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The effects of STAT activators in the crosstalk of gp130 cytokines in adipocytes

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THE EFFECTS OF STAT ACTIVATORS AND THE CROSSTALK OF GP130 CYTOKINES IN ADIPOCYTES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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by

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LIST OF ABBREVIATIONS

ADD1 - adipocyte differentiation and determination
AOX - acyl CoA oxidase
AP-1 - activating protein 1
C/EBP - CAAAT Enhancer Binding Protein
CLC - cardiotrophin-like cytokine
CNTF - ciliary neurotrophic factor
CT-1 - cardiotrophin-1
EMSA - electrophoretic mobility shift assays
EPI - epididymal
ERK - extracellular signal-related kinase
FAS - fatty acid synthase
FFAs - free fatty acids
GLUT4 - glucose transporter 4
GH - growth hormone
gp130 - glycoprotein 130
IL-6 - interleukin 6
IL-11 - interleukin 11
IL-27 - interleukin 27
IRS - insulin receptor substrate
JAK/STAT - janus kinase/signal transducer and activator of transcription
KLF - krüppel-like factor
LDL - low-density lipoprotein
LIF- leukemia inhibitory factor
LPL- lipoprotein lipase
MHO- metabolically healthy but obese
MCP-1- monocyte chemoattractant protein 1
MAPK- mitogen activated protein kinase
NIDDM- non-insulin dependent diabetes mellitus
NP- neuropoietin
OSM- oncostatin M
PDC- pyruvate dehydrogenase complex
PDK- pyruvate dehydrogenase kinase
PPAR- peroxisome proliferator-activated receptor
PRL- prolactin
RP- retroperitoneal
SDS-PAGE- sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SOCS- suppressors of cytokine signaling
SREBP- sterol regulatory element binding protein
STAT- signal transducer and activator of transcription
SUMO- Small Ubiquitin-like Modifier
T2DM- type 2 diabetes mellitus
TGs- triglycerides
TNFα- tumor necrosis factor alpha
TZD- thiazolidinedione
ABSTRACT

Obesity is characterized by an overabundance of fat cells, or adipocytes, and is currently a global epidemic. Adipocytes store energy, respond to insulin, and participate in diverse endocrine signaling pathways by secreting adipokines. Obesity leads to the dysregulation of adipocyte function, which could lead to numerous metabolic diseases, such as Type II diabetes. Understanding the mechanisms that regulate adipocyte development and involvement in endocrine signaling will lead to a greater understanding of the importance of fat metabolism in full-body health. Many lines of evidence demonstrate the importance of various transcription factors in regulating adipogenic processes, and one of which is the Signal Transducer and Activator of Transcription (STAT) proteins. Work by a variety of laboratories has demonstrated that STAT proteins, particularly STAT5A, are activated and induced during adipogenesis and play an important role in adipose tissue development. Novel studies highlighted in this dissertation demonstrate that pyruvate dehydrogenase kinase (PDK)-4, a known regulator of glycolysis, is highly induced in adipocytes by growth hormone (GH) or prolactin (PRL) in a STAT5 dependent manner. Under these conditions, the induction of PDK4 is accompanied by insulin resistance. Because adipocytes also express other STATs, we observed the effects of other STAT activators on adipocytes both in vitro and in vivo. Adipocytes are responsive to gp130 cytokines, and these cytokines have diverse functions in adipocytes, as well as other tissues. All gp130 cytokines share glycoprotein 130 as a common transducer protein in their functional receptor complex and typically activate STAT3. Several gp130 cytokines differentially affect adipogenesis and insulin-stimulated glucose uptake, as well as crosstalk with other members of the gp130 family to alter one another’s signaling. Novel studies highlighted in these studies demonstrate that adipocytes both in vitro and in vivo are responsive to neuropoietin
(NP), which can inhibit adipogenesis and negatively regulate insulin signaling. Importantly, NP does not activate the LIF receptor, although previously reported to do so, and does not crosstalk with other gp130 cytokines. An in-depth analysis of STATs, as well as their activators, will enable us to understand how their role in adipocytes could contribute to the pathogenesis of metabolic disease.
CHAPTER 1
GENERAL INTRODUCTION

1.1 Adipocytes

Adipocytes are tightly packed cells that form and store fat in adipose tissue. They are highly specialized in function and play a critical role in energy homeostasis in vertebrate organisms (reviewed in (1)). The size and distribution of adipocyte stores in the body greatly affect metabolism in human disease. Excessive growth in adipose tissue mass and adipocyte size are hallmarks of obesity, which is the primary disease of fat cells. Obesity modifies the endocrine and metabolic functions of adipose tissue, which contribute to various metabolic complications. Hence, obesity is a major risk factor for the development and/or progression of many diseases; most notably non-insulin dependent diabetes mellitus (NIDDM) (2), cardiovascular disease, and atherosclerosis (reviewed in (3)). Obesity is becoming increasingly prevalent in the United States (4) and is strongly correlated with an increase in NIDDM, or type 2 diabetes mellitus (T2DM) (reviewed in (5)). Likewise, 80% of type 2 diabetics are overweight or obese (reviewed in (6)). The study of adipose tissue, specifically adipocytes, is essential for understanding the metabolic abnormalities associated with obesity.

One of the primary functions of adipose tissue is to synthesize and store excess energy as triglycerides (TGs), or lipid, as well as to release it in the form of free fatty acids (FFAs) and glycerol during fasting (reviewed in (1)). The mature adipocytes within the adipose tissue expand as they take up lipid, and preadipocytes differentiate (adipogenesis) to become mature adipocytes that are capable of additional fat uptake and storage. In addition to its role as an energy reservoir, the adipose tissue also possesses other distinct properties: endocrine function via the release of adipokines (cytokines or hormones synthesized and released by adipose tissue)
and sensitivity to insulin. The discovery that leptin, a satiety factor that regulates food intake and energy metabolism, is an adipokine, was the first evidence of adipose tissue as an endocrine organ (7). Since then, numerous other adipokines that exert diverse effects on metabolism have been discovered (reviewed in (8)). These endocrine signals allow the adipocyte to regulate the metabolism of not only other fat cells, but also cells that are located in numerous other tissues. The fact that a subset of these adipokines—leptin (7), adiponectin (9), and resistin (10)—can only be secreted from adipose tissue contributes to the importance of its endocrine function. Many of these vital adipokines are involved in the metabolic disorders associated with obesity and insulin resistance.

The regulation of glucose metabolism is essential for metabolic homeostasis, and insulin, which enhances overall glucose disposal, is the dominant hormone that maintains this control. Though most cells are responsive to insulin, in the fed state, insulin stimulates glucose uptake into only two main target tissues: one of which is adipose tissue. Insulin resistance is the failure of target tissues to respond to physiological levels of insulin (reviewed in (11)) and is a key element in the pathogenesis of T2DM (12;13). This severe metabolic disorder often results in hyperglycemia (excessive blood glucose levels) and can lead to major complications, including neuropathy, nephropathy, and retinopathy.

In recent years, adipose tissue has been increasingly recognized for its active role in the maintenance of glucose homeostasis, as well as whole-body metabolic homeostasis. This idea is supported by studies demonstrating that a lack of or severe reduction in adipose tissue causes insulin resistance in both mice (14) and humans, as in the case of lipodystrophy (15)(reviewed in (16)). Likewise, it is well known that excess adipose tissue is associated with decreased sensitivity to insulin. Hence, normal glucose homeostasis is contingent upon functional adipose
tissue of a proper amount. An impaired capacity to properly store excess dietary fat intake due to too much or too little adipose tissue may result in insulin resistance. The mechanisms linking obesity, namely adipose tissue metabolism, to insulin resistance are intensely investigated, and all involve the inability of adipocytes to function in their innate fashion. Taken together, these findings suggest that adipose tissue dysfunction plays a vital role in the pathogenesis of obesity-related T2DM.

Adipose tissue expansion through both increased adipocyte recruitment (hyperplasia) and adipocyte enlargement (hypertrophy) is critical for the safe storage of lipid. An increasingly popular hypothesis that correlates with insulin resistance is adipose tissue expandability, which postulates that each individual’s adipose tissue may have a maximum capacity to efficiently store fat (reviewed in (17)). Hence, an individual will remain metabolically healthy, even if they become obese, as long as the adipose tissue can safely accommodate the excess caloric intake. Any loss of capacity for storing fat can promote insulin resistance. When this maximum storage capacity is reached, lipid spillage occurs, causing increased circulating levels of FFAs. Many lines of evidence demonstrate a strong association of obesity and insulin resistance with high circulating FFA levels (reviewed in (18)). Consequently, various non-adipose tissues, such as skeletal muscle, liver, and the pancreas, accumulate excess ectopic lipid stores (lipotoxicity), which may cause insulin resistance, as they lack the capacity to efficiently oxidize the excess fatty acids (reviewed in (19)). Several studies support the hypothesis of adipose tissue expandability and its implications in disease. For example, studies in mouse models have demonstrated that over expression of adiponectin in the ob/ob mouse caused an expansion of fat mass, which led to an improvement in insulin sensitivity (20). In human subjects, studies have shown that the PPARγ agonists, thiazolidinediones (TZDs), improve insulin sensitivity and
diabetes (21) and increase fat mass (22), supporting the idea that stimulating adipose tissue expansion is associated with improved metabolism. In addition, several lines of evidence show that individuals who are metabolically healthy but obese (MHO) have significantly increased fat mass, but remain insulin sensitive and have no metabolic complications (23;24), presumably due to the expansion of their adipose tissue. A better understanding of the mechanisms underlying adipose tissue development, function, and expansion is required to improve the identification of successful therapeutic approaches.

Studies also suggest that as adipocytes reach their maximum storage capacity, their endocrine function is compromised, which yields derangements in adipokine secretion and insulin resistance (reviewed in (25)). Human studies reveal a positive correlation between large, lipid-filled adipocytes, insulin resistance, and altered adipokine secretion (26). In obese individuals, the adipose tissue is engorged with TGs, and the adipocytes cannot efficiently store additional lipid (reviewed in (19)). Therefore, the adipocytes become large and hypertrophied, unresponsive to normal biological signals, and dysfunctional in the secretion of signaling factors. Adipocyte size is linked to insulin sensitivity, for adipocyte hypertrophy is often observed in obesity, as well as T2DM (27;28). Likewise, human studies demonstrated that weight loss results in reduced adipocyte size (29).

It is well known that chronic, low-grade inflammation, characterized by abnormal adipokine production, is positively correlated with obesity, insulin resistance, and T2DM. Adipocyte hypertrophy stimulates the release of pro-inflammatory cytokines, which elicit this effect (reviewed in (3)). Though the mechanisms underlying this inflammatory state have not been fully elucidated, evidence suggests that macrophages, which are one of the numerous non-adipocyte cells located in the adipose tissue, contribute to the production of inflammatory factors
(reviewed in (19) and (30)). Studies have shown that in obese individuals, hypertrophied adipocytes synthesize and secrete large amounts of monocyte chemoattractant protein (MCP)-1 (31), which enhances macrophage infiltration into adipose tissue (32). The additional macrophage recruitment results in a chronic pro-inflammatory state stimulated by the release of several cytokines, including tumor necrosis factor (TNF)α (33) and interleukin (IL)-6 (34). In line with these findings, obese patients were shown to contain substantially more macrophages in their adipose tissue than lean subjects (35), and obesity-related insulin resistance is correlated with increased secretion of inflammatory cytokines (36). Obese individuals also display decreased levels of the insulin-sensitizing and anti-inflammatory factor adiponectin (37-39), as well as significantly increased circulating levels of leptin, suggesting insensitivity to endogenous leptin production (40). The inflammatory molecules have detrimental effects on adipose tissue function, such as inhibition of both adipogenesis (41;42) and expression of important adipocyte genes, including glucose transporter (GLUT)4, which is essential for insulin-regulated glucose disposal (43). Both TNFα and IL-6 have been shown to increase lipolysis in adipocytes (44;45), which may lead to increased levels of circulating FFAs and possibly insulin resistance. Consequently, thiazolidinedione (TZD) treatment of obese rats stimulated an increase in the number of small adipocytes and a decrease in the number of large adipocytes, causing improvement of the adipokine signaling profile and normalization of increased levels of TNFα (46). In turn, weight loss has been shown to cause decreased concentration of MCP-1 (47), as well as a reduction in adipocyte hypertrophy and macrophage infiltration in adipose tissue (48;49), which would collectively improve the inflammatory profile. Taken together, these findings support the hypothesis that the inflammatory response contributes to dysfunctional
metabolism in obesity via the altered release of adipokines, and this response may be mediated, in part, by the infiltration of macrophages in adipose tissue.

1.2 The Transcriptional Control of Adipogenesis

Obesity and its related disorders result in dysregulation of the mechanisms that control the expression of metabolic genes in adipocytes. Therefore, understanding adipocyte differentiation is relevant not only for gaining insight into the pathogenesis of metabolic diseases, but also for identifying proteins or pathways which might be appropriate targets for pharmacological interventions. In the last twenty years, significant advances towards an understanding of the regulatory processes involved in adipocyte differentiation have largely been made by the identification of several transcription factors that regulate the differentiation of fat cells and/or are involved in the induction and maintenance of adipocyte gene expression.

Interestingly, the majority of studies that have identified transcriptional regulators of adipogenesis have been performed in vitro. These studies have been primarily conducted in the 3T3-L1 or 3T3-F442A preadipocyte cell lines that were originally generated in the laboratory of Dr. Howard Green at Harvard University (50;51). In the last thirty-two years, these cells lines have been used by thousands of investigators world-wide. In vivo, adipocytes have three primary characteristics, which include lipid storage, insulin sensitivity, and endocrine properties. The 3T3-L1 cells have all three of these notable characteristics of fat cells. In addition, many adipocyte specific genes have been identified using this cell line. Many cell types cannot be adequately studied in vitro because the cultured cells do not possess the properties of adipocytes in vivo. However, the preadipocyte cell lines that were developed by Dr. Green have been an extremely useful model system for adipocyte biologists, and the data obtained in these cells has been validated from less mechanistic in vivo studies in the last decade.
In the late 1980’s, a commentary by Steven McKnight and Dan Lane indicated that C/EBPα was a metabolic regulator of energy metabolism (52). Since that time numerous studies have confirmed the role of C/EBPα and other C/EBP family members in energy balance and defined roles for these transcription factors in adipocyte differentiation (reviewed in (53)). In 1994, two prominent laboratories independently identified PPARγ as an important modulator of adipocyte differentiation. Studies by Dr. Mitch Lazar’s group observed the induction of PPARγ during adipogenesis (54), and experiments by Dr. Bruce Speigelman’s laboratory revealed that PPARγ was a transcription factor which bound to an enhancer element in the aP2 promoter and conferred its fat specific expression (55). The early 1990s was also the time that Brown and Goldstein identified SREBP-1 (56), also termed ADD1, whose expression was also observed to play a role in adipocyte determination by the Speigelman laboratory (57). Since this time, several other transcription factors have been found to play an important role in adipocyte differentiation. This section will focus on the transcription factors mentioned above and other important transcription factors that are regulated during adipocyte differentiation and are known to contribute to this process.

Studies of the aP2 gene or use of its regulatory sequences have led to significant discoveries in adipocyte biology. aP2 is an abundantly expressed adipocyte gene that was first discovered in 1984 (58). One of the earliest studies on PPARγ identified it as a transcription factor that bound to an enhancer element in the aP2 promoter and conferred its fat-specific expression. Since its discovery, the aP2 promoter has been used by hundreds of laboratories to construct transgenes that have largely fat-specific expression. A lesser known historical fact is that c-fos was also shown to bind to the aP2 promoter just 124 base pairs upstream of the
transcriptional start site (59). As means of introduction, this was the first study to propose that AP-1 proteins were regulators of adipocyte gene expression.

**Activating Protein (AP-1) Transcription Factors:** Members of the activating protein-1 (AP-1) family of transcription factors are well-known regulators of cellular proliferation and differentiation. AP-1 is a collective term referring to dimeric transcription factors composed of c-Jun, JunB, JunD and c-Fos, FosB, Fra-1, or Fra-2 subunits that bind to a common DNA site, the AP-1-binding site (reviewed in (60). As indicated above, studies by the Spiegelman laboratory indicated that c-fos was involved in the modulation of aP2 expression (59). Several years later, it was shown that the expression of c-jun, c-fos, jun-B, fos-B, and fra-1 was induced immediately after the induction of adipocyte differentiation (61;62). Although the role of individual AP-1 family members in adipogenesis has not been elucidated, there is strong evidence to indicate the importance of these transcription factors in vivo. Transgenic mice were generated that express a dominant-negative protein that prevents the DNA binding of B-ZIP transcription factors of both the C/EBP and Jun families under the control of the adipose-specific aP2 enhancer/promoter. These mice have no white adipose tissue throughout life (14). Collectively, these studies suggest that the induction and expression of AP-1 transcription factors are important in fat cell differentiation.

**Krüppel-like Factors (KLFs):** Krüppel-like zinc finger transcription factors (KLFs) are known to play diverse roles in cell differentiation and development in mammals. One protein in the KLF family, KLF15, was shown to be highly induced during the differentiation of 3T3-L1 preadipocytes into adipocytes (63). Inhibition of KLF15 function or expression with a dominant negative mutant or via RNA interference results in an inhibition of adipogenesis in 3T3-L1 cells (63). These studies also revealed that KLF15 could confer adipogenesis in non-precursor cells.
and result in the induction of PPARγ expression. Similar to KLF15, KLF5 expression is also highly induced during adipocyte differentiation in 3T3-L1 cells, and embryonic fibroblasts obtained from heterozygote KLF5(+/−) mice exhibit reduced adipogenesis (64). Another member of this family, KLF2, has been shown to be a negative regulator of adipogenesis and has the ability to attenuate PPARγ expression (65). KLFs are not only involved in lipid accumulation, but also appear to play a role in the ability of the adipocyte to be insulin sensitive, as indicated by studies showing that KLF15 is important in the expression of GLUT4 (66). KLF4 is also induced early in the differentiation process and can transactivate the C/EBPβ promoter in cooperation with Krox20 (67), another zinc finger transcription factor shown to stimulate adipogenesis (68). Other members of the KLF family have been shown to have differential effects on adipogenesis; hence, emerging studies on the role of KLFs in adipocyte development will be critical for the identification of target genes in fat cells, as well as the likely discovery of both redundant and non-redundant functions of these transcription factors in adipocytes.

**Sterol Regulatory Element Binding Proteins (SREBPs):** In 1993, studies by the Spiegelman group identified a basic helix-loop-helix transcription factor that was expressed in adipocytes and whose expression was increased during adipogenesis (57). The protein was called ADD1 for adipocyte differentiation and determination. Two months later, this protein was labeled SREBP-1 by the Brown and Goldstein’s laboratories, who named the transcription factor for its ability to bind sterol responsive elements within the promoter of the low-density lipoprotein (LDL) receptor gene (56). It is now known that there are three SREBP isoforms (SREBP-1a, -1c, and -2) that have been well characterized in the last fifteen years. SREBP-1a and -1c are transcribed from the same gene, each by a distinct promoter, and the predominant SREBP-1 isoform in liver and adipose tissue is SREBP-1c. SREBP-2 is relatively selective in transcriptionally activating
cholesterol biosynthetic genes, and SREBP-1c has a greater role in regulating genes associated with fatty acid synthesis (reviewed in (69). Although there is clear evidence that SREBP is an insulin modulated transcription factor that is involved in the regulation of genes associated with cholesterol and lipid metabolism, studies indicate that SREBPs may not be critical for adipogenesis. Mice deficient in SREBP-1 do not have a significantly decreased amount of white adipose tissue, but SREBP-2 levels were increased suggesting it might compensate for SREBP-1 (70). These in vivo studies are supported by additional transgenic studies where the SREBP-1 deficient mice were crossed with ob/ob (leptin deficient) mice, and it was found that SREBP-1 was not required for the development of obesity. These observations demonstrated that SREBP-1 regulation of lipogenesis was highly involved in the development of fatty livers but was not a determinant of obesity in this animal model (71). However, ectopic expression of a dominant-negative SREBP-1c was shown to attenuate adipocyte differentiation (72). In addition, over expression of SREBP-1c enhanced the adipogenic activity of PPARγ (72) and other studies suggest that SREBP-1c contributes to the generation of PPARγ ligands (73). In summary, in vitro studies support a role for SREBP-1 in adipogenesis, whereas in vivo studies indicate that SREBPs are not required for the production or expansion of adipose tissue.

**CAAAT Enhancer Binding Proteins (C/EBPs):** C/EBP transcription factors were the first family of proteins shown to play a critical role in the differentiation of fat cells in vitro. Today, we know that transgenic mice lacking both C/EBPβ and C/EBPδ or C/EBPα alone have defective adipocyte differentiation (74;75). Prior to these in vivo observations, the cascade of induction of these three C/EBP family members was revealed by McKnight and collaborators who showed that C/EBPs β and δ were induced immediately after the induction of differentiation, whereas C/EBPα expression did not occur until four to five days after the initiation of differentiation (76).
This group also demonstrated that C/EBPs β and δ were responsible for inducing C/EBPα expression (77). Ectopic expression studies conducted by several laboratories demonstrated the adipogenic capabilities of C/EBPα or C/EBPβ alone, or in the presence of C/EBPδ (77-80). Today, C/EBPα and PPARγ are considered the two primary transcription factors that mediate adipogenesis. However, cells lacking C/EBPα are capable of adipogenesis, but are not insulin sensitive (81;82). There is also evidence to indicate that C/EBPs may play a role in the induction of a PPARγ ligand (83). In summary, both in vitro and in vivo studies indicate a substantial role for C/EBPβ, C/EBPδ and C/EBPα in adipogenesis. Although C/EBPα may not be required for lipid accumulation, this transcription factor clearly plays a role in conferring insulin sensitivity in adipocytes.

**Peroxisome Proliferator-Activated Receptors (PPARs):** Although a number of transcription factors, including those mentioned above, have been shown to have profound effects on fat cell differentiation and the expression of adipocyte genes, only one adipocyte transcription factor has been shown to be necessary for adipogenesis. Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear hormone receptor superfamily that is required for the development of adipocytes, and deletion of PPARγ in mice results in placental dysfunction and embryonic lethality (84;85). As mentioned above, PPARγ was originally identified as a transcription factor induced during differentiation that bound to the enhancer element within the aP2 promoter (86). Since that time, there have been multiple studies on the role of PPARγ in adipocytes. A remarkable finding in 1995 was that the insulin sensitizing drugs thiazolidinediones were ligands for PPARγ (87). These were some of the first molecular studies to indicate the importance of adipose tissue in insulin resistance. Many investigators were surprised to learn that activating a transcription factor whose expression was highly enriched in
fat cells could contribute to whole animal insulin sensitivity. It is now known that adipocytes secrete several hormones that can affect the activity of other tissues; however, the studies of PPARγ have revealed that modulation of this transcription factor can contribute to systemic insulin resistance. Although the PPARγ null mice are embryonic lethal (85), transgenic mice lacking PPARγ specifically in adipose tissue exhibit greatly reduced sized fat pads and insulin resistance in fat and liver (88). However, PPARγ heterozygote mice(+/−) have enhanced insulin sensitivity (89). Together, these studies suggest the amount of PPARγ in adipose tissue is physiologically relevant. In the last several years, several studies have examined pathways that are involved in regulating the levels of PPARγ. In particular, the ubiquitin-proteasome system has emerged as an important regulator of PPARγ proteins (90;91). In addition, a role for the ubiquitin-like protein, SUMO (Small Ubiquitin-like Modifier) in regulating PPARγ has been demonstrated by several groups (92-95). The phosphorylation of PPARγ by MAPKs is also an important modulator of the activity of this transcription factor (reviewed in (96). A very recent study confirmed that PPARγ knockdown prevented adipocytes differentiation, but was not required for maintenance of the adipocyte differentiation state after the cells had undergone adipogenesis (97). These findings are supported by many anecdotal observations that indicate that PPARγ is decreased as adipocytes age in vitro. In addition, the increase of life span via caloric restriction results in the induction of Sirt1, a transcriptional modulator with deacetylase activity, that represses PPARγ activity in vivo (98). This study also demonstrated that the repression of PPARγ by Sirt1 was evident in 3T3-L1 adipocytes. Collectively, studies by numerous laboratories demonstrate the adipogenic capabilities of PPARγ. However, the role of this transcription factor in mature adipocytes is not well understood, and recent studies suggest that this transcription factor is not required for maintenance of the adipocyte following
adipogenesis (97). Nonetheless, PPARγ expression and activity is controlled at multiple levels, including alternative promoter usage, tissue limited expression, phosphorylation, acetylation, ubiquitylation, and SUMOylation. The multiple regulatory features of this transcription factor suggest that controlling the amount and activity of PPARγ is important. The role of PPARγ in the development and treatment of diabetes is well established (reviewed in (99)), and the importance of PPARγ in humans is indicated by several loss-of-function mutations in the PPARγ gene that cause lipodystrophy and diabetes (100-102) (reviewed in (103)).

**Signal Transducers and Activators of Transcription (STATs):** The janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway was identified by studying the transcriptional activation of genes in response to cytokine/growth factors (reviewed in (104-106)). Cytokines induce a broad range of effects on a number of biological processes and activate receptors that do not contain any obvious cytoplasmic catalytic domains. To date, there are four members in the JAK kinase family (JAKs 1-3 and Tyk 2) that have been identified and shown to associate with cytokine/growth factor receptors and result in the activation of a signal transduction cascade involving STAT proteins.

The STAT family of mammalian transcription factors is comprised of seven proteins (STATs 1, 2, 3, 4, 5A, 5B, 6) which, in response to stimulation of various receptors, are phosphorylated on tyrosine residues causing their translocation to the nucleus. Each STAT family member shows a distinct pattern of activation by different ligands, and upon nuclear translocation can regulate the transcription of particular genes. The order of events for STAT activation can be briefly described as follows: 1) Ligand binds cell surface receptor; 2) receptor associates with a JAK family member; 3) JAK tyrosine phosphorylates receptor 4) STAT associates with phosphorylated receptor and is phosphorylated by JAK 5) STATs form dimers
and translocate to the nucleus; and 6) STATs bind DNA and modulate transcription. STATs have been shown to bind distinct DNA sequences, and this binding regulates the transcription of specific genes (reviewed in (105;107)). Since the tissue distribution and function of each STAT is unique, the regulation of tissue-specific genes may be a physiological role for these proteins (reviewed in (108)). This hypothesis is supported by numerous reports which demonstrate that specific STATs are activated differently by growth factors and cytokines, and STAT activation can be cell type dependent. In addition, transgenic knockout experiments have revealed crucial roles for each known mammalian STAT (reviewed in (105)), and cell-specific functions for STAT family members have been identified (reviewed in (106)). STATs have been shown to play a role in many processes, including oncogenesis, vascular disease, myeloproliferative disorders, diabetic nephropathy, and energy balance.

Two STAT family members, STAT5A and STAT5B, are highly related, but are encoded for by two separate genes (109). Murine STAT5A and 5B have 96% sequence similarity, and the major difference between the two proteins is at the C-terminus, where the final eight residues are different. Also, STAT5B is 12 residues shorter than STAT5A. Interestingly, STATs 5A and 5B have been shown to have both essential and nonessential, or redundant, roles in cytokine responses (110). Several studies indicated that STAT5 proteins can be activated by a number of different cytokines, yet STAT5 null mice have a phenotype that is consistent with these two STAT5 proteins being primarily activated by growth hormone (GH) and prolactin (PRL) (110).

In addition to its role in growth, GH plays a role in energy metabolism. Over ninety years ago, it was shown that the anterior pituitary was the source of a factor that exerted profound effects on glucose metabolism. It is now known that GH plays a critical role in modulating a variety of metabolic pathways, including lipolysis, lipogenesis, glucose uptake,
protein synthesis (reviewed in (111)). Overall, GH promotes growth of lean tissue while reducing accumulation of fat in adipose tissue. Mice with GH excess are lean and resistant to diet induced obesity, but are severely insulin resistant (112;113). In contrast, mice with chronic GH deficiency (114;115) have increased insulin sensitivity despite increased adiposity (112;114;115). Increased adipose tissue mass occurs in GH-deficient children and adults (116-118), and treatment of GH-deficient patients with exogenous GH can improve lean body mass while reducing fat mass (119-121). These advantageous effects of GH have generated interest in using GH or analogs in the treatment of several types of obesity (122-124), despite the likelihood that GH treatment may exacerbate glucose intolerance and hyperinsulinemia (reviewed in (125;126)). Several studies have shown that activation of STATs 5A and 5B are critical for a variety of GH functions, including changes in metabolism, body growth, and sex-dependent gene regulation (110)(reviewed in (125;126)).

PRL is a peptide hormone primarily known for its role in mammary gland development during lactation, but it has been shown to have pleiotrophic effects in a variety of tissues (reviewed in (127)). PRL activates multiple signal transduction pathways, including MAPK (reviewed in (128)) and PI3K reviewed in (129)); however, the JAK/STAT pathway is the predominant signaling cascade activated by PRL, resulting in the nuclear translocation of STAT5 proteins (reviewed in (130)). The regulation of mammary tissue by PRL is well described, but there is also evidence that PRL modulates adipose tissue. The prolactin receptor (PRL-R) is expressed in both mouse (131) and human adipose tissue (reviewed in (132)) and is induced during adipogenesis (133). Ectopic expression of the PRL-R in NIH-3T3 cells confers adipocyte conversion and activation of the aP2 promoter in a PRL-dependent manner (134). Collectively, these observations strongly suggest a role for PRL in the regulation of adipocite
function. Furthermore, the occurrence of obesity has been correlated with hyperprolactinomas in humans (135). In addition to these adipogenic effects, PRL has been shown to induce lipolysis in rabbits (136) and mouse adipose tissue explants (137). Studies have shown that PRL reduces lipoprotein lipase (LPL) activity in cultured human adipocytes (reviewed in (132)) and decreases the activity of LPL and fatty acid synthase (FAS) in adipose tissue of lactating mice (138). Thus, PRL exerts adipogenic and anti-lipogenic effects on adipose tissue in a variety of species, and several of which may be mediated by STAT proteins.

In the last decade, several groups have studied the modulation and function of STAT proteins during adipogenesis and in mature fat cells. The first studies on STAT expression in 3T3-L1 cells revealed that STATs 1, 5A and 5B were highly induced during murine adipogenesis (139). Similar results were observed during the in vitro differentiation of human preadipocytes (140). In addition, the ectopic expression of C/EBPs β and δ in non-precursor cells results in an induction of adipogenesis (79) that is accompanied by an induction in STAT5A and STAT5B protein levels (141). These two STAT proteins were also coordinately regulated with both PPARγ and C/EBPα in differentiating 3T3-L1 cells under a variety of different conditions (142). In 3T3-F44A preadipocytes, the ability of GH to modulate adipogenesis was attenuated by STAT5 anti-sense oligonucleotides (143). Also, constitutively active STAT5 was capable of replacing the requirement for GH in adipogenesis of these cells (144). Moreover, ectopic expression of STAT5A has been shown to confer adipogenesis in 3T3-L1 preadipocytes (145) and in two different non-precursor cell lines (146). Interestingly, STAT5B was not capable of conferring adipogenesis in non-precursor cells (146). Transgenic deletion of STAT5A, STAT5B, or both STAT5 genes in mice resulted in significantly reduced fat pad sizes compared to wild-type mice (110). Also, in primary cultures of adipose tissue from these animals, GH did not
stimulate lipolysis as it did in adipocytes from wild-type animals (147) suggesting that some of the effects of GH on fat metabolism are dependent on STAT5 proteins. It should be noted that the increased expression of STAT5 proteins is not typically observed until after the induction of C/EBPα and PPARγ (refer to Figure 1.1), yet the activation of STAT5 proteins in preadipocytes occurs prior to the induction in expression of PPARγ in 3T3-L1 cells (146). In fact, both STAT5 proteins are tyrosine phosphorylated and translocate to the nucleus within 15 minutes after the induction of adipogenesis of preadipocytes (146;148). Coupled with the observations in STAT5 null mice, which have fat pads of one-fifth normal size (110), the data suggest that activation of STAT5 proteins may be an important driver of adipogenesis both in vitro and in vivo. This hypothesis is also supported by work indicating that one of the PPARγ promoters can be modulated by STAT5 (149), suggesting that STAT5 activation might drive adipogenesis by inducing PPARγ expression. In summary, work by a variety of laboratories in the last decade has demonstrated that STAT5 proteins, particularly STAT5A, are activated and induced during adipogenesis and play an important role in adipose tissue development.

In addition to the transcription factors described above that promote adipogenesis, there are several other transcription factor families that have been shown to have positive or negative effects on adipocyte differentiation (reviewed in (53;150)). The expression and activity of these transcription factors play a profound role in modulating a vast array of target genes that are important in conferring lipid accumulation, insulin sensitivity, and endocrine properties in mature fat cells. The identification and characterization of the target genes of adipogenic transcription factors has provided critical information in understanding the role of these proteins in both adipocyte differentiation and in adipose tissue. Adipogenesis occurs as a result of a transcriptional cascade that involves the tightly regulated induction of numerous transcription
factors, including STAT5, SREBP-1c, PPARγ and several members of the C/EBPs and KLF families (refer to Figure 1.1). In addition, many of these adipogenic factors possess the ability to regulate one another’s gene expression, and there is substantial evidence to support functional redundancy in the ability of some of these transcription factor families to confer adipocyte development in vitro and in vivo. The future study of transcription factors that modulate adipocyte development and function is important and will likely continue to provide insight into our understanding of metabolic diseases including obesity and type II diabetes.

![Figure 1.1 Transcription factor activation or expression during adipogenesis](image)

**1.3 Identification of STAT5 Target Genes**

As previously stated, adipogenesis occurs as a result of a transcriptional cascade that involves a tightly regulated induction of key transcription factors- one of which is STAT5. It is critical to identify the target genes of these adipogenic transcription factors in order to understand adipocyte gene expression and fat cell function. STAT5 proteins play a vital role in the regulation of genes involved in lipid metabolism and insulin resistance in adipocytes, and this
section will focus on specific target genes that have been identified. The identification and
characterization of these target genes has provided critical information in understanding the role
of STAT proteins in both differentiating and mature fat cells.

It is widely accepted that STAT proteins have cell-specific functions. Studies on STAT5
proteins have revealed their importance in adipogenesis in vitro and in vivo (110;146). In
addition to their role in promoting adipocyte differentiation, STAT5 proteins also exert effects on
mature adipocytes. Recent studies have focused on the identification of STAT5 target genes in
adipocytes. The promoter for acyl CoA oxidase (AOX), the rate limiting enzyme in peroxisomal
fatty acid β-oxidation, contains a STAT5 binding site that modulates its gene expression in fat
cells (151). Another study indicates that GH also exerts stimulatory effects on adipogenesis
through STAT5A and 5B by enhancing the transcriptional activity of C/EBPβ/δ and PPARγ
(149). Transfection studies demonstrated that aP2 promoter activity can be activated by STAT5
(134). Yet, other studies have shown that STAT5 mediates the inhibition of aP2 expression in rat
primary preadipocytes (152). These were the first studies to suggest that STAT5 proteins could
act as a transcriptional repressor. Another study has also revealed that STAT5 can act as a
transcriptional repressor in adipocytes: a STAT5A binding site in the murine FAS promoter has
been shown to mediate the repression of FAS transcription that occurs with PRL treatment (153).

It is well known that PRL and GH are important modulators of lipid metabolism and are
also potent inducers of STAT5 in adipocytes (134;154). Hence, many of the metabolic actions of
these hormones could be mediated by STAT5’s direct modulation of target genes. Unfortunately, relatively few STAT5 target genes have been identified in adipocytes. Nonetheless, we hypothesize that several other STAT5A target genes will be identified that play a role in lipid or glucose metabolism.
1.4 gp130 Cytokines

In addition to STATs 5A and 5B, other STAT proteins have been shown to be expressed in adipocytes (139). Therefore, our studies also focus on plausible roles for these STATs, as well as their activators. The Interleukin (IL)-6 family of cytokines is a group of functionally and structurally related proteins that include Interleukin-6 (IL-6), Interleukin-11 (IL-11), Interleukin-27 (IL-27), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), novel neurotrophin-1/B cell stimulating factor-3 or cardiotrophin-like cytokine (CLC), and neuropoietin (NP) (155;156)(reviewed in (157)). All members of this family utilize glycoprotein 130 as a common transducer protein in their functional receptor complex that is required for signaling; therefore, the IL-6 family of cytokines is commonly referred to as the gp130 cytokines. These cytokines are involved in the regulation of numerous biological processes, including hematopoiesis, immune response, inflammation, differentiation, mammalian reproduction, cardiovascular action, and neuronal survival (reviewed in (157)). Gp130 cytokine signal transduction involves the activation of the JAK/STAT pathway, typically activating STAT3 and, to a lesser extent, STAT1. Though originally characterized as having unique properties, gp130 cytokines are known to function in a pleiotropic and redundant manner. The shared usage of signal transducers in the receptor complexes may explain the functional redundancy of gp130 cytokines. Gp130 is ubiquitously expressed in every cell type, which allows for the pleiotropic nature of the cytokines on a variety of cells. Also, though all of these cytokines share gp130 in their receptor complex, they are differentially regulated in multiple tissues, due to the presence of other receptor components that are required for their activation, including the LIF receptor and the cytokine’s specific α receptor (refer to Figure 1.2). Interleukin-6 (IL-6) and interleukin-11 (IL-11) first bind to IL-6 receptor α
and IL-11 receptor α (158), respectively, and then recruit a gp130 homodimer complex for signaling. Interleukin-27 (IL-27) engages a gp130/WSX-1 heterodimeric receptor complex (155). Leukemia inhibitory factor (LIF) and oncostatin M (OSM) directly bind the gp130/LIF receptor (LIFR) complex to induce signal transduction; OSM can alternatively utilize a gp130/OSM receptor (OSMR) complex. Cardiotrophin-1 (CT-1) recruits an alpha receptor (159), which to date has not been characterized, and also signals via gp130/LIFR. Ciliary neurotrophic facor (CNTF), cardiotrophin-like-cytokine (CLC), and neuropoietin (NP) all recruit CNTFRα and then induce the formation of a gp130/LIFR heterodimer for signal transduction (156;160;161).

In recent years, gp130 cytokines have become a focus of attention in the scientific community, as they have been implicated as potential therapeutic targets in obesity (162). Studies have shown that adipocytes are responsive to gp130 cytokines (154;163-167); however, their functions, as well as their complex signaling cascades, in adipose tissue have been only partially elucidated. Findings in our laboratory indicate that several gp130 cytokines have differential effects on adipogenesis (166-168), as well as insulin-stimulated glucose uptake (165-168). In addition, gp130 cytokines have the unique ability to crosstalk with other members of the gp130 family to alter one another’s signaling (169). An in-depth analysis of the mechanisms underlying gp130 signaling and crosstalk will enable us to further understand their role in adipocytes.

**Research Focus:** Overall, the goal of fat cell research is to analyze adipocytes both in cell culture and in animal models to determine how modifications in their function can contribute to metabolic diseases, namely obesity and T2D. Specifically, our lab observes the modulation of important transcription factors that regulate fat cell development and are involved in both the
induction and/or maintenance of adipocyte gene expression. The work included in this dissertation will highlight the differential actions of various activators of the STAT family of transcription factors in adipocytes. STAT proteins have been shown to have significant roles in adipocyte development and function. In addition to modulating genes associated with lipid metabolism, our novel studies indicate that STAT5 can also modulate a gene associated with glucose metabolism. Pyruvate dehydrogenase kinase (PDK)-4, a known regulator of glycolysis, is highly induced in adipocytes by GH or PRL in a STAT5 dependent manner (170), and this induction is associated with insulin resistance.

Target genes modulated by other STAT proteins, such as STAT3, have not been fully characterized. However, our laboratory has demonstrated that gp130 cytokines, which are potent STAT3 activators, exert numerous differential effects on adipocytes. The gp130 family has been shown to affect important adipocyte properties, including insulin sensitivity and fat cell
development. Our novel data reveal that NP has inhibitory effects on adipogenesis, insulin signaling, and insulin-stimulated glucose uptake. Importantly, although several gp130 cytokines possess the ability to alter the subsequent signaling of other family members both in vitro and in vivo, NP does not crosstalk in the same manner. We also demonstrate that NP does not activate or induce the degradation of the LIFR, though previously reported to do so, and this may be the reason for NP’s unique action.
CHAPTER 2

THE STAT5A-MEDIATED INDUCTION OF PYRUVATE DEHYDROGENASE KINASE 4 EXPRESSION BY PROLACTIN OR GROWTH HORMONE IN ADIPOCYTES

2.1 Introduction

It is well known that growth hormone (GH) and prolactin (PRL) induce signaling via the JAK-STAT pathway. In particular, STAT5 proteins are potently activated by these hormones (reviewed in (171)). GH is known to have profound effects on lipid metabolism (reviewed in (172)). The effects of PRL have been well characterized in mammary tissues, yet there is also evidence demonstrating that this hormone can affect adipose tissue in mice and humans (131)(reviewed in (132). Yet, few molecular targets for the STAT5-mediated actions of GH and PRL on adipocytes have been identified. Although, multiple lines of recent evidence suggest that STAT5 proteins can modulate adipocyte function (110;141;143-147), very few studies have identified direct STAT5 target genes in adipocytes. We recently observed that the GH and PRL inhibition of fatty acid synthase (FAS) transcription was mediated by a STAT5A binding site in the rat FAS promoter (153). Hence, our current efforts have been to identify other genes associated with glucose or lipid metabolism that are directly modulated by STAT5 proteins in adipocytes. In this study, we present data demonstrating that pyruvate dehydrogenase kinase (PDK)4 is a STAT5A target gene.

PDK is family of kinases that negatively regulate the activity of the pyruvate dehydrogenase complex (PDC) (reviewed in (173)). There are four tissue specific isoforms of PDK, PDK1-4, that have been identified in mammals (174), and each has different patterns of

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gene expression (174-176). The specificity in distribution, expression, and activity of each PDK isoform contributes to the long-term regulation of PDC in a given tissue, and thus, in part, regulates glucose metabolism. There are several conditions that result in the short term regulation of PDC activity (177)(reviewed in (178;179)). The long term regulation of PDK that occurs in starvation (reviewed in (179))(180) and diabetes involves an increase in the amount of PDK protein, which results in stable increases in PDK activity (181).

An induction of PDK4 leads to decreased glucose oxidation, which results in hyperglycemia. PDK4 expression has also been shown to be induced by high-fat feeding in skeletal muscle (182). A study in nondiabetic Pima Indians, a group prevalently stricken with obesity and type 2 diabetes, has demonstrated that increased levels of muscle PDK4 expression positively correlate with increased fasting plasma levels of insulin and negatively correlate with insulin-mediated glucose uptake (183). It has also been documented that insulin has the ability to suppress PDK4 expression in skeletal muscle and hepatoma cells (183-185). Increased insulin sensitivity in postobese patients has also been shown to be associated with decreased PDK4 expression (186).

In our present study, we examined the modulation of PDK4 in adipose tissue. Our studies clearly demonstrate that PRL and GH can induce the expression of PDK4 in 3T3-L1 adipocytes. Our studies also show that insulin pretreatment has the ability to attenuate the ability of these hormones to induce PDK4 expression. In addition, we identified a hormone sensitive region in the murine PDK4 promoter and characterized a STAT5 binding site in this region, which mediates the hormonal affects on PDK4 expression. Also, when PDK4 protein was induced, we observed an inhibition in insulin stimulated glucose uptake. In summary, our studies indicate that PDK4 can be induced in adipocytes and may play a role in insulin resistance.
2.2 Materials and Methods

Materials. Dulbecco’s modified Eagle medium (DMEM) was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, and calf serum was purchased from Biosource. PDK4 antibodies were purchased from Abgent. Porcine and human growth hormone and sheep prolactin were purchased from Sigma. STAT5A antibodies were purchased from Santa Cruz. Active MAPK was purchased from Promega. \([\alpha^{32}\text{P}]\) dCTP was purchased from Perkin-Elmer and Amersham Biosciences. Deoxynucleotide thymine triphosphate, dATP, and dGTP were purchased from Amersham Biosciences. Oligonucleotides were purchased from Integrated DNA Technologies. DNAse polymerase I large (Klenow) fragment was purchased from Promega.

Cell Culture. Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in DMEM containing 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% FBS, 0.5 mM 3-isobutyl-methylxanthine, 1 \(\mu\)M dexamethasone, and 1.7 \(\mu\)M insulin (MDI). After 48 h, this medium was replaced with DMEM supplemented with 10% FBS, and the cells were maintained in this medium until utilized for experimentation. For serum deprivation, cells that were between 7-10 days post MDI were placed in DMEM supplemented with 0.15% fatty-acid free and growth factor depleted BSA for 16-20 hours.

Preparation of Whole Cell Extracts. Cell monolayers were rinsed with PBS and then harvested in a non-denaturing buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 \(\mu\)M phenylmethylsulfonyl fluoride, 1 \(\mu\)M pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 \(\mu\)M leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 15,000 rpm at 4°C for 15
min. Supernatants containing whole cell extracts were analyzed for protein content by BCA analysis (Pierce) according to the manufacturer’s instructions.

**RNA Analysis.** Total RNA was isolated from cell monolayers with Trizol (Invitrogen) according to manufacturer’s instructions with minor modifications. For Northern blot analysis, 15 μg of total RNA was denatured in formamide and electrophoresed through a formaldehyde/agarose gel. The RNA was transferred to Zeta Probe-GT (Bio-Rad) in a buffer containing 75 mM sodium citrate tribasic, 10 mM NaOH and 750 mM NaCl. Probes were labeled by random priming using Klenow fragment and [α-32P] dCTP.

**Plasmid Constructs:** A 973 nucleotide segment of the PDK4 promoter (-949 to +24) was cloned from murine Sv/129 liver genomic DNA using the following PCR primers: forward ATGACCGCTAGCATGTTTC; reverse GGTGAAGGGTTGACACTTGG. Nucleotides were added to the 5’ ends of each primer so that the forward primer could be digested by KpnI and the reverse primer by SacI for directional ligation into the multiple cloning site of pGL3-basic KpnI/SacI to create pGL3-PDK4pro. The STAT element in pGL3-PDK4pro (TTCTTGGAA) was mutated to (TTTAATGGAA) using the QuickChange II Site Directed Mutagenesis Kit (Stratagene) with the following primer and its antisense, CACGCTCCGCGGTGAGATTTATGGAAACAGTTTCTGGCTAG, to create pGL3-PDK4mut. Plasmids were sequenced for verification of wild type and mutagenic nucleotide sequences using Big Dye Terminator Extension Reactions (ABI).

**Transfections and Luciferase Reporter Assays:** 3T3-L1 adipocytes in 6-well plates were transfected on day 3 of differentiation following addition of DMEM containing 10% fetal bovine serum and 425 nM insulin. Either 2.5 μg of pGL3-PDK4pro or 2.5 μg of pGL3-PDK4mut was cotransfected with 250 ng of pRL-CMV/renilla vector to control for transfection efficiency in
each well using Fugene 6 (Roche). After 32h, cells were serum-deprived overnight in DMEM with 0.15% BSA, then untreated or treated with prolactin for 2h or 4h and harvested. Cell lysates were assayed for firefly and renilla luciferase using the Dual Luciferase Reporter System (Promega). Relative light units (RLU) were calculated by dividing firefly luciferase activity values by renilla luciferase.

**Preparation of Nuclear and Cytosolic Extracts.** Cell monolayers were rinsed with PBS and then harvested in a nuclear homogenization buffer (NHB) and centrifuged to isolate cytosolic and nuclear extracts as previously described (169).

**Gel Electrophoresis and Immunoblotting.** Proteins were separated in 6% polyacrylamide (National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (187) and transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk overnight at 4°C. Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and enhanced chemiluminescence (Pierce).

**Electrophoretic Mobility Shift Analysis.** Double-stranded oligonucleotides were filled-in with [α-32P] dCTP using Klenow. Binding reactions were performed with nuclear extracts, according to Ritzenthaler et al (188). The sequence of the oligonucleotide used was 5’-GTGAGATTCTTGGAAACAGTT-3’. Protein-DNA complexes were resolved and visualized, as previously described (153). For supershift analysis, nuclear extracts were preincubated with 4 μg of antibody for 1 h at room temperature.

**Determination of [3H] 2-Deoxyglucose Uptake** - The assay of [3H] 2-deoxyglucose uptake was performed as previously described (189). Prior to the assay, fully differentiated 3T3-L1 adipocytes were serum deprived for 4 hours. Uptake measurements were performed in triplicate.
under conditions where hexose uptake was linear. The results were corrected for nonspecific uptake, and absorption was determined by $[^3]$H 2-deoxyglucose uptake in the presence of 5 μM cytochalasin B. Nonspecific uptake and absorption was always less than 10% of the total uptake.

2.3 Results

Several previous studies have demonstrated an induction of PDK4 in skeletal muscle (182;184); but, the induction of PDK4 expression in adipocytes has not been previously examined. In the course of studying activators of STAT5A, we observed an induction of PDK4 mRNA by PRL. Total RNA was isolated from fully differentiated 3T3-L1 adipocytes after treatment with PRL for the times indicated. PRL is known to induce the expression of SOCS (suppressors of cytokine signaling)-3. As shown in Figure 2.1, the efficacy of PRL was demonstrated via the induction of SOCS-3, which occurred following a 30 min treatment. In addition, PRL induced the expression of PDK4 mRNA as evidenced by the robust expression at 2 hours. Between 2 and 6 hours, the induced PDK4 mRNA was apparent. In addition, PRL did not have any substantial effect on the expression of PDK2. Ethidium bromide staining of 28S and 18S RNA was included as a loading control.

To further examine the modulation of PDK4 by PRL, fully differentiated 3T3-L1 adipocytes were treated with various doses of PRL for 2 hours. As shown in Figure 2.2, PRL treatment resulted in a dose dependent increase in PDK4 mRNA. Physiological levels of prolactin, 0.5 nM (12 ng/ml), were capable of inducting PDK4 mRNA. An analysis of β-actin mRNA levels is included as a loading control.

Because PRL is a potent STAT 5 activator, we also examined the effect of growth hormone (GH), another hormone known to activate STAT 5 in adipocytes. As shown in Figure 2.3, a 2-h treatment with sheep PRL or porcine GH resulted in an induction of PDK4 mRNA.
Figure 2.1 Prolactin induces PDK4 expression in a time dependent manner in 3T3-L1 adipocytes. Total RNA was isolated from serum deprived 3T3-L1 adipocytes following 20 nM (480 ng/ml) prolactin treatment for the times indicated. Untreated cells (0) were also harvested at the start of the time course. Fifteen μg of total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis with radiolabeled probes for PDK4, PDK2, and SOCS-3. Ethidium bromide staining of 28S and 18S RNA is also included. This is a representative experiment independently performed four times.

Figure 2.2. Prolactin induces PDK4 expression in a dose dependent manner in 3T3-L1 adipocytes. Total RNA was isolated from serum deprived 3T3-L1 adipocytes following prolactin treatment with the doses indicated. Untreated cells (0) were also harvested at the start of the experiment. Fifteen μg of total RNA was subjected to Northern blot analysis. This is a representative experiment independently performed three times.
Figure 2.3 Both Prolactin and porcine Growth Hormone induce PDK4 expression in 3T3-L1 adipocytes. Total RNA was isolated from serum deprived 3T3-L1 adipocytes following a 2 hour treatment with 0.5 nM prolactin (P), human growth hormone (h), or porcine growth hormone (p). Untreated cells (CTL) were also harvested at the start of the experiment. Fifteen μg of total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis with radiolabeled probes for PDK4 and β-actin. Ethidium bromide staining of 28S and 18S RNA (gel photo) is also shown. This is a representative experiment independently performed four times.

Further analysis revealed that PRL and pig GH resulted in an equivalent level of STAT5 phosphorylation (Figure 2.4). Human GH was also capable of activating STAT5 to a lesser extent, yet did not have any effect on PDK4 (Figure 2.3). Interestingly, all of the hormones resulted in equivalent levels of extracellular signal-related kinase (ERK) 1 and 2 (active MAPK) activation.

To further examine the specificity of the induction of PDK4, we examined the effects of several STAT1 and STAT3 activators. We have previously shown that these cytokines are potent activators of STAT1 or STAT3 or both of these STATs in 3T3-L1 adipocytes, but do not activate STAT5 proteins (154;169). Total RNA was isolated from serum deprived 3T3-L1 adipocytes after a 2-h treatment with ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), interferon γ (IFNγ), or PRL. As shown in Figure 2.5, we observed that
PRL, but not any of the other cytokines, induced PDK4 expression. β-actin is included as a loading control. The efficacy of the cytokines was confirmed by examining STAT activation (data not shown).

Because previous studies suggest that insulin has the ability to suppress PDK4 expression in other tissues (183-185), we examined the PRL- and GH-mediated induction of PDK4 mRNA after an insulin pretreatment in adipose tissue. Fully differentiated 3T3-L1 adipocytes were treated with porcine GH or PRL for 2 hours in the presence or absence of a 20 minute insulin pretreatment. The robust activation of PDK4 mRNA following the treatment with either PRL or GH was evident with no insulin pre-incubation; but the induction of PDK4 mRNA was substantially attenuated by the insulin pretreatment (Figure 2.6).
Figure 2.5 STAT1 and STAT 3 activators do not induce PDK4 expression in 3T3-L1 adipocytes. Total RNA was isolated from serum deprived 3T3-L1 adipocytes following 2 nM ciliary neurotrophic factor (CNTF), 0.2 nM cardiotrophin-1 (CT-1), 0.5 nM leukemia inhibitory factor (LIF), 100 U/ml interferon γ (IFNγ), or 0.5 nM prolactin treatment. Untreated cells (CTL) were also harvested at the start of the experiment. Fifteen μg of total RNA was subjected to Northern blot analysis. This is a representative experiment independently performed two times.

Figure 2.6 The induction of PDK4 by prolactin or growth hormone is attenuated by insulin pretreatment in 3T3-L1 adipocytes. Total RNA was isolated from serum deprived 3T3-L1 adipocytes following 2 hour treatments with 0.5 nM porcine GH or 0.5 nM PRL in the presence or absence of a 20 minute 50 nM insulin pretreatment. Fifteen μg of total RNA was subjected to Northern blot analysis. This is a representative experiment independently performed three times.
Because PRL and GH are potent activators of STAT5, we searched the murine PDK4 promoter for STAT binding motifs in the promoter region 2.5 kb upstream of the transcription start sites. Typically, STAT proteins bind a core palindromic sequence, TTC NNN GAA, and selective variation can occur in particular nucleotide positions (190). One consensus STAT binding motif was identified at -389 in the PDK4 promoter. An oligonucleotide probe containing 9-bp of the core STAT motif with 6-bp of flanking sequence on either end was incubated with cytoplasmic or nuclear extracts from PRL treated 3T3-L1 adipocytes. To evaluate this potential STAT5 binding site, we performed electrophoretic mobility shift assays (EMSAs). As shown in Figure 2.7, we observed PRL-induced binding when the labeled probe was incubated with nuclear extracts from PRL treated adipocytes. Specific binding to the probe was also confirmed by results demonstrating that cytosolic proteins did not bind to this site and pre-incubation with unlabeled probe decreased binding intensity (Figure 2.7, last lane).

Pre-incubation of nuclear extracts with specific antibodies to individual STAT proteins in a supershift EMSA indicated that STAT5A was present in the binding complex induced by PRL (Figure 2.8). In addition, STAT1 and STAT3 did not participate in binding to the STAT element in the PDK4 promoter. Our data clearly demonstrate that the -389 to -378 region of the murine PDK4 promoter binds nuclear PRL-activated STAT5A proteins in vitro.

To determine whether this region of the PDK4 promoter contributed to the regulation of PDK4 by STAT5 activators in living cells, we performed site-specific mutagenesis to alter two bp within the murine PDK4 promoter/luciferase construct. We have shown that this mutation abolished binding of PRL-induced proteins to this site (data not shown). Transfection of the wild-type and mutant constructs into 3T3-L1 cells revealed that the basal level of luciferase activity was not significantly affected by mutation of the STAT5A site that we identified in the
Figure 2.7 Prolactin induces binding to a potential STAT site at -389 to -378 of the mouse PDK4 promoter in vitro. Cytosolic and nuclear extracts were prepared from differentiated serum deprived 3T3-L1 adipocytes that were untreated or treated with PRL for 15 min. For each sample, 10 μg of protein were incubated with 50,000 cpm/ml of a -389 to 378 32P-labeled probe of the murine PDK4 promoter. The protein-DNA complexes were resolved by EMSA. For cold competition, nuclear extracts were preincubated with an excess of the indicated unlabeled oligonucleotides (10 μM). The PRL-sensitive shift is indicated with an arrow. This is a representative experiment independently performed three times.
Figure 2.8 STAT 5A, but not STATs 1 or 3, was present in the binding complex induced by Prolactin in vitro. Cytosolic and nuclear extracts were prepared from 3T3-L1 adipocytes treated with PRL for 15 min. For supershift analysis, 10 μg of protein were preincubated with 4 μg of the indicated antibody and then incubated with 50,000 cpm/ml of the indicated 32P-labeled probe of the -389 to -378 site in the mouse PDK4 promoter. The protein-DNA complexes were resolved by EMSA. This is a representative experiment independently performed two times.

PDK4 promoter (Figure 2.9). In addition, a 2-4 fold increase in luciferase activity was observed following a 2 hour PRL or GH (porcine) treatment and measurement of the wild-type construct. In addition, the construct containing the mutated STAT5 site was unresponsive to induction by GH or PRL. Thus, these data clearly indicate that the -389 to -378 site of the PDK4 promoter is sensitive to GH and PRL, and these data also suggest that this site confers the positive regulation of PDK4 by GH- and PRL-activated STAT5A protein complexes.

The expression and modulation of PDK4 in skeletal muscle is well documented, yet, the induction of PDK4 in adipocytes has not been previously observed. Because our studies indicate that PRL increased PDK4 mRNA levels, we hypothesized that PDK4 protein levels might be increased following PRL treatment. Hence, we exposed fully differentiated serum deprived 3T3-
Figure 2.9 Prolactin and growth hormone induce the activity of the mouse PDK4 promoter in 3T3-L1 cells via a STAT5 binding site. Proliferating 3T3-L1 cells were transiently transfected with murine PDK4 promoter (-949 to +24)/luciferase constructs (wildtype or mutant) and the TK/renilla vector to control for transfection efficiency. After 48 hours of transfection, cells were stimulated with PRL or GH for two hours. Relative light units (RLU) were calculated by dividing firefly luciferase activity by renilla luciferase activity. Results are shown as +/- standard deviation. For each experiment, three plates of cells were used for each particular condition. In addition, each experiment was performed in triplicate on three independent batches of cells with similar results.

L1 adipocytes to PRL and isolated whole cell extracts to examine PDK4 expression. Chronic treatments (22 hours or more) did not result in any substantial changes in PDK4 protein levels (data not shown). Since the effects of STATs on gene expression are usually transient, we examined the ability of shorter PRL treatments to modulate PDK4 expression. As shown in Figure 2.10, we observed a significant induction of PDK4 protein levels following a 6 and 8 hour treatment with PRL. After 24 hours, this induction was not substantial. We did observe some fluctuation in the time in which prolactin-induced levels of PDK4 protein. In some experiments, we observed a substantial induction at 4 hours whereas in most experiments, the levels of PDK4 protein were the greatest after an 8-10 hour treatment. However, in all of our experiments, there
was no significant effect observed after a 22 hour PRL treatment. The efficacy of the PRL treatment was demonstrated by showing the activation of STAT5 and ERKs 1 and 2 (active MAPK).

Because PRL induced PDK4 proteins levels, we hypothesized that glucose uptake might be regulated under these conditions. The results in Figure 2.11 demonstrate that a 10 minute treatment with insulin results in a fivefold increase in glucose uptake in fully differentiated 3T3-L1 adipocytes. Also, a 6 hour treatment with PRL resulted in a substantial decrease (2.5 fold) in insulin-stimulated glucose uptake. The induction of PDK4 protein under these conditions was confirmed by Western Blot analysis (data not shown).

Figure 2.10 Prolactin induces the expression of PDK4 protein in 3T3-L1 adipocytes in a time dependent manner. Fully differentiated 3T3-L1 adipocytes were serum deprived overnight and then exposed to prolactin for the times indicated. One hundred μg of protein from whole cell extracts were loaded into the gel for each sample. The samples were subjected to SDS-PAGE, and then were transferred to nitrocellulose for immunoblot analysis. This is a representative experiment independently performed four times.
Figure 2.11 The effects of PRL on insulin stimulated glucose uptake in 3T3-L1 adipocytes. Serum deprived, fully differentiated 3T3-L1 adipocytes were untreated or incubated with 0.5 nM PRL for 6 hours. Cells were then exposed to 50 nM insulin or saline control for 10 minutes. Glucose uptake was measured as described in Experimental Procedures. Values shown represent the mean +/- S.E. (light bars = saline control, dark bars = insulin treatment) of triplicate determinations from four independent experiments.

2.4 Discussion

The novel findings in this study include data demonstrating that PDK4 levels are induced in adipocytes after stimulation with two STAT5 activating hormones, the identification of a hormone responsive region in the murine PDK4 promoter, and the characterization of a STAT5 binding site in this region. These results strongly suggest that STAT5A directly activates the expression of PDK4 in adipocytes. Moreover, our data indicate that PRL and STAT5A could induce conditions of insulin resistance by inducing PDK4 expression and attenuating insulin-stimulated glucose uptake.

Although GH is a well-known modulator of metabolism and is a known affector of insulin action (reviewed in (172))(191), the role of PRL in these processes is less understood.
PRL is primarily a pituitary secreted hormone, yet in humans, PRL is produced and secreted from additional tissues, such as human glandular and adipose breast tissues (192), and human decidua (193), and is a circulating hormone (reviewed in (194)). The presence of PRL receptors in almost all organs, including adipocytes (131), contributes to prolactin’s diverse roles. Recently, a novel human adipocyte cell line, LS14, has been developed that produces and responds to PRL (195). In morbidly obese patients, the amount of PRL secreted from subcutaneous adipose tissue is much less than in lean patients, but, surprisingly, there are no apparent differences in PRL secretion between men and women (reviewed in (194)).

Studies have also implicated PRL as a contributor to insulin resistance. One study conducted in lean pregnant women demonstrated that PRL could cause insulin resistance in adipocytes by suppressing adiponectin production and secretion (196). This mechanism is plausible, because low levels of adiponectin can be associated with obesity and insulin resistance. Although the crosstalk between PRL and leptin action is not well understood, PRL has been shown to suppress insulin-induced leptin secretion in cultured adipocytes (197). PRL also decreases insulin-stimulated glucose uptake in pregnant rat adipose tissue (198). Likewise, severe hyperprolactinemia has been shown to induce an insulin-resistant state by decreasing insulin binding in human subjects (199). Our data presents another plausible mechanism by which PRL may cause insulin resistance in adipocytes by modulating PDK4 levels.

Previous studies support similar or overlapping functions for PRL and GH in mammary gland metabolism (200). Our studies revealed that sheep PRL and porcine GH behaved similarly to modulate PDK4 levels in murine adipocytes. As is commonly known for PRL action, we observed a bell-shaped curve rather than linear dose-dependence relationship, and higher doses of the hormone were inhibitory. Interestingly, human GH had no substantial effects on PDK4,
despite its ability to weakly activate STAT5A and strongly activate ERKs 1 and 2. In multiple independent experiments, we demonstrated the induction of PDK4 by sheep PRL and porcine GH, but not human GH in 3T3-L1 adipocytes. This data is intriguing, since human GH did result in some activation of STAT5A, but did not induce PDK4 levels. A BLAST analyses revealed that human GH and mouse GH have a 69.7% similarity (58.3% identity). However, porcine GH and mouse GH have a 94.4% similarity (89.8% identity). Hence, we hypothesize that the human GH does not engage the murine receptor normally due to the substantial difference in homology. Alternatively, the activation of STAT5 by sheep PRL and porcine GH may include an additional factor(s) that participates in the induction of PDK4 expression, and this factor is not similarly modulated by human GH signaling in murine adipocytes.

Although it has been shown that insulin has the ability to suppress PDK4 expression in various other tissues (183-185), our studies demonstrate that insulin has the ability to suppress the PRL- and GH- induced PDK4 expression in adipocytes. Because the basal levels of PDK4 are low in adipocytes, we did not observe a strong repression of PDK4 mRNA following insulin treatment alone (Figure 2.6). Our studies provide evidence to support antagonistic cross-talk between PRL or GH and insulin, as well as the possible role of PRL in insulin resistance in adipose tissue. In addition, our results indicate that a 6 hour PRL treatment results in an inhibition of insulin-sensitive glucose uptake. This data is complemented by studies showing that reduced PDK4 expression is associated with increased insulin sensitivity (186). In summary, the presence of PDK4 protein in adipocytes correlates with an attenuation of insulin-sensitive glucose uptake and likely plays a role in the ability of prolactin to induce insulin resistance.

Studies have shown that other transcription factors, such as FOXO, also bind to the PDK4 promoter and induce PDK4 expression in response to starvation in the skeletal muscle of
mice (201). Glucocorticoids, which are implicated in insulin resistance, also increase the expression of PDK4, and insulin antagonizes these effects (185). Additional studies show that insulin suppresses the PDK4 induction mediated by glucocorticoids by inactivation of FOXO transcription factors, which are required for full transcriptional activation of the PDK4 gene by glucocorticoids (202). The transcriptional co-activator peroxisome proliferator-activated γ coactivator (PGC)1α has also been shown to induce the expression of PDK4 in primary rat hepatocytes and ventricular myocytes (203). In C2C12 myoblasts, PGC1α induces PDK4 expression in a manner that is dependent on ERRα (204). Hence, in muscle, the modulation of PDK4 can be influenced by FOXO transcription factors and PGC1α. Our studies demonstrate a novel induction of PDK4 in adipocytes that is mediated by a STAT 5A binding site in the mPDK4 promoter. Other studies from our laboratory have shown that adipocyte STAT 5A can inhibit the expression of fatty acid synthase (FAS) (153) and bind to the promoter of the acyl Co-A oxidase (AOX) gene, which correlates with an increase in AOX expression (151). Hence, the role of STAT5A in mature adipocytes appears to be one that attenuates lipid accumulation and favors lipid oxidation. Interestingly, our studies on PDK4 also suggest that STAT 5A may contribute to energy homeostasis by decreasing glucose oxidation and increasing insulin resistance in adipocytes. The differential capabilities of STAT 5A are apparent from several studies that demonstrate that in various preadipocytes or non-precursor cells that STAT5A can promote lipid accumulation and adipogenesis (144-146). Although the mechanisms that account for the ability of STAT 5A to have lipogenic or lipolytic effects have not been clearly defined, collectively these studies indicate that STAT 5A is likely an important contributor to maintenance of energy balance, particularly in response to hormones.
Because PDK inhibitors have been shown to be effective potential therapeutics in improving glycemic control and type 2 diabetes (reviewed in (205)), these kinases warrant more in-depth analysis. In particular, PDK4 proves to be an object of necessary consideration because it is expressed in substantial amounts in skeletal muscle and adipose tissue. We have observed that PRL and GH can induce the expression of PDK4 in adipocytes and positively regulate the murine PDK4 promoter. Our identification of a STAT5 binding site in the promoter of PDK4 characterizes a novel mechanism of regulating its expression. In summary, we hypothesize that the regulation of PDK4 by PRL and GH via STAT 5A is likely an important contribution to the maintenance of energy homeostasis.
CHAPTER 3

NEUROPOIETIN ATTENUATES ADIPOGENESIS AND INDUCES INSULIN RESISTANCE IN ADIPOCYTES

3.1 Introduction

In recent years, gp130 receptor ligands, notably ciliary neurotrophic factor (CNTF), have been implicated as potential targets for obesity therapeutics (reviewed in (162)). It is well known that obese individuals are usually leptin resistant, despite increased production of endogenous leptin (40)(reviewed in (206). Hence, leptin is not a viable cytokine therapeutic. Therefore, the characterization of cytokine-mediated anti-obesity mechanisms that act independently of leptin may be utilized for the treatment of obesity. Because gp130 and leptin receptor (LRβ) possess substantial sequence homology (207;208) and both activate the JAK/STAT pathway (209) and other signal transduction pathways that are important in energy balance (210-213), gp130 cytokines may provide a mechanism to evade leptin resistance. Also, recent studies have shown that CNTF can have anti-obesogenic effects in both high fat diet induced and db/db mice (214), indicating that the effects of CNTF are not dependent on a functional leptin receptor. In addition, leptin does not stimulate the tyrosine phosphorylation of gp130 to activate the JAK/STAT pathway (209). These data suggest that neither gp130 nor LRβ signal through each other’s ligand. Likewise, the efficacy of the gp130 cytokine as a therapeutic would not be altered due to the leptin resistant phenotype.

CNTF was originally characterized through its ability to maintain the survival of parasympathetic and motor neurons (reviewed in (215)). The anti-obesogenic properties of CNTF were discovered in clinical trials examining its effects on progression of the

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neurodegenerative disease amyotrophic lateral sclerosis (216). This observation led to many studies to understand the effects of CNTF on weight-loss. Early studies revealed that the CNTF receptors and LRβ were colocalized in the hypothalamus, and CNTF administration activated or suppressed the same genes as leptin in the arcuate nucleus (217). These studies suggested similar roles for these cytokines in neuronal signaling pathways that affect satiety. Also, CNTF treatment of leptin-deficient ob/ob and leptin-resistant db/db mice as well as mice with diet-induced obesity and partial resistance to leptin was found to reduce adiposity, hyperphagia, and hyperinsulinemia (217). Subsequent studies revealed that Axokine, a human recombinant form of CNTF, also improved the phenotype of obese, leptin resistant rodents (218). Collectively, these studies provide evidence to support the use of gp130 cytokines, specifically CNTF, as potential obesity therapeutics.

Though all gp130 cytokines share the transmembrane protein gp130 as a transducer in their receptor complex (reviewed in (157), the other components of each respective complex are variable. Interleukin-6 (IL-6) and interleukin-11 (IL-11) first bind to IL-6 receptor α and IL-11 receptor α, respectively, and then recruit a gp130 homodimer complex for signaling. Interleukin-27 (IL-27) engages a gp130/WSX-1 heterodimeric receptor complex (155). Leukemia inhibitory factor (LIF) and oncostatin M (OSM) directly bind the gp130/LIF receptor (LIFR) complex to induce signal transduction; OSM can alternatively utilize a gp130/OSM receptor (OSMR) complex. Cardiotrophin-1 (CT-1) recruits an alpha receptor (159), which to date has not been characterized, and also signals via gp130/LIFR. CNTF, cardiotrophin-like-cytokine (CLC), and neuropoietin (NP) all recruit CNTFRα and then induce the formation of a gp130/LIFR heterodimer for signal transduction (156;160;161). The requirement of NP to utilize CNTF
signaling receptors was supported by studies which showed that NP did not signal in cells expressing only LIFR and gp130, but not CNTFRα (156).

CNTF-deficient mice develop normally and exhibit only a mild loss of motor neurons later in adulthood (219), whereas newborn mice lacking CNTFRα display a dramatic loss of motor neurons, fail to initiate the feeding process, and die shortly after birth (220). These studies provided evidence for the existence of an alternative ligand for the CNTF receptor complex which led to the initial characterization of NP, a 22 kDa member of the gp130 cytokine family (156). Though not detected in adult tissues, NP is expressed in embryonic mouse neuroepithelia at a time during development when both CNTF and CLC are absent (156). NP, like CNTF, can also mediate motor neuron survival (156). Importantly, the actions of NP are dependent on the presence of the tripartite functional receptor complex for CNTF that includes CNTFRα, LIFR and gp130 (156;160). Due to the functional similarities between NP and CNTF and their utilization of the same tripartite receptor complex, we hypothesized that NP might mimic the anti-obesogenic properties of CNTF. We have previously shown that CNTF treatment induces STAT3 activation in adipocytes in vitro and in vivo and that CNTFRα is expressed in adipose tissue and regulated in obese/type II diabetic rats (165).

Because CNTF induces weight loss (216-218) and does not result in the development of insulin resistance in adipocytes (165) and NP utilizes the same tripartite receptor complex as CNTF (156), we examined the ability of NP to modulate adipocyte differentiation and insulin action. Although the roles of NP in the nervous system have been examined, the effects of NP on adipocytes have not been studied. Our results demonstrate that both 3T3-L1 adipocytes and rat adipose tissue are responsive to NP treatment. Our studies also indicate that NP specifically activates STAT3 Tyr705 in vivo and in vitro. We also observed the induction of SOCS-3 mRNA
in 3T3-L1 adipocytes and MAPK (ERK 1 and 2) activation in vivo following NP treatment. In addition, our studies demonstrate that NP can inhibit adipogenesis and negatively affect insulin signaling in cultured adipocytes. Overall, our findings indicate that adipocytes are highly responsive to NP. However, unlike CNTF, our results indicate that NP is not a suitable anti-obesity therapeutic since it inhibits insulin action in adipocytes.

3.2 Materials and Methods

Materials – Dulbecco’s Modified Eagle’s Media (DMEM) was purchased from Sigma. Bovine and fetal bovine (FBS) sera were purchased from Atlanta Biological. Mouse recombinant NP, mouse recombinant CT-1, mouse recombinant OSM, and rat recombinant CNTF were all purchased from R&D Systems. Mouse recombinant LIF was purchased from BD Transduction. GH (porcine) and insulin (bovine) were purchased from Sigma. STAT1 and STAT5A were polyclonal IgGs purchased from Santa Cruz. STAT3 was a monoclonal IgG purchased from BD Transduction. The phospho-specific monoclonal antibody for STAT3 (Y705) was purchased from BD Transduction. The highly phospho-specific polyclonal antibodies for IRS1 (Y896) and Akt (S473) were purchased from Biosource and Cell Signaling, respectively. The ERK polyclonal antibody and the PPARy monoclonal antibody were both purchased from Santa Cruz. The polyclonal Active MAPK antibody was purchased from Promega. Adiponectin polyclonal antibody was purchased from Affinity Bioreagents. DNase I and Trizol were both purchased from Invitrogen. Nitrocellulose and Zeta Probe-GT membranes were purchased from Bio-Rad. The BCA kit and the enhanced chemiluminescence kit were purchased form Pierce. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.
Cell Culture – Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in DMEM containing 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). After 48 h, this medium was replaced with DMEM supplemented with 10% FBS and cells were maintained in this medium until utilized for experimentation. In some experiments, cells were induced to differentiate in the presence of NP and treated at various times after the addition of the differentiation cocktail.

Preparation of Whole Cell Extracts – Cell monolayers of 3T3-L1 adipocytes were harvested in a non-denaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Igepal CA-630, 1 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate and frozen. Next, the samples were thawed and centrifuged at 13,000 rpm at 4°C for 10 minutes. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit according to the manufacturer’s instructions.

Preparation of Nuclear and Cytosolic Extracts – Untreated and NP treated serum deprived 3T3-L1 adipocytes were harvested in a nuclear homogenization buffer containing 20 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM MgCl2. Igepal CA-630 was added to a final concentration of 0.15%, and cells were homogenized with 16 strokes in a Dounce homogenizer. The homogenates were centrifuged at 10000 x g for 8 min. Supernatants were saved as cytosolic extract, and the nuclear pellets were resuspended in half the volume of nuclear homogenization buffer and were centrifuged as before. The pellet was then resuspended in an extraction buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 25%
glycerol. Both cytosolic and nuclear samples were frozen until time for analysis and later thawed at room temperature. Two hundred units of DNase I was added to each nuclear sample and the tubes were inverted and incubated an additional 10 min at room temperature. Finally, the samples were subjected to centrifugation at 10,000 x g at 4°C for 10 minutes. Cytosolic fractions and supernatants containing nuclear extracts were analyzed for protein content using a BCA kit (Pierce).

**Rodent Adipose Tissue Isolation** – Animals were euthanized by cervical dislocation, and tissues were immediately removed and frozen in liquid nitrogen. Frozen tissues were homogenized in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1mM EGTA, 1mM EDTA, 1% Triton-X 100, 0.5% Igepal CA-630, 1 μM PMSF, 1 μM pepstatin, and 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 minutes at 1000 x g to remove any debris and insoluble material and then analyzed for protein content. All animal studies were carried out with protocols which were reviewed and approved by institutional animal care and use committees.

**Gel Electrophoresis and Western Blot Analysis** – Proteins were separated in 7.5% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (187) and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked overnight in 4% milk at 4°C. Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence.

**RNA Analysis** – Total RNA was isolated from cell monolayers with Trizol according to the manufacturer’s instructions with minor modifications. For Northern blot analysis, 20 μg of total RNA was denatured in formamide and electrophoresed through a formaldehyde/agarose gel.
The RNA was transferred to Zeta Probe-GT, cross-linked, hybridized, and washed as previously described (43). Probes were labeled by random priming using Klenow fragment and $[^{32}\text{P}] \text{dATP}$.  

**Determination of $^3\text{H}$-labeled 2-Deoxyglucose Uptake** – The assay of 2- [$^3\text{H}$] deoxyglucose was performed as previously described (189). Prior to the assay, mature 3T3-L1 adipocytes were serum deprived for 2 h and then incubated in the presence or absence of insulin (50 nM) for 10 min and the presence or absence of NP for 6h. Glucose uptake was initiated by addition of 2-[$^3\text{H}$] deoxyglucose at a concentration of 0.1 mM 2-deoxyglucose in 1 $\mu\text{Ci}$ 2-[$^3\text{H}$] deoxyglucose in Krebs-Ringer-Hepes buffer and incubated for 3 min at room temperature. Glucose uptake is reported as [$^3\text{H}$] radioactivity, corrected for nonspecific diffusion (5 $\mu\text{M}$ cytochalasin B) and normalized to total protein content as determined by BCA analysis. Nonspecific uptake and absorption was always less than 10% of the total uptake. Uptake measurements were performed in triplicate under conditions where hexose uptake was linear.  

### 3.3 Results  

To determine if adipocytes were responsive to NP, we examined the ability of this gp130 cytokine to activate STAT proteins in adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with several different gp130 cytokines including CT-1, LIF, OSM, and NP for 10 minutes. As shown in Figure 3.1, NP activated STAT3 Tyr$^{705}$ phosphorylation in a manner equivalent to CT-1, LIF, and OSM. Because growth hormone (GH) is not a gp130 cytokine and is not a potent activator of STAT3 tyrosine phosphorylation in these cells (164), this growth factor was used as a negative control. The levels of STAT3 protein were equivalent in each sample.
Figure 3.1 Neuropoietin activates STAT3 as potently as other gp130 cytokines. Fully differentiated 3T3-L1 adipocytes were treated for 10 min with either CT-1 (1 nM), LIF (1 nM), OSM (1 nM), NP (1 nM), or GH (.5 nM). Following the treatment, whole cell extracts were prepared, and one hundred fifty μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The detection system was HRP-conjugated secondary antibodies and enhanced chemiluminescence. This is a representative experiment independently performed three times.

To investigate the specificity of NP action in adipocytes, serum-deprived fully differentiated 3T3-L1 adipocytes were treated with various doses of NP (0.12 nM, 0.25 nM, 0.5 nM, 1 nM, 2 nM, and 2.5 nM) or insulin for 10 min. Although we detected low levels of STAT3 activation with 0.25 nM and 0.5 nM NP, we observed substantial activation with 1 nM, 2 nM, and 2.5 nM NP treatments (Figure 3.2). These results indicate that NP activated STAT3 Tyr\(^{705}\) in a dose-dependent manner, with maximal activation at 1 nM.

Figure 3.2 Neuropoietin activates STAT3 in a dose-dependent manner. Fully differentiated 3T3-L1 adipocytes were treated for 10 min with the various doses of NP or Insulin (50 nM). Following the treatment, whole cell extracts were prepared, and one hundred fifty μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
Because insulin induces the serine, but not the tyrosine, phosphorylation of STAT3 (164;221), this peptide hormone was used as a negative control. ERK (MAPK) protein levels are shown to demonstrate even loading.

Previous studies have demonstrated that, in addition to STAT3, STATs 1, 5A, 5B, and 6 are expressed in both cultured and native adipocytes (139). Therefore, we examined the ability of NP to activate other adipocyte expressed STATs. Fully differentiated 3T3-L1 adipocytes were treated with NP for different times, ranging from 0.3 to 5 h. The cells were homogenized and fractionated into nuclear and cytosolic extracts. The activation of STATs was observed by examining the presence of these proteins in the nucleus. Based on the results in Figure 3.3, NP did not activate STATs 1 and 5A, as the levels of these STATs in the nucleus of NP treated cells were similar to untreated control cells. In contrast, we observed a robust time dependent activation and nuclear translocation of STAT3.

Because various members of the gp130 cytokine family are known to induce Suppressors of Cytokine Signaling (SOCS)-3 (222), we examined the ability of NP to induce SOCS-3 expression in fully differentiated 3T3-L1 adipocytes. As shown in Figure 3.4, NP treatment resulted in a transient induction of SOCS-3 mRNA that was detectable at 30 minutes and maximum expression occurred after a 1 hr treatment. Both aP2 and β-actin mRNA levels were unaffected by the NP treatments. Ribosomal RNAs are shown to demonstrate even loading of total RNA.

The data in Figures 3.1-3.4 clearly indicated that NP activates STAT3 and SOCS-3 in adipocytes in vitro; hence, we wanted to determine if adipocytes in vivo were responsive to NP. Sprague Dawley rats were injected with either NP (0.20 μg/g animal) or vehicle (saline) and then sacrificed 15 minutes following the injection. As shown in Figure 3.5, NP injection induced the
Figure 3.3 Neuropoietin induces the translocation of STAT3 to the nucleus. Cytosolic and nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes following a treatment with 1 nM NP for the times indicated. One hundred fifty μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Each figure represents an experiment independently performed three times.

Activation of STAT3 Tyr<sup>705</sup> in both the epididymal and retroperitoneal adipose tissues, but not in skeletal muscle. NP also induced the activation of MAPK (ERKs 1 and 2) in adipose tissue, but not skeletal muscle. The inability of NP to induce STAT3 tyrosine phosphorylation in skeletal muscle was not due to decreased levels of STAT3 expression.

Since gp130 cytokines have been reported to have differential effects on adipogenesis (166;168;223), we investigated the ability of NP to modulate adipocyte differentiation. As shown in Figure 3.6, 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of exogenously added NP and whole cell extracts were harvested at the indicated time points. Adipogenesis was assessed by examining the induction of PPARγ and adiponectin. NP attenuated adipogenesis, as evidenced by the decrease in expression of both PPARγ and adiponectin at 72 h and 96 h after the induction of differentiation.
Figure 3.4  Neuropoietin induces SOCS-3 mRNA. Fully differentiated 3T3-L1 adipocytes were treated with 1 nM NP for the times indicated. Following the treatment total RNA was collected from the cells and twenty μg of each total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis. This is a representative experiment independently performed three times.

Figure 3.5  Neuropoietin induces STAT3 tyrosine phosphorylation in adipose tissue in vivo. Six week old male Sprague Dawley rats were given an intraperitoneal injection of NP (0.20 μg/g animal) or vehicle (saline) control. In each individual experiment, at least two animals were used for each condition. Fifteen minutes after the injection the rats were sacrificed and both retroperitoneal and epididymal fat pads and skeletal muscle tissues were immediately removed and frozen in liquid nitrogen. Two hundred fifty μg of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed three times.
Figure 3.6 Neuropoietin attenuates the differentiation of 3T3-L1 adipocytes. Whole cell extracts were prepared from 3T3-L1 preadipocytes or 3T3-L1 cells induced to differentiate in the presence (+) or absence (-) of NP (1 nM). Cells were treated every 24 h with a fresh bolus of NP. Two hundred μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

We also examined the ability of NP to modulate adipogenesis in the absence of insulin. The preadipocytes were induced to differentiate with normal differentiation media (MDI) or media minus insulin (MD) and harvested 96 h post induction. As shown in Figure 3.7, NP was capable of blocking the induction of both PPARγ and adiponectin without insulin present in the induction cocktail. Because cells treated with MD media exhibited less differentiation, we used a greater protein concentration than in the normally differentiated cells.

To compare the effect of NP to other gp130 cytokines, 3T3-L1 preadipocytes were induced to differentiate in the presence of NP, as well as CNTF, CT-1, OSM, and GH. As shown in Figure 3.8, both NP and OSM greatly attenuated adipogenesis as shown by the decreased expression of PPARγ and adiponectin, while CNTF and CT-1 had no effect on adipocyte marker expression and was similar to untreated cells. We have previously shown that CT-1 (166) and CNTF (165) do not inhibit adipogenesis. These results also confirm recent
studies showing that OSM can inhibit murine (223) and human (224) adipocyte differentiation. It is also known that GH does not affect the adipogenesis of 3T3-L1 cells (225). STAT3 expression does not change during adipogenesis of murine (142) or human (140) cells and was used as a loading control.

![Western blot analysis](image)

Figure 3.7 Neuropoietin attenuates the differentiation of 3T3-L1 adipocytes in the presence or absence of insulin. 3T3-L1 cells were induced to differentiate in the presence (+) or absence (-) of NP (1 nM). Cells were treated every 24 h with a fresh bolus of NP. Two samples were induced to differentiate with normal (MDI) cocktail, while the other samples were induced with differentiation cocktail minus insulin (MD). Whole cell extracts were harvested 96 h after the induction of differentiation. Ninety μg of samples with normal MDI and two hundred μg of samples with MD media were subjected to Western blot analysis. This is a representative experiment independently performed three times.

To demonstrate the specificity of the ability of NP to negatively affect adipogenesis, we investigated whether the attenuation was dose and time dependent. In Figure 3.9, 3T3-L1 preadipocytes were induced to differentiate in the presence of NP at the various doses indicated and harvested 4 days after the induction of adipogenesis. Cells were treated every 24 h with a fresh bolus of NP. Although there were no changes in protein expression at .25 nM NP, we observed a decrease in PPARγ and adiponectin expression at 0.5 nM, with the greatest attenuation of adiponectin at 1 nM and PPARγ at 2 nM.
Figure 3.8 Neuropoietin and Oncostatin M attenuate the differentiation of 3T3-L1 adipocytes. Whole cell extracts were prepared from 3T3-L1 preadipocytes or 3T3-L1 cells induced to differentiate in the presence of either NP (1 nM), CNTF (1 nM), CT-1 (1 nM), OSM (1 nM), or GH (0.5 nM). Cells were treated every 24 h with a fresh bolus of each specific cytokine or growth factor. Two hundred μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Each figure represents an experiment independently performed at least three times.

In Figure 3.10, 3T3-L1 preadipocytes were induced to differentiate in the presence of NP at different times post the induction of adipogenesis. Cells were treated every 24 h with a fresh bolus of NP after their initial treatment. Our data indicated that a decrease in expression of adipogenic markers occurred when NP was added between 0 h and 48 h post induction. When NP was added 72 h or 96 h post induction, we did not observe a change in PPARγ or adiponectin levels. The efficacy of NP was assessed by examining the activation of STAT3 Tyr705, and STAT3 protein levels were observed to demonstrate even loading in both experiments.

Because other gp130 cytokines have been reported to have effects on insulin signaling, we examined how NP affects insulin action. As shown in Figure 3.11, mature 3T3-L1 adipocytes were pretreated with NP (+) for the times indicated, and then exposed to an acute 10 min treatment of insulin. As expected, a 10 min insulin treatment resulted in the activation of
Figure 3.9 Neuropoietin inhibits adipogenesis in a dose dependent manner. Whole cell extracts were prepared from 3T3-L1 preadipocytes or 3T3-L1 cells induced to differentiate in the presence of NP at the various doses indicated. Cells were treated every 24 h with a fresh bolus of NP. One hundred fifty μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

IRS-1 Tyr$^{896}$ and Akt Ser$^{473}$, which are substrates and indicators of insulin signaling. However, pretreatment with NP prior to insulin stimulation resulted in a decrease in IRS-1 Tyr$^{896}$ signaling at 10 min, and we observed this inhibition with each NP pretreatment. Although we did not see a decrease in Akt Ser$^{473}$ signaling after the 10 min NP pretreatment, we observed a significant attenuation in Akt activation after 2 hrs with NP pretreatment. The efficacy of the NP was demonstrated by confirming the activation of STAT3 Tyr$^{705}$. A longer exposure of the film revealed lower levels of STAT3 phosphorylation following longer NP pretreatment. ERK (MAPK) protein levels were examined to demonstrate even loading.

Because NP negatively affects insulin signaling in adipocytes, we examined the effects of NP on insulin signaling in preadipocytes. As shown in Figure 3.12, confluent 3T3-L1
Figure 3.10 Neuropoietin inhibits adipogenesis in a time dependent manner. Whole cell extracts were prepared from 3T3-L1 preadipocytes or 3T3-L1 cells induced to differentiate in the presence of NP (1 nM) at different times post MDI. Cells were treated every 24 h with a fresh bolus of NP after their initial treatment. One hundred fifty μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Each figure represents an experiment independently performed three times.

preadipocytes were pretreated with NP (+) for the times indicated, and then exposed to an acute 10 min treatment of insulin. Although a 10 min insulin treatment resulted in the activation of IRS-1 Tyr<sup>896</sup>, pretreatment with NP for various times prior to insulin stimulation resulted in a decrease in IRS-1 Tyr<sup>896</sup> phosphorylation. The efficacy of NP was demonstrated by observing the activation of STAT3 Tyr<sup>705</sup>. ERK (MAPK) protein levels demonstrate even loading.

Since we observed a decrease in insulin signaling with NP pretreatment, we examined the ability of NP to regulate insulin-stimulated glucose uptake in 3T3-L1 adipocytes. As shown in Figure 3.13, a 10 min treatment with insulin resulted in a nearly 5-fold increase in glucose uptake as compared to the vehicle treated cells. However, following a 6 h pretreatment with NP, we observed a substantial decrease in insulin-stimulated glucose uptake compared with controls. The lack of insulin stimulated uptake was not due to a significant increase in basal glucose uptake.
Figure 3.11 Neuropoietin inhibits insulin signaling. Fully differentiated 3T3-L1 adipocytes were pre-treated with NP (1 nM) for the times indicated. Next, the cells were then treated for 10 min with insulin (50 nM). Following the treatment, whole cell extracts were prepared, and one hundred μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed three times.

Figure 3.12 Neuropoietin inhibits insulin signaling in preadipocytes. Confluent 3T3-L1 preadipocytes were pre-treated with NP (1 nM) for the times indicated. Next, the cells were treated for 10 min with insulin (50 nM). Following the treatment, whole cell extracts were prepared, and one hundred twenty μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed three times.
Figure 3.13  Neuropoietin inhibits insulin-stimulated glucose uptake. Fully differentiated 3T3-L1 adipocytes were serum deprived for 2 h and then treated with NP (1 nM) for 6 h. After the pretreatment, cells were stimulated with insulin (50 nM) for 10 min. Glucose uptake was initiated by addition of 2-[\(^{3}\)H] deoxyglucose. The glucose uptake values shown represent the mean +/- S.E. of triplicate determinations from four independent experiments.

The data in Figures 3.11-3.13 clearly indicated that NP negatively affects insulin signaling in adipocytes in vitro; therefore, we wanted to determine if NP could attenuate insulin signaling in adipose tissue in vivo. C57B1/6J mice were injected with either vehicle (saline) or insulin, with or without a pre-injection of NP. The mice were treated for 15 min with insulin alone or following either a 30 min or 90 min NP pretreatment.

As shown in Figures 3.14 and 3.15, a 15 min insulin treatment resulted in the activation of Akt Ser\(^{473}\). Although we did not see a decrease in Akt Ser\(^{473}\) signaling after the 30 min NP pretreatment, we observed a significant attenuation in Akt activation after 1.5 h with each NP pretreatment in both the epididymal and retroperitoneal adipose tissues. The efficacy of NP was assessed by the activation of STAT3 Tyr\(^{705}\), and ERK (MAPK) protein levels were observed to demonstrate even loading.
Figure 3.14 Neuropoietin attenuates insulin signaling in adipose tissue in vivo. Seven-week old male C57B1/6J mice were given an intraperitoneal injection of either vehicle (saline) control, or insulin (5U/kg), with or without a pre-injection of NP (0.20 µg/g animal). Two animals were used for each condition. The mice were treated with insulin alone or following either a 30 min or 90 min NP pretreatment. After the mice were injected with insulin for 15 min, they were sacrificed, and epididymal fat pads were immediately removed and frozen in liquid nitrogen. Two hundred fifty µg of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed twice on separate groups of mice.

Figure 3.15 Neuropoietin attenuates insulin signaling in adipose tissue in vivo. Seven-week old male C57B1/6J mice were given an intraperitoneal injection of either vehicle (saline) control, or insulin (5U/kg), with or without a pre-injection of NP (0.20 mg/g animal). Two animals were used for each condition. The mice were treated with insulin alone or following either a 30 min or 90 min NP pretreatment. After the mice were injected with insulin for 15 min, they were sacrificed, and retroperitoneal fat pads were immediately removed and frozen in liquid nitrogen. One hundred fifty µg (retroperitoneal) of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed twice on separate groups of mice.
3.4 Discussion

Due to recent studies that implicate gp130 cytokines, specifically CNTF, as potential therapeutic targets for obesity, we investigated the actions of NP in adipocytes both in vitro and in vivo. Even though sequence analysis suggests that the np gene has evolved into a pseudogene in humans (156), we hypothesized that NP, due to similar functional roles with CNTF, could induce anti-obesogenic effects and be considered a potential therapeutic treatment. Although NP signals via the same tripartite receptor complex as CNTF, our findings demonstrate both similar and opposing roles for NP as compared to CNTF. In adipocytes, both NP and CNTF activate STAT3 Tyr\(^{705}\) in vivo and in vitro and induce the nuclear translocation of STAT3 but not STATs 1 and 5A in vitro. Our studies also indicate that NP treatment, like CNTF, resulted in ERKs 1 and 2 activation in vivo (209). As shown in Figure 3.4, NP and CNTF (166) are also alike in their ability to induce SOCS-3 mRNA in 3T3-L1 adipocytes. SOCS-3 proteins have been shown to play a role in both leptin resistance (226) and insulin resistance (227). Interestingly, SOCS-3 is induced by all known gp130 cytokines (166;222;226;228-231), as well as additional hormones, including insulin (232), GH (233), and leptin (226). However, many inducers of SOCS-3, including CTNF, do not induce insulin resistance. It is striking that so many cytokines, in addition to NP and CNTF result in the activation of STAT3, ERKs 1 and 2 and SOCS-3, yet the action of these cytokines have different responses despite the activation of the same signaling proteins.

Although our observations indicated that NP induced the same signaling proteins as CNTF in adipocytes, we observed different effects of NP, as compared to CNTF, on both adipogenesis and insulin action. Our results demonstrated that NP can inhibit adipogenesis in a dose and time dependent manner in 3T3-L1 cells, whereas CNTF does not attenuate
adipogenesis in this model system (165). The different effects do not appear to be due to altered levels of STAT3 activation as equivalent levels of NP and CNTF results in similar activation of STAT3 (data not shown). CT-1, LIF, and OSM activate STAT3 in a manner equivalent to NP (Figure 3.1), but only OSM and NP inhibit differentiation (Figure 3.8). In addition, the ability of NP to block adipogenesis does not seem to be dependent on the presence of insulin or the negative effects on insulin action. Although NP can attenuate insulin signaling in both preadipocytes (Figure 3.12) and adipocytes (Figure 3.11), our data indicate that NP could inhibit adipocyte differentiation in the absence of insulin (Figure 3.7). Of all the gp130 cytokines, NP is the most structurally and functionally related to CNTF and CT-1, yet neither CNTF nor CT-1 attenuate adipogenesis (165;166). These observations suggest the specificity of these gp130 cytokines to modulate adipocyte differentiation involves more than their ability to activate STAT3 and ERK and induce SOCS-3 expression and indicate that NP possesses unique signaling abilities that differ from CNTF despite use of the same receptor complex.

Despite the presence of CNTFRα in skeletal muscle (160), our results demonstrate that acute NP treatment failed to activate STAT3 Tyr705 in skeletal muscle in vivo, in contrast to CNTF which induced STAT3 Tyr705 phosphorylation in the skeletal muscle of rodents (165). In addition, CNTF has been shown to increase fat oxidation and reduce insulin resistance via an AMP-activated protein kinase (AMPK)-dependent mechanism in skeletal muscle (214). It is possible that NP could also mediate signal transduction in skeletal muscle via other signaling pathways that are independent of the JAK/STAT pathway, as we did not test this hypothesis in our experiments.

Importantly, our studies indicate that NP treatment not only attenuates insulin signaling in vitro, as demonstrated by decreased activation of both IRS-1 Tyr896 and Akt Ser473, but also in
vivo, as evidenced by the reduction in Akt activation. In comparison, CNTF treatment was shown to act synergistically with insulin to increase the activation of both IRS-1 Tyr\textsuperscript{896} and Akt Ser\textsuperscript{473} in cultured adipocytes (165) and in vivo studies demonstrated that CNTF could improve insulin sensitivity in diet-induced and genetic models of obesity (214). In addition to our findings that NP negatively regulates insulin signaling proteins, we demonstrated that NP treatment impaired insulin-stimulated glucose uptake in cultured adipocytes. Because CNTF treatment of cultured adipocytes did not result in insulin resistance (165), these results clearly indicate significant and opposing roles between NP and CNTF.

This represents the first study to examine the role of NP in adipocytes. Interestingly, we observed distinct responses between NP and CNTF in adipocytes and skeletal muscle despite studies which showed that both of these cytokines act through the same signaling receptor complex (156;160). In summary, our findings indicate that NP can affect adipogenesis and insulin sensitivity in fat cells both in vitro and in vivo. Based on our present data, NP, unlike CNTF, is not a suitable anti-obesity therapeutic since it inhibits insulin action and negatively affects glucose uptake in adipocytes.
4.1 Introduction

The Interleukin (IL)-6 family of cytokines is a group of functionally and structurally related proteins that consists of Interleukin-6 (IL-6), Interleukin-11 (IL-11), Interleukin-27 (IL-27), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), novel neurotrophin-1/B cell stimulating factor-3 or cardiotrophin-like cytokine (CLC), and neuropoietin (NP) (reviewed in (234)). Though originally identified as having distinct properties, these cytokines often have pleiotropic and redundant biological effects (reviewed in (235)). The IL-6 cytokine family regulates a variety of complex cellular processes, such as hematopoiesis, immune response, inflammation, differentiation, mammalian reproduction, cardiovascular action, and neuronal survival (reviewed in (157)). Their signal transduction involves the activation of the JAK/STAT pathway, typically activating STAT3 and, to a lesser extent, STAT1. In addition, the MAPK (ERK) pathway can be activated.

Because all members of this family utilize glycoprotein 130 as a common signal transducer within their receptor complex for signaling, the IL-6 family is commonly referred to as the gp130 cytokines (reviewed in (235)). The shared usage of signal transducers in the receptor complexes may account for the functional redundancy of gp130 cytokines. In addition, gp130 is ubiquitously expressed in every cell type, which allows for the pleiotropic nature of these cytokines in a variety of cells. Although gp130 cytokines are highly redundant in mediating biological functions, the differences in their activity reside in the additional receptor components utilized by each member. These cytokines are differentially regulated in multiple tissues, due to the tissue-specific expression of other receptor components that are required for
their activation, including the LIF receptor (LIFR) and the cytokine’s specific α receptor. Likewise, the responsiveness of a given cell type to these cytokines is tightly regulated by the presence of the cytokine-specific receptor complex, and specific characteristics emerge for each cytokine. IL-6 and IL-11 first bind to the IL-6 receptor α and IL-11 receptor α (158), respectively, and then the complex associates to a gp130 homodimer complex for signaling. IL-27 engages a gp130/WSX-1 heterodimeric receptor complex (155). LIF and OSM require the gp130/LIF receptor (LIFR) complex to mediate signal transduction; OSM can alternatively utilize a gp130/OSM receptor (OSMR) complex. CT-1 recruits an alpha receptor (159), which to date has not been characterized, and also signals via gp130/LIFR. CNTF, CLC, and NP all recruit CNTFRα and then induce the formation of a gp130/LIFR heterodimer for signal transduction (156;160;161).

In recent years, studies have shown that several gp130 cytokines activate both the JAK/STAT and MAPK (ERKs 1 and 2) signaling pathways in adipocytes (154;163-167). Findings from our laboratory indicate that several gp130 cytokines have differential effects on adipogenesis (166-168), as well as insulin-stimulated glucose uptake (165-168). However, their functions, as well as their complex signaling cascades, in adipose tissue have been only partially elucidated. Previous studies from our lab indicate that gp130 cytokines have the unique ability to crosstalk with other members of the gp130 family to alter one another’s signaling (169), and this inhibition of signaling correlates with the degradation of the LIFR and its loss at the plasma membrane. In this follow-up study, our novel data demonstrate that NP, a gp130 cytokine characterized as signaling via the LIFR, does not have the capability to crosstalk with other gp130 family members. Several gp130 cytokines have the ability to block NP signaling, but NP does not inhibit the signaling of other gp130 family members because it does not result in LIFR
phosphorylation and degradation. We also demonstrated that mOSM crosstalks in a distinct manner from hOSM, which can be attributed to LIFR activation. Collectively, our data show that gp130 cytokines exert differential crosstalk signaling capabilities in both cultured adipocytes in vitro and adipose tissue in vivo, and the inhibitory crosstalk is not dependent on new protein synthesis or the activation of MAPK.

4.2 Materials and Methods

**Materials** – Dulbecco’s Modified Eagle’s Media (DMEM) was purchased from Sigma. Bovine and fetal bovine (FBS) sera were purchased from Atlanta Biological. Mouse recombinant NP, mouse recombinant CT-1, mouse recombinant OSM, mouse recombinant OSMRβ antibody were all purchased from R&D Systems. Recombinant human OSM was purchased from Invitrogen, and mouse recombinant LIF was purchased from BD Transduction. Cycloheximide was purchased from Sigma, and 4G10 was purchased from Upstate. Both STAT3 and phospho-specific STAT3 (Y705) monoclonal antibodies were purchased from BD Transduction. Both the MAPK (ERK) and LIFR polyclonal antibodies and the PPARγ monoclonal antibody were all purchased from Santa Cruz. The polyclonal anti-active MAPK (ERK1/2) antibody and U0126 inhibitor were purchased from Promega. Protein-A agarose was purchased from Repligen Corporation. Nitrocellulose was purchased from Bio-Rad. The BCA kit and the enhanced chemiluminescence kit were purchased from Pierce. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

**Cell Culture** – Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in DMEM containing 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). After 48 h,
this medium was replaced with DMEM supplemented with 10% FBS and cells were maintained in this medium until utilized for experimentation.

**Preparation of Whole Cell Extracts** – Cell monolayers of 3T3-L1 adipocytes were harvested in a non-denaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Igepal CA-630, 1 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate, and then frozen. Next, the samples were thawed and centrifuged at 10,000 x g at 4°C for 10 minutes. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit according to the manufacturer’s instructions.

**Rodent Adipose Tissue Isolation** – Animals were euthanized by cervical dislocation, and tissues were immediately removed and frozen in liquid nitrogen. Frozen tissues were homogenized in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Igepal CA-630, 1 μM PMSF, 1 μM pepstatin, and 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 minutes at 1,000 x g to remove any debris and insoluble material and then analyzed for protein content. All animal studies were carried out with protocols which were reviewed and approved by institutional animal care and use committees.

**Immunoprecipitations (IPs) of LIFR or OSMRβ from Adipocyte Extracts** – Cell monolayers of 3T3-L1 adipocytes were harvested under non-denaturing conditions following treatment with respective cytokines, and the protein content of the whole-cell extracts was analyzed as described above. The protein extracts were preincubated with protein-A agarose, and the resulting supernatants were then incubated with 5 μg of either the LIFR antibody or the OSMRβ antibody for 2 hours at 4°C. Protein-A agarose was added to the mixture, and the samples were rotated for
an additional hour. Bound LIFR or OSMRβ and any associated proteins were isolated by pelleting this mixture. The pellets were rinsed twice with phosphate-buffered saline (PBS), and the bound proteins were eluted from the agarose by incubation at 100°C for 10 minutes after the addition of Laemmli sample buffer. These samples were separated by sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis and analyzed by Western blotting with 4G10 antibody.

**Gel Electrophoresis and Western Blot Analysis** – Proteins were separated in 7.5% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (187) and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked overnight in 4% milk at 4°C. Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence.

### 4.3 Results

Previous studies from our laboratory demonstrated that gp130 cytokines have the ability to crosstalk with other members in the gp130 family, which results in altering of subsequent signaling (169). In recent studies, we have shown that a newly discovered gp130 cytokine, Neuropoietin (NP), can activate STAT3 tyrosine phosphorylation in adipocytes both in vitro and in vivo (167). Therefore, we wanted to examine the ability of NP to modulate the signaling of other gp130 cytokines. In addition, previous crosstalk studies were performed using human oncostatin M (hOSM). Evidence shows that murine OSM (mOSM) signals differently than hOSM in murine cells and utilizes the OSM receptor β (OSMRβ), as opposed to the leukemia inhibitory factor receptor (LIFR), in the receptor complex (236). Hence, we wanted to confirm these observations in 3T3-L1 cells and examine the ability of mOSM to crosstalk with other
gp130 family members. We pretreated fully differentiated 3T3-L1 adipocytes with several different gp130 cytokines (CT-1, LIF, and NP) for 6-hr, followed by an acute 10 min treatment with CT-1, LIF, mOSM, or NP. To ensure the efficacy of each cytokine, the activation of STAT3 Tyr\textsuperscript{705} was observed with no pretreatment. As shown in Figures 4.1-4.2, a 6-hr pretreatment with CT-1 or LIF completely blocked the STAT3 activation induced by a 10 min treatment of CT-1, LIF, or NP, but did not attenuate mOSM. In addition, we observed the same pattern of inhibition in the activation of MAPK (ERKs 1 and 2). Our data from Figure 4.3 demonstrate that STAT3 activation by CT-1, LIF, and mOSM was not attenuated by a 6-hr NP pretreatment. Therefore, several gp130 cytokines have the ability to block NP signaling, but NP does not inhibit the signaling of other gp130 family members. ERK (MAPK) protein levels are shown to demonstrate even loading in each experiment.

![Figure 4.1 CT-1 pretreatment inhibits CT-1, LIF, and NP signaling, but not mOSM, in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes received no pretreatment or were preincubated with 1 nM CT-1 for 6 hr. Next, the cells were treated for 10 min with 1 nM CT-1, 1 nM LIF, 1 nM mOSM, or 1 nM NP. After the acute treatment, whole cell extracts were prepared, and 150 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently four times.](image-url)
Figure 4.2 LIF pretreatment inhibits CT-1, LIF, and NP signaling, but not mOSM, in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes received no pretreatment or were preincubated with 1 nM LIF for 6 hr. Next, the cells were treated for 10 min with 1 nM CT-1, 1 nM LIF, 1 nM mOSM, or 1 nM NP. After the treatment, whole cell extracts were prepared, and 150 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently four times.

Figure 4.3 NP pretreatment does not inhibit signaling of other gp130 cytokines. Fully differentiated 3T3-L1 adipocytes received no pretreatment or were preincubated with 1 nM NP for 6 hr. Next, the cells were treated for 10 min with 1 nM CT-1, 1 nM LIF, 1 nM mOSM, or 1 nM NP. After the treatment, whole cell extracts were prepared, and 150 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently four times.

In order to observe the differences in crosstalk signaling between mOSM and hOSM and other gp130 cytokines, we pretreated with CT-1 or LIF for 6 hr, followed by a 10 min treatment with either mOSM or hOSM. The activation of STAT3 Tyr\textsuperscript{705} by acute 10 min treatments of each cytokine, with no pretreatment, was observed to show efficacy of signaling. Our results show that neither a CT-1 nor a LIF pretreatment inhibited mOSM STAT3 activation; however,
both CT-1 and LIF attenuated hOSM signaling, as evidenced by the decrease in STAT3 Tyr705. ERK (MAPK) protein levels are shown to demonstrate even loading.

Figure 4.4 CT-1 and LIF attenuate hOSM signaling, but not mOSM signaling. Fully differentiated 3T3-L1 adipocytes were pretreated with 1 nM CT or 1 nM LIF for 6-hr. Next, the cells were treated for 10 min with 1 nM mOSM, or 1 nM hOSM. After the treatment, whole cell extracts were prepared, and 160 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently two times.

Data from Figures 4.1-4.4, as well as other studies (169), demonstrate that the gp130 cytokines CT-1, LIF, and hOSM crosstalk to inhibit one another’s signaling; however, both NP and mOSM have distinct crosstalk capabilities. In order to further examine the crosstalk ability of mOSM, hOSM, and NP, we pretreated 3T3-L1 adipocytes with these cytokines for 6-hr. Next, we treated the cells with NP, mOSM, or hOSM for 10 min. Our data in Figure 4.5 show that hOSM, but not mOSM, inhibited NP signaling, as evidenced by the decreased activation of STAT3 Tyr705 and MAPK. Conversely, NP did not attenuate either mOSM or hOSM; only NP signaling was blocked. ERK (MAPK) protein levels are shown to demonstrate even loading.

The mechanisms underlying the crosstalk of the IL-6 family of cytokines have not been fully elucidated. Therefore, we wanted to further investigate plausible factors that may be involved and impact the interaction between gp130 cytokines.
Figure 4.5 mOSM and NP crosstalk differently than other gp130 cytokines. Fully differentiated 3T3-L1 adipocytes were pretreated with 1 nM mOSM, 1 nM hOSM, or 1nM NP for 6-hr. Next, the cells were treated for 10 min with 1 nM mOSM, 1 nM hOSM, or 1nM NP. After the treatment, whole cell extracts were prepared, and 160 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently two times.

Various lines of evidence show that gp130 cytokines induce suppressors of cytokine signaling (SOCS) proteins (166-168;222). These proteins negatively regulate cytokine signal transduction, and expression is dependent on new protein synthesis. Therefore, to determine if the inhibitory crosstalk exerted by gp130 cytokines could be a result of newly synthesized SOCS proteins, we pretreated fully differentiated 3T3-L1 adipocytes for 4-hr with CT-1, LIF, or NP in the presence or absence of cycloheximide, an inhibitor of new protein synthesis. Next, we treated the cells with an acute 10 min treatment of CT-1. Adipocytes with no pretreatment were observed to show the efficacy of CT-1 signaling. As indicated in Figure 4.6, the same pattern of crosstalk was observed in the presence or absence of cycloheximide. Both CT-1 and LIF blocked the signaling of an acute CT-1 treatment, as evidenced by a drastic decrease in STAT3 Tyr705 and MAPK activation. In addition, NP pretreatment did not inhibit CT-1 signaling. Both STAT3 and ERK (MAPK) protein levels are shown to demonstrate even loading. The expression of PPARγ, a labile transcription factor, is included to show efficacy of the cycloheximide.
Figure 4.6 Crosstalk of gp130 cytokines is not dependent on new protein synthesis. Fully differentiated 3T3-L1 adipocytes were pretreated with 1 nM CT-1, 1 nM LIF, or 1nM NP for 4-hr in the presence or absence of a 30 min 5 μM cycloheximide preincubation. The cells were then treated (+) for 10 min with 1 nM CT-1. After the treatment, whole cell extracts were prepared, and 150 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently four times.

In addition to the JAK/STAT pathway, gp130 cytokines can also activate the MAPK (mitogen-activated protein kinase) cascade (reviewed in (157)). In order to determine whether the crosstalk of gp130 family members is dependent on the activation of ERKS 1 and 2, we pretreated fully differentiated 3T3-L1 adipocytes with CT-1, NP, or LIF for 3-hr in the presence or absence of U0126, a selective ERK 1 and 2 inhibitor. Cells were then given an acute 15 min treatment (+) of LIF or NP. The efficacy of both LIF and NP signaling was observed in adipocytes with no pretreatment. As demonstrated in Figures 4.7-4.10, the absence of MAPK activation had no effect on the crosstalk signaling of gp130 cytokines. Figures 4.7 and 4.8 indicate that both CT-1 and LIF pretreatments blocked an acute treatment of LIF, as evidenced by the decrease in both STAT3 Tyr\textsuperscript{705} and MAPK activation.
Figure 4.7 Crosstalk of gp130 cytokines is not dependent on the activation of MAPK. Fully differentiated 3T3-L1 adipocytes were pretreated with 1 nM CT-1 or 1nM NP for 3-hr in the presence or absence of a 30 min 5 μM U0126 preincubation. The cells were then treated (+) for 10 min with 1 nM LIF. After the treatment, whole cell extracts were prepared, and 150 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently three times.

Figure 4.8 Crosstalk of gp130 cytokines is not dependent on the activation of MAPK. Fully differentiated 3T3-L1 adipocytes were pretreated with 1 nM LIF for 3-hr in the presence or absence of a 30 min 5 μM U0126 preincubation. The cells were then treated (+) for 10 min with 1 nM LIF. After the treatment, whole cell extracts were prepared, and 150 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently three times.
Likewise, NP pretreatment had no effect on LIF’s activation of STAT3 or MAPK. In addition, NP signaling was greatly attenuated by pretreatments with CT-1, LIF, and NP, which corresponds with our previous experiments (Figures 4.9 and 4.10). In each experiment, STAT3 and MAPK protein levels are shown to demonstrate even loading, and the efficacy of the U0126 inhibitor was validated by the decrease in active MAPK following a 30 min preincubation.

Figures 4.9 Crosstalk of gp130 cytokines is not dependent on the activation of MAPK. Fully differentiated 3T3-L1 adipocytes were pretreated with 1 nM CT-1 or 1nM NP for 3-hr in the presence or absence of a 30 min 5 μM U0126 preincubation. The cells were then treated (+) for 10 min with 1 nM NP. After the treatment, whole cell extracts were prepared, and 150 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently three times.

The data from Figures 4.1-4.10 evaluate gp130 cytokine signaling in 3T3-L1 adipocytes in vitro; hence, we wanted to further examine the interactions among these cytokines in vivo. To conduct these studies, Sprague Dawley rats were injected with either vehicle (saline), CT-1 (0.1μg/g animal), or LIF (0.1 μg/g animal), with or without an 8-hr pre-injection of LIF. The animals were sacrificed 15 minutes following the second injection. As shown in Figure 4.11 and 4.12, both a CT-1 and a LIF injection induced the activation of STAT3 Tyr705 and MAPK in both
Figures 4.10 Crosstalk of gp130 cytokines is not dependent on the activation of MAPK. Fully differentiated 3T3-L1 adipocytes were pretreated with 1 nM CT-1, 1 nM LIF, or 1 nM NP for 3-hr in the presence or absence of a 30 min 5 μM U0126 preincubation. The cells were then treated (+) for 10 min with 1 nM LIF or 1 nM NP. After the treatment, whole cell extracts were prepared, and 150 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment performed independently three times.

In addition to observing the interaction of CT-1 and LIF in vivo, we examined the crosstalk signaling of NP and mOSM in adipose tissue in vivo. Previous studies from our lab showed that NP can induce STAT3 tyrosine phosphorylation in vivo (167); however, NP interaction with other gp130 cytokines has not been previously observed. Sprague Dawley rats were injected with either vehicle (saline), NP (0.1μg/g animal), or mOSM (0.1μg/g animal), with or without a 5 hr pre-injection of NP. The animals were sacrificed 20 minutes following the second injection. As shown in Figure 4.13, both a NP and a mOSM injection induced the
Figures 4.11 and 4.12. LIF treatment in vivo blocks subsequent CT-1 and LIF signaling in rodent fat pads. Six week old male Sprague Dawley rats were given an intraperitoneal injection of vehicle (saline) control, CT-1 (0.1 μg/g animal), or LIF (0.1 μg/g animal), with or without an 8 hr preinjection of LIF. Fifteen minutes after the second injection the rats were sacrificed and both retroperitoneal and epididymal fat pads were immediately removed and frozen in liquid nitrogen. 220 μg of retroperitoneal and 350 μg of epididymal tissue extract were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The data in each figure is generated from the same exposure of the same blot. Each figure is a representative experiment independently performed three times.
activation of STAT3 Tyr$^{705}$ in retroperitoneal adipose tissue. Our data indicate that a NP pretreatment did not inhibit the mOSM induction of STAT3 tyrosine phosphorylation; but, the NP induced STAT3 Tyr$^{705}$ was abolished. These findings correlate with previous in vitro data (Figure 4.5). MAPK protein levels are shown to demonstrate even loading.

Figures 4.13  NP pretreatment inhibits NP signaling, but not mOSM in adipose tissue in vivo. Six week old male Sprague Dawley rats were given an intraperitoneal injection of vehicle (saline) control, NP (0.1μg/g animal), or mOSM (0.1μg/g animal), with or without a 5 hr preinjection of NP. Twenty minutes after the second injection the rats were sacrificed and the retroperitoneal fat pad was immediately removed and frozen in liquid nitrogen. 150 μg of retroperitoneal tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed three times.

Our in vivo studies demonstrate gp130 cytokine signaling in only epididymal and retroperitoneal fat pads. In order to gauge our studies in white adipose tissue, we analyzed brown adipose tissue (BAT), skeletal muscle, and liver extracts to test their sensitivity to gp130 cytokines. Tissue samples were extracted from Sprague Dawley rats following an injection with either vehicle (saline), CT-1 (.1μg/g animal), LIF (.1μg/g animal), NP (.1μg/g animal), or mOSM (.1μg/g animal). Figures 4.14 and 4.15 show that CT-1, LIF, NP, and mOSM induced STAT3 tyrosine phosphorylation, as well as MAPK activation, in only retroperitoneal and epididymal adipose tissue, but not in BAT, skeletal muscle, or liver. Hence, our data indicate that gp130 cytokine action is highly enriched in adipose tissue. MAPK protein levels are shown to demonstrate even loading, relative to the amount of protein added for each tissue.
Figures 4.14 and 4.15  CT-1, LIF, NP, and mOSM induce STAT3 tyrosine phosphorylation in only retroperitoneal and epididymal adipose tissue extracts. Six week old male Sprague Dawley rats were given an intraperitoneal injection of vehicle (saline) control, CT-1 (0.1μg/g animal), LIF (0.1μg/g animal), NP (0.1μg/g animal), or mOSM (0.1μg/g animal). Fifteen minutes after the injection the rats were sacrificed and the epididymal, retroperitoneal, brown adipose tissue (BAT), skeletal muscle, and liver extracts were immediately removed and frozen in liquid nitrogen. 220 μg of epididymal, retroperitoneal, and BAT extracts and 440 μg of skeletal muscle and liver extracts were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed three times.
Because we observed specificity of gp130 family signaling in adipose tissue, we wanted to ensure that our findings were not due to the lack of essential receptor complex components in each tissue. It is well known that many gp130 cytokines utilize the leukemia inhibitory factor receptor (LIFR) as an important signal transducer in the receptor complex (reviewed in Heinrich (157)). Therefore, we examined the expression of the LIFR in retroperitoneal, epididymal, BAT, skeletal muscle, and liver extracts that were utilized for our in vivo studies. As shown in Figure 4.16, a substantial amount of the LIFR was present in each tissue. Ironically, LIFR was more highly expressed in other tissues, as compared to adipose tissue (retroperitoneal), yet these tissues were not responsive to the gp130 cytokines.

Figure 4.16 The LIFR is expressed in brown adipose tissue (BAT), skeletal muscle, and liver, as well as adipose tissue. Extracts were taken from six week old Sprague Dawley rats that were given only a vehicle (saline) injection. The rats were sacrificed, and the epididymal, retroperitoneal, brown adipose tissue (BAT), skeletal muscle, and liver extracts were immediately removed and frozen in liquid nitrogen. 200 μg of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed two times.

Previous studies by Zvonic et al. postulated that the inhibitory crosstalk among certain gp130 cytokines in adipocytes occurs as a result of specific degradation of the LIFR via a lysosome-mediated pathway (169). These studies also demonstrated that LIF, CT-1, and hOSM had profound effects on LIFR degradation; hence, subsequent signaling by certain gp130 cytokines was attenuated due to the lack of the LIFR. In the studies shown above, we observed that NP pretreatment does not inhibit signaling of other gp130 family members; therefore, NP crosstalks differently than other gp130 cytokines. Hence, we wanted to determine if NP resulted
in decreased LIFR levels in 3T3-L1 adipocytes. We treated fully differentiated 3T3-L1 adipocytes with cycloheximide (CH), in the presence or absence of LIF or NP. As shown in Figure 4.17, the expression levels of LIFR in adipocytes treated with LIF dramatically decreased within 1 hr; however, NP treatment did not cause a substantial decrease in LIFR expression. The efficacy of both LIF and NP was shown by the activation of STAT3 Tyr\(^{705}\). We also observed that NP failed to cause a decrease in LIFR expression in OP9 mouse stromal cells, a newly characterized adipocyte cell culture model (237) (data not shown).

![Figure 4.17](image)

Figure 4.17 NP does not induce the degradation of the LIFR. Fully differentiated 3T3-L1 adipocytes were treated with either 1 nM LIF or 1 nM NP in the presence of 5 μM cycloheximide (CH). Whole cell extracts were prepared from adipocytes that were untreated (0) or treated at the times indicated. 125 μg of extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. This is a representative experiment independently performed three times.

Gp130 cytokines are known to induce the tyrosine phosphorylation of their recruited signal transducer receptors, including the LIFR, upon ligand binding. Because data in Figure 4.17 indicate that NP does not induce LIFR degradation, we examined the ability of NP to induce the tyrosine phosphorylation of the LIFR, since other studies have reported that NP signals via the LIFR (156). The ability of gp130 cytokines to phosphorylate the LIFR was examined by immunoprecipitating LIFR from whole cell extracts isolated from cells treated with LIF, mOSM,
hOSM, or NP. Phosphorylation of the LIFR was observed by western blotting with 4G10, an anti-phosphotyrosine antibody. As shown in Figure 4.18, both LIF and hOSM, but not NP, induced the phosphorylation of the LIFR, and this effect was not attributed to differences in LIFR expression. The lack of LIFR phosphorylation by mOSM treated cells supports previous studies that revealed an alternative receptor complex in murine cells for this cytokine. Collectively, our data demonstrate that NP does not cause the degradation or tyrosine phosphorylation of the LIFR as we have observed for other gp130 cytokines. The efficacy of each cytokine was shown by the substantial activation of STAT3 Tyr$^{705}$.

Figure 4.18  NP does not cause the tyrosine phosphorylation of the LIFR. Whole cell extracts were isolated from fully differentiated 3T3-L1 adipocytes that were harvested after no treatment (CTL) or an acute 6 minute treatment with 1 nM LIF, 1 nM mOSM, 1 nM hOSM, or 1nM NP. Each extract was subjected to immunoprecipitation (IP) with an antibody directed against the LIFR and Western blotting analysis with 4G10, an anti-phosphotyrosine antibody. 700 μg of protein was used for IP, and 90 μg of protein was used for Western blotting. One IP contained buffer, but not extract, and is labeled as Mock. This is a representative experiment independently performed four times.

Because our findings indicated that NP does not induce phosphorylation of the LIFR, we wanted to determine if NP would cause the tyrosine phosphorylation of another gp130 cytokine signaling receptor component. The oncostatin M receptor β (OSMRβ) is similar in structure to the LIFR and is utilized by mOSM for signal transduction. The ability of both mOSM and NP to
tyrosine phosphorylate the OSMRβ was examined by immunoprecipitating cytokine-treated whole cell extracts with an antibody directed against OSMRβ and analyzing these precipitates with western blotting to observe the presence of tyrosine phosphorylated proteins, using a 4G10 anti-phosphotyrosine antibody. As shown in Figure 4.19, mOSM, but not NP, induced the tyrosine phosphorylation of the OSMRβ, and this effect was not attributed to differences in OSMRβ expression. The efficacy of each cytokine was shown by the substantial activation of STAT3 Tyr705.

Figure 4.19 NP does not cause the tyrosine phosphorylation of the OSMRβ. Whole cell extracts were isolated from fully differentiated 3T3-L1 adipocytes that were harvested after no treatment (CTL) or an acute 6 minute treatment with 1 nM mOSM, or 1nM NP. Each extract was subjected to immunoprecipitation (IP) with an antibody directed against the OSMRβ and Western blotting analysis with 4G10, an anti-phosphotyrosine antibody. 600 μg of protein was used for IP, and 90 μg of protein was used for Western blotting. One IP contained buffer, but not extract, and is labeled as Mock. This is a representative experiment independently performed three times.

4.4 Concluding Summary

Although the mechanisms underlying the actions of gp130 cytokines have not been fully elucidated, it is now common knowledge that members of the gp130 family activate STAT proteins, as well as the MAPK (ERK) pathway in adipocytes. Previous studies from our laboratory highlighted the ability of several gp130 cytokines to crosstalk in an inhibitory fashion with specific gp130 family members in adipocytes as a result of specific degradation of the
LIFR. Our follow-up studies evaluated the crosstalk signaling of several gp130 cytokines both in vitro and in vivo. Novel data show that NP does not inhibit the signaling of other gp130 cytokines, presumably because it does not result in LIFR phosphorylation and degradation. Likewise, mOSM crosstalks in a distinct manner from hOSM, which can be attributed to LIFR activation. In contrast, many gp130 cytokines, specifically CT-1, LIF, and hOSM, signal in an antagonistic fashion with one another. We also demonstrate that this crosstalk inhibition is not dependent on the activation of MAPK or new protein synthesis.

A common feature of many gp130 cytokines is their shared use of not only gp130, but also LIFR proteins as components of their receptor complex. Some of the most interesting characteristics of gp130 cytokines involve their pleiotropic and redundant functions, and many lines of evidence show that these features can be partly attributed to the presence of the signal transducers in the receptor complex. Our data support the idea that the unique crosstalk ability of gp130 cytokines could also be due to differential usage of receptor components. Several gp130 family members that utilize the LIFR, including CT-1, LIF, and hOSM, have the ability to crosstalk with one another in an antagonistic fashion. Studies show that following gp130 cytokine treatment, LIFR is degraded after internalization (169;238;239), which results in the loss of LIFR from the plasma membrane and the inability for subsequent signaling. Although NP was originally characterized as a cytokine that required the LIFR for signal transduction (156), our novel data indicate that NP does not, in fact, phosphorylate or degrade the LIFR (Figure 4.17) in the same manner as other gp130 cytokines. Hence, these observations suggest that NP does not crosstalk and inhibit the signaling of other gp130 cytokines because this crosstalk is associated with LIFR phosphorylation. Since NP propagates its signal through the JAK/STAT and MAPK pathways in a manner similar to other gp130 cytokines, our data suggest
that the lack of signal inhibition may reside at the level of the receptor. We postulate that NP may utilize an alternative signal transducer in its receptor complex, in addition to gp130 and CNTFRα. Although we do not know the identity of this NP receptor(s), our data indicate that the LIFR and OSMRβ (Figure 4.19) are not involved.

We also further examined the mechanistic actions of gp130 cytokines by investigating potential factors that could impact gp130 crosstalk interaction. Although previous studies in our laboratory demonstrated that a loss in the induction in SOCS mRNA by gp130 members coincided with an impairment of signaling by gp130 cytokine pretreatments, we demonstrate that gp130 inhibitory crosstalk is not dependent on newly synthesized SOCS proteins. Therefore, the antagonistic signaling of these cytokines is not the result of additional protein function, but most likely the action of each respective cytokine to bind and induce the degradation of a receptor component. In addition, though the activation of MAPK (ERKs 1 and 2) by gp130 cytokines may serve as an essential pathway in other cellular actions, our data show that gp130 crosstalk is not dependent on MAPK activation (Figures 4.7-4.10). The mechanisms underlying the crosstalk of gp130 cytokines are poorly understood; however, we have demonstrated that two well-established gp130-activated signaling proteins are not the cause of the observed crosstalk.

To our knowledge, no other laboratories have investigated the interactions of various gp130 cytokines in adipose tissue in vivo. Interestingly, our data indicate that gp130 family members crosstalk in vivo in the same manner as in vitro, and these effects are likely very important in adipose tissue since it is highly responsive to cytokine exposure compared to other insulin sensitive tissues (Figures 4.11-4.15). LIF pretreatment inhibited both LIF and CT-1 subsequent treatments. In addition, NP pretreatment had no effect on an acute treatment of mOSM. The fact that gp130 cytokines not only activate signaling pathways in vivo, but also
inhibit subsequent signaling of other gp130 family members confirm that a detailed analysis of crosstalk mechanisms is relevant for a more detailed perspective of gp130 cytokine function. In addition, it is well known that in humans there are circulating levels of some gp130 cytokines, notably IL-6 and CT-1 (reviewed in (162))(240), which greatly contribute to metabolism. Therefore, it is necessary to understand how these cytokines function and interact, as well as the inhibitory crosstalk that gp130 cytokines exert on their respective family members. Collectively, our data provide a basis for future analyses of cytokine interaction; nonetheless, further studies are needed to examine how these processes could possibly contribute to or affect metabolic disease states that are characterized by the expression of multiple cytokines, including those belonging to the gp130 family.

Although few STAT target genes have been identified, numerous lines of evidence suggest an important role for these transcription factors in adipocytes. Our studies have characterized a new STAT5A target gene, PDK4, which plays a critical role in glucose metabolism. This data provides additional evidence for the importance of STATs in regulating genes that regulate not only adipocyte function, but also full-body metabolism. Though the mechanisms underlying the action of STAT3 activators have not been fully elucidated, our novel studies implicate differential actions of these cytokines in adipocytes. The importance of STAT3 in fat cell metabolism is supported by data indicating that cytokine activators exert unique, yet significant effects on both adipogenesis and insulin sensitivity. In addition, the metabolic implications of gp130 cytokine crosstalk remain to be discovered; yet, we speculate that these interactions may greatly affect the physiological action of these cytokines. Future research will hopefully lead to insights into the molecular mechanisms regulating whole-body homeostasis and may contribute to understanding the metabolic defects underlying obesity and T2D.
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