immunoblotting**

Cell monolayers were washed with phosphate-buffered saline (PBS) and scraped into ice-cold lysis buffer containing PBS, 300 mM NaCl, 0.5% Triton-X, as well as protease and phosphatase inhibitors (HALT; Fisher Scientific) added to lysis buffer prior to cell harvest. Cell lysates were sonicated, centrifuged (16,000 x g, 5 min, 4°C) and supernatant transferred to a fresh tube. Bicinchoninic acid assay (Pierce, Rockford, IL) was used to determine protein concentration. Cell lysates were re-suspended in loading buffer containing 0.20 M Tris, pH 6.8, 10% sodium dodecyl sulfate (SDS), 40% glycerol, 20% β-mercaptanol, and 0.01% bromophenol blue and heated for 5 min at 80°C. Proteins were resolved on SDS-polyacrylamide gel electrophoresis gels (PAGE) and transferred to Nitrocellulose membranes (Genesee Scientific). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies for phospho-JNK (Cell Signaling Technology; 4668), phospho-ERK (Cell Signaling Technology; 4370), total JNK (Santa Cruz Biotechnology; sc-571), or total ERK (Santa Cruz Biotechnology; sc-153). Horseradish Peroxidase (HRP)-conjugated secondary antibodies (Southern Biotech) were used at a concentration of 1:2000. SuperSignal West Pico chemiluminescence system (Thermo Scientific) and a ChemiDoc XRS+ imager (BioRad) were used to detect protein.
**Real-Time qRT-PCR**

Total RNA was assessed via triazol (Qiagen) following the manufacturer’s protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer and reverse-transcribed to cDNA using the Verso cDNA Synthesis Kit (Thermo Scientific; AB-1453). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and RT enzyme was added to 500 ng RNA and RNase-free water.

PCR amplification was run utilizing the BioRad CF96X qPCR instrument (BioRad) that consisted of enzyme activation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 sec combined with annealing at 55°C for 30 sec and extension at 72°C for 1 minute to examine DUSP gene expression (Table 1). Data were recorded and analyzed with Sequence Detector Software (BioRad) and graphs visualized with GraphPad Prism software. All data were presented as mean ± standard error of the mean (SEM) and representative. Data were normalized to 18S previously validated by this lab as a suitable reference gene under these experimental conditions (Ferguson et al., 2010).
RNA Interference

Short interfering RNAs (siRNAs) for dusp5 specific sequences as well as non-targeting sequences were transfected using Lipofectamine 3000 transfection reagent according to manufacturer’s (Thermo Scientific) protocol. Briefly, 3T3-L1 adipocytes were differentiated in 6-well culture dishes. Growth medium was then replaced with DMEM under serum-deprived conditions prior to addition of 3 µl of Lipofectamine 3000 reagent and either 100 nM dusp5 specific siRNAs or non-targeting siRNA control for 3 hours. Growth medium was subsequently switched to fresh growth medium with FBS for overnight incubation prior to stimulation with TNFα. Cells lysate was harvested for RNA or protein as described above.

Table 1. DUSP, adipocyte, and inflammatory genes analyzed in this study.

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<td>M3/6, hVH5</td>
<td></td>
<td>Mm00456230_m1</td>
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</tr>
<tr>
<td>Dusp10</td>
<td>MKP-5</td>
<td></td>
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<tr>
<td>Dusp16</td>
<td>MKP-7, MKP-M</td>
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<tr>
<td></td>
<td>reference gene</td>
<td>18 ribosomal RNA</td>
<td>X03205</td>
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DUSP group (GP), MAPK binding domain (MKB), nuclear localization sequence (NLS), nuclear export sequence (NES), ERK (E), JNK (J), p38 (P), threshold cycle (C_T) measured in lean adipose tissue.
Statistics

Statistical analyses were conducted using GraphPad Prism software. Differences in DUSP expression under high fat diet were determined via student’s t-test where a p-value of <0.05 was considered significant. All other data were analyzed using ANOVA, with Tukey's post-hoc analysis used when the p value for the respective parameter was statistically significant (p < 0.05).

RESULTS

**DUSPs 1, 5 and 9 are induced in response to diet-induced obesity.** Little is known regarding the role for DUSPs on adipocyte biology. As such, we first assessed the expression for these phosphatases in an animal model of diet-induced obesity. For these studies, C57BL6/J male mice were placed on either a control or high fat diet (60% kcal from fat) for either 12 or 18 weeks starting at 6 weeks of age representing an early stage (Stage I) or late stage (Stage II) of obesity in which body mass would increase over time (Table 2). Control mice were given a low fat diet containing 10% kcal from fat and the same protein content as the mice given the high fat diet. As expected, the body weight of mice fed a high fat diet at stage I (18 weeks of age) increased as compared to control animals, while animals at stage II (24 weeks of age) had a greater increase in body weight compared to controls (Table 2). An examination of DUSP mRNA expression was conducted for mice at both Stage I and Stage II for both the lean (low fat diet) and obese (high fat diet) mice. The total RNA was prepared from white adipose tissue (WAT) and quantitative polymerase chain reaction (qPCR) was performed to assess DUSP mRNA abundance and gene expression (Fig. 2.1). The expression of several DUSPs (DUSPs -1, -2, -5, and -9) shows a statistically significant increase during Stage I when compared to control mice (Fig. 2.1). Several
of these DUSPs (DUSPs -1, -5, and -9) remained significantly elevated during Stage II of obesity (24 weeks of age) (Fig. 2.1). Of these DUSPs, DUSP 1 and DUSP 9 have been more extensively studied previously with regard to obesity and insulin resistance, while nothing is currently known regarding DUSP 5.

<table>
<thead>
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<th>Table 2. Anthropometrics of mice in this study.</th>
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<td>stage</td>
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<td>diet-induced</td>
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<td>I</td>
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Percent difference in body weight (BW) between lean and obese mice (%Δ).

**Figure 2.1. Adipose tissue specific regulation of DUSPs and inflammatory mediators during the development of obesity under conditions of diet-induced obesity.** Total RNA was prepared from white adipose tissue (WAT) harvested during development of diet-induced obesity (DIO), C57BL/6J males were given ad libitum access to a high fat diet (HFD; 60% kcal) starting at six wks of age. Control males were fed a control low fat diet (LFD) containing 10% kcal from fat and the same protein content as the HFD. RNA was isolated WAT at A) 18 wks and B) 24 wks of age. Relative DUSP mRNA abundance was measured via qRT-PCR and statistical significance determined by student’s t-test (p<0.05). All data were normalized to 18S rRNA and expressed relative to lean littermates.

**TNFα increased ERK and JNK phosphorylation in adipocytes.** Intracellular signaling cascades such as the MAPKs link inflammatory stimuli to inflammatory gene expression and
insulin resistance. To assess this, 3T3-L1 preadipocytes were differentiated to fully mature adipocytes that accumulate lipid. Mature adipocytes were then exposed to increasing doses of TNFα (Fig. 2.2.A). TNFα was used as an inflammatory agonist. Cell lysates were harvested 15 minutes after stimulation by TNFα. Immunoblotting was used to analyze phospho-ERK, phospho-JNK, and phospho-p38 (Fig. 2.2.A). As shown, phosphorylation increased with increasing doses of TNFα, with ED₅₀ estimated at 100pM; this dose was chosen for all remaining experiments. Adipocytes were next stimulated with 100 pM TNFα and cell lysates collected over time post- TNFα stimulation (0, 15, 30, 60, 120, and 180 minutes). Immunoblotting was used to examine levels of phospho-ERK, total ERK, phospho-JNK, and total JNK (Fig. 2.2.B). The levels of phosphorylation in each condition are depicted in figure 2.2.B. As shown, both phospho-ERK and phospho-JNK expression was elevated at 15 minutes post-stimulation by TNFα and rapidly dephosphorylated with expression levels decreased to baseline by 30 minutes. Of note, phospho-ERK was biphasic as phosphorylation increased at 15 minutes, returned to baseline and increased again at the 180 minute post- TNFα stimulation. Finally, TNFα stimulation did not change total ERK or total JNK expression, demonstrating that changes in phosphorylation were the result of the addition or removal of phosphate groups and not changes in protein abundance.
Figure 2.2. MAPK signaling and DUSP expression in adipocytes in response to TNFα. 

A) Adipocytes were stimulated with increasing doses of TNFα and cell lysates harvested at 15 min post-TNFα stimulation. Phospho-ERK, JNK, and p38 were examined by immunoblotting. 

B) Adipocytes were stimulated with TNFα (100 pM) and cell lysates collected over time post-TNFα and immunoblotted for phospho-ERK and JNK as well as total ERK and JNK. Total RNA was harvested over time from adipocytes stimulated with TNFα (100 pM). DUSP mRNA expression was assessed by qRT-PCR. DUSPs were grouped as mentioned in the Lit Review (Fig. 1.2): A) group I, B) group II, and C) group III. All data were normalized to 18S rRNA and expressed relative to untreated cells. Genes were selected as 'inducible' when upregulated above a 2-fold criterion indicated by the dashed line.
DUSP1, 5 and 16 are induced in adipocytes in response to TNFα in a manner consistent with MAPK dephosphorylation. As obesity is commonly characterized by chronic, low grade inflammation (e.g. TNFα), we next wished to assess if DUSP-mediated induction was due to obesity or increases in adipose tissue inflammation. To address this question, total RNA was harvested over time from adipocytes stimulated with 100 pM of TNFα. DUSPs were categorized into designated groups based on cell localization, substrate specificity, and protein structure (Kondoh and Nishida, 2007). Group I DUSPs here localize in the nuclear compartment (DUSPs -1, -2, -4, and -5). Group II DUSPs localize to the cytoplasm and contain nuclear export signals (DUSPs -6, -7, and -9). Group III DUSPs can localize to either the cytoplasm or the nucleus (DUSPs -8, -10, and -16). Quantitative PCR was run to examine DUSP mRNA expression, where genes were determined significant when increased over a two-fold induction; indicated by the red dashed line (Fig. 2.2). These data were normalized to 18S rRNA and shown as expression relative to untreated cells. As shown in figure 2.2 C, D, and E, only DUSPs 1, 5, and 16 were induced (i.e upregulated) above a two-fold increase in response to TNFα. Of these three DUSPs, only DUSP 1 and DUSP 5 were seen previously to be increased in experiments of diet-induced obesity (Fig. 2.1). As stated, DUSP 1 has previously been studied with regards to obesity, inflammation and insulin resistance; DUSP 5 has not been previously studied in adipose tissue and as such, remaining experiments focused on DUSP 5.

**DUSP5 deletion exacerbates TNFα-induced ERK1/2 phosphorylation in adipocytes.** DUSP5 is a nuclear, ERK1/2-specific phosphatase (Mandl, Slack, and Keyse, 2005). To determine if phosphatases regulate MAPK dephosphorylation, we initially pretreated adipocytes with pervanadate for 30 minutes before stimulation with 100 pM TNFα. Pervanadate is a known
protein-tyrosine phosphatase inhibitor (Huyer et al., 1997). Cell lysates were collected over time post- TNFα stimulation and immunoblotted to assess phospo-ERK, total ERK, phospo-JNK, and total JNK (Fig. 2.3.A). TNFα-induced phosphorylation of ERK and JNK was exacerbated in response to phosphatase inhibition, while no changes were observed for total protein expression. These results confirm that phosphatases regulate MAPK dephosphorylation and suggest the potential for DUSP5 in the regulation of ERK1/2 signaling.
Figure 2.3. DUSP5 regulates ERK dephosphorylation in response to TNFα. A) 3T3-L1 adipocytes were pretreated with pervanadate 30 min prior to stimulation with TNFα (100 pM) and cell lysates harvested over time post-TNFα. Phospho-ERK and JNK as well as total ERK and JNK were examined by immunoblotting. Adipocytes were transfected with a non-targeting control siRNA or two independent siRNAs specific for DUSP5 for 48 hrs prior to TNFα (100 pM) stimulation. B) Total RNA was harvested 30 min post-TNFα stimulation and qRT-PCR used to examine DUSP5 mRNA expression. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to unstimulated control siRNA. Statistical differences were determined by ANOVA, with Tukey’s post-hoc analysis performed when the p value for the respective parameter was statistically significant (p < 0.05). C) Cell lysates were collected over time post-TNFα stimulation and immunoblotted for phospho-ERK and JNK as well as total-ERK and JNK.
To definitively address a role for DUSP5 as a regulator of ERK1/2 dephosphorylation in adipocytes, loss of function studies were performed. Mature 3T3-L1 adipocytes were transfected with small interfering RNAs (siRNAs) to target two independent regions of the DUSP5 gene (siDUSP5#1 and siDUSP5#2) or an siControl for 48 hours prior to stimulation with 100 pM TNFα. Cell lysates were harvested for RNA at 30 minutes or protein collected over time post-TNFα. Using qPCR, we demonstrate significant loss of TNFα-induced DUSP5 gene expression with both siRNAs targeting the DUSP5 gene (Fig. 2.3.B), indicating successful gene knockdown. Consistent with the postulate that DUSP5 regulates ERK1/2 dephosphorylation, loss of DUSP5 exacerbated TNFα-stimulated ERK1/2 phosphorylation (Fig. 2.3.C) with regards to signaling magnitude and duration. More importantly, loss of DUSP5 did not however alter JNK phosphorylation, demonstrating that DUSP5 is an ERK-specific phosphatase in adipocytes. These date clearly demonstrate that DUSP5 regulates ERK1/2 phosphorylation in adipocytes in response to TNFα.

**DISCUSSION**

Collectively, these data suggest that DUSP5 plays a key role in the regulation of adipose tissue inflammation associated with obesity. We propose a model in which TNFα-induced phosphorylation of JNK and ERK drive inflammatory gene expression, while TNFα-induced DUSP5 expression inhibits ERK1/2 phosphorylation to mitigate adipose tissue inflammation (Fig. 2.4).
Obesity rates are increasing in Westernized nations, particularly the United States (Greenberg and Obin, 2006). Obesity is characterized by chronic, low grade inflammation in the body, particularly in adipose tissue. Inflammation is regulated by the phosphorylation and dephosphorylation of various MAPKs that work to induce expression of proinflammatory genes.
Both ERK and JNK have been identified as key MAPKs involved in the inflammatory pathway (Gil, Aguilera, Gil-Campos, and Cañete, 2007; Greenberg and Obin, 2006). Indeed, we report that both ERK1/2 and JNK are phosphorylated in adipocytes in response to TNFα, suggesting that these intracellular signaling cascades link external cues such as TNFα to internal biological function within the cell. Consistent with this, inhibition of MAPK signaling protects animals from diet-induced obesity, inflammation and insulin resistance (Jeffrey et al., 2007; Wu et al., 2006; Zhang et al., 2003; Xu et al, 2003; Emanuelli et al., 2008), suggesting that endogenous dephosphorylation of these ERK and JNK by phosphatases plays a key role in attenuating cellular stress.

Recent literature suggests that DUSPs play an important role in innate and adaptive immunity (Gregoire, Smas, and Sul, 1998). Moreover, loss-of-function and gain-of-function studies have highlighted a critical role for DUSPs in obesity and diabetes (Jeffrey et al. 2007). DUSP9 overexpression, for instance, has been shown to attenuate insulin resistance in adipocytes (Emanuelli et al., 2008), while mice deficient in DUSP1 succumb to glucose intolerance (Zhang et al., 2003). In keeping with these reports, we publish that loss of DUSP5 exacerbated TNFα-induced ERK1/2 phosphorylation. As ERK inhibition has been reported to block inflammatory gene expression (Bost, Aouadi, Caron, and Binetruy, 2005), our data would suggest that DUSP5 regulates ERK-dependent inflammation in adipose tissue. Together, these data highlight the potential for phosphatases in the regulation of adipose tissue inflammation and insulin resistance.

In conclusion, we propose that DUSP5 regulates inflammation through the regulation of ERK1/2 signaling. Obesity is a major risk factor for the development of type 2 diabetes, hypertension and cardiovascular disease (Gregoire, Smas, and Sul, 1998). Complications from these obesity-related comorbidities result in approximately 300,000 deaths per year in the United
States (Mokdad et al., 2003). Foods, nutraceuticals or therapies designed to upregulate DUSP5 expression have the potential to inhibit obesity-mediated inflammation and as such improve peripheral insulin resistance and type 2 diabetes. Since obesity is a growing concern in the world today and is associated with multiple comorbidities, knowledge gained from our studies could promote the advancement of therapeutic targets that have the potential to benefit the health of a large percentage of the population.

CHAPTER 3: FUTURE IMPLICATIONS

EPILOGUE

One year ago, I began searching for a research lab in the nutrition department when I met Dr. Bradley Ferguson. As both a premedical and nutrition science student, I have had many classes that have emphasized the detrimental affects obesity can have on the body. As a result, I quickly became interested in his research on obesity and insulin resistance. Thankfully, Dr. Ferguson was extremely supportive and allowed me to begin exploring the impact of DUSP5 under conditions of diet-induced obesity, inflammation and insulin resistance for my honors thesis. Throughout the past year, we have worked at the molecular level to delineate a role for TNFα on MAPK signaling and DUSP expression in adipocytes as well as, elucidate a role for inducible DUSPs on MAPK signaling.

Our findings suggest potential therapeutic applications for the treatment of obesity induced insulin resistance. I hope that these findings will be furthered to eventually develop a realistic, effective treatment for obesity-induced type 2 diabetes. In order for this to occur, further research is necessary. This could include using mouse models to knock out the DUSP5
gene. Adipocytes could be harvested from the mice and analyzed for MAPK activation and inflammation. It is expected that elimination of the DUSP5 gene would cause an increase in inflammation. In addition, since skeletal muscle cells are one of the body’s main storage sites for glucose, experiments can be run to study if DUSP5 also plays a role in MAPK dephosphorylation in these cells. In this case, the C2C12 muscle cell line can be used to examine if ERK dephosphorylation occurs as readily in these cells due to influence by DUSP5 as it did in adipocytes.

Our findings could also be expanded to test specifically for insulin resistance in cells. Insulin resistance could be induced in cells, just as it is in the body in cases of type 2 diabetes, and the role of DUSP5 could be elucidated from there. In this case, I could explore whether or not cells become more sensitive to insulin with increasing expression of DUSP5. Experiments could begin with the 3T3-L1 cell line, then move on to mouse models of insulin resistance. If these experiments are successful, human trials could ultimately be conducted to create a therapeutic treatment for insulin resistance in type 2 diabetes based on the inflammatory pathway related to DUSP5.

Those studying nutrition on a molecular level would find this research particularly appealing. My findings can be used to expand the knowledge base on the mechanisms of obesity-induced inflammation at a biochemical level. These findings can then be expanded to cases of insulin resistance due to obesity-induced type 2 diabetes. If time and funding were abundant, pharmaceutical companies could pursue this research in order to develop a safe, effective treatment for type 2 diabetes. Since this mechanism would target insulin resistance at a very specific gene, this treatment could potentially avoid some of the side effects of type 2 diabetes.
medications that are currently on the market. Ultimately, our findings in this research study could be used to benefit physicians and consumers alike.
REFERENCES


