Effects of gp130 cytokines on adipocytes

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EFFECTS OF GP130 CYTOKINES ON ADIPOCYTES

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Sanjin Zvonić
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ABSTRACT

Members of the gp130 cytokine family are known for their pleiotropic roles in various cell types. Our work has focused on their actions in adipocytes.

CNTF administration has been shown to ameliorate most complications associated with obesity and type 2 diabetes through an unknown mechanism. In this study CNTF is shown to activate JAK/STAT and MAPK signaling, both in vitro and in vivo. In 3T3-L1 adipocytes, chronic CNTF also regulates the expression of SREBP-1, FAS, IRS-1 and GLUT4. Acute CNTF administration enhances the activation of IRS-1 and Akt by insulin. Our data also demonstrate that the expression of CNTF-specific receptor, CNTFRα, is decreased during adipocyte differentiation, but that this protein is expressed in several other tissues, and the level of CNTFRα glycosylation varies between different tissues. Most importantly, the expression of CNTFRα is dramatically increased in the fat pads of obese and diabetic animals.

Even though CT-1 is mostly known for its actions in cardiomyocytes, the work presented demonstrates that, in fat cells, CT-1 can potently induce JAK/STAT and MAPK signaling. Neither CT-1 nor CNTF affected adipocyte differentiation, but chronic CT-1 did induce a decrease in FAS and IRS-1 protein expression. Both CNTF and CT-1 also induced a transient increase in SOCS-3 and a transient decrease in PPARγ mRNA levels, in a MAPK-independent manner.

Our studies also demonstrate that several gp130 cytokines, namely CT-1, LIF and OSM, have the ability to block signaling by other gp130 cytokines, but not GH. These cytokines can also dramatically reduce the half-life of LIFR protein in adipocytes, and
this change in LIFR stability correlates with the ability of CT-1, LIF and OSM, to block subsequent gp130 cytokine signaling. The loss of LIFR protein can be prevented by the lysosome inhibitors leupeptin and chloroquine, but not by proteasome inhibitors.

In summary, these studies demonstrate that gp130 cytokines have a wide spectrum of effects on both cultured and native adipocytes, and that their actions may be important in various aspects of adipocyte physiology.
1.1 Adipocyte Research: Why and How?

Economic growth and modernization, which occurred in nations with established market economies over the last few decades, has in most cases lead to the adoption of a "western" lifestyle. In such economies, the modernization of agricultural production and food processing has lead to the dramatic increase of food availability and energy density per capita (Seidell, 2000). Workplace mechanization and the changes in transportation have caused an increasing percentage of population to lead very sedentary lives, and have contributed to the alarmingly high rates of obesity and related health problems in these nations (Seidell, 2000). According to the World Health Organization's (WHO) World Health Report the prevalence of obesity in established market economies (Europe, USA, Canada, etc.) is, on average, in the range of 15-20% of the total population. There is also a growing trend of obesity among the population in the nations with developing economies (Seidell, 2000). In the US, the prevalence overall is about 20%, with variations within certain racial and ethnic subgroups (Flegal et al., 1998).

Obesity-related diseases, such as hypertension and type 2 diabetes (Non Insulin-Dependent Diabetes Mellitus, or NIDDM), are becoming a great burden, both financially and in terms of their management and prevention (Wolf and Colditz, 1998). The WHO estimates that by year 2025 about 300 million people will be affected by type 2 diabetes, primarily due to obesity and related lifestyle factors (Seidell, 2000). The incidence of type 2 diabetes is tightly linked to obesity, as 80-85% of all type 2 diabetics are also obese (BMI > 30 kg/m²) (Must et al., 1999). At the same time, type 2 diabetes is
characterized as a condition marked by insulin resistance in otherwise insulin-sensitive tissues, especially skeletal muscle and adipose. Therefore, understanding the physiology of adipose (fat) tissue, in both healthy and diseased states, is crucial for understanding the etiology of obesity and type 2 diabetes, and for developing new strategies to combat the world-wide epidemic of these diseases.

Until recently, adipose tissue was considered to be an inert energy depot used for storage or release of lipid, under appropriate hormonal stimuli. However, the work performed in the field of adipocyte biology within the last decade has revealed new functions of adipose tissue. We now know that, apart from storing excess energy, adipose tissue contributes to the fine tuning of energy homeostasis, and other physiological functions (Friedman and Halaas, 1998). This is achieved through their specific secreted gene products which include leptin, tumor necrosis factor (TNF) -α, adiponectin, resistin, interleukin 6 (IL-6), and others (Guerre-Millo, 2002). It has also been demonstrated that the production of these signals is regulated by feeding, fasting, and obesity (Hamilton et al., 1995; Hotamisligil et al., 1995; Lefebvre et al., 1998). These findings are mostly derived from the studies in white adipose tissue (WAT).

Another type of fat, the brown adipose tissue (BAT), is present in very limited quantity in humans, while more abundant in other animals. The physiological actions of BAT dramatically contrast that of WAT. The primary role of BAT is to dissipate energy rather than storing it (Lowell and Flier, 1997). This is achieved through increased mitochondrial biogenesis and the expression of uncoupling protein (UCP) -1. UCP-1 interrupts the established proton gradient of the respiratory chain across the inner mitochondrial membrane. The result is generation of heat instead of ATP production.
Another misconception about fat tissue is that people are born with all the adipocytes they will have as adults. However, current research clearly demonstrates that the production of new fat cells (adipogenesis) occurs throughout the life of the organism. Adipogenesis can be a result of normal cell turnover or the means of producing additional fat mass for excess energy storage and weight gain (Prins and O'Rahilly, 1997). In the former case, present adipocytes accumulate lipid until they reach the physical limit of their size. At that point, new adipocytes are derived from their cellular precursors, and they start accumulating the excess lipid (Miller, Jr. et al., 1984).

To study adipocyte physiology in vitro, researchers use either the immortalized, predetermined, clonal preadipocyte cell lines, or stromovascular cells cultured from fractionated animal fat pads. Most of the in vitro work with adipocytes utilizes the murine 3T3-L1 and 3T3-F442A adipocyte lines. These cells were cloned from the non-clonal Swiss 3T3 cells, and, even though morphologically identical to fibroblasts, are committed to adipocytic lineage and develop into fat cells when stimulated with prodifferentiative agents (Green and Meuth, 1974; Green and Kehinde, 1975). Compared to other cell lineages, the physiology of cultured fat cells in vitro exhibits very high identity to that of fat in vivo. Cultured adipocytes closely resemble native fat cells in vivo and express almost all adipocyte-specific genes, secrete fat-specific proteins, are insulin sensitive, and respond to all the signals which impact fat tissue.

Cultured preadipocytes are induced to differentiate by a cocktail of hormones (MDI) containing methyl-isobutyl-xanthine (MIX), dexamethasone (Dex), and insulin. Insulin and insulin-like growth factor (IGF) -1 are potent inducers of adipogenesis, increasing the number of cells that undergo differentiation (Girard et al., 1994). They also
increase the amount of lipid stored in each cell and have strong anti-apoptotic activity (Kiess and Gallaher, 1998). The effects of these two factors are mediated through the IGF-1 receptor, since preadipocytes express very limited levels of insulin receptor (Reed and Lane, 1980). Insulin and IGF-1 induce adipogenesis mostly through activation of the RAS/MAPK signaling pathway. Activated RAS has been shown to induce adipogenesis in the absence of other hormonal stimuli (Benito et al., 1991). Insulin and IGF-1 also activate protein kinase B (PKB, or Akt), which has been implicated as a possible mediator of differentiation (Magun et al., 1996). Dexamethasone (Dex) is a synthetic glucocorticoid hormone that activates the glucocorticoid receptor (GR). Transcriptional targets of GR that are involved in adipogenesis are still unclear, but may involve the proadipogenic transcription factors CCAAT/Enhancer-Binding Protein (C/EBP)–δ, and a negative regulator of adipogenesis, preadipocyte factor (pref)-1 (Wu et al., 1996; Smas et al., 1999; Cao et al., 1991). Methyl-iso-butyl-xanthine (MIX), a cAMP phosphodiesterase inhibitor, results in the elevation of cAMP levels, which has proadipogenic property (Yarwood et al., 1995). MIX-induced increase in cAMP also leads to the expression of the proadipogenic transcription factors C/EBP-β, and CREB (Cao et al., 1991; Reusch et al., 2000). Even though all three components of MDI are capable of inducing adipogenesis alone, their synergistic effects, in the presence of FBS, induce robust differentiation which results in fat cells highly similar to native adipocytes.

The addition of MDI to cultured preadipocytes leads to cell growth arrest. The arrest is followed by 1-2 rounds of subsequent cell division known as clonal expansion. The onset of differentiation is marked by the transient induction of proadipogenic transcription factors C/EBP-β and δ (Darlington et al., 1998). This is followed by
increased expression of proadipogenic transcription factors including Peroxisome Proliferator-Activated Receptor (PPAR) –γ (Chawla et al., 1994; Tontonoz et al., 1994), C/EBP-α (Shao and Lazar, 1997), and Signal Transducer and Activator of Transcription (STAT) 5 (Floyd and Stephens, 2003), which can act as the central regulators of adipogenesis. Increased transcriptional activity of these factors results in accumulation of gene products that characterize the adipocyte phenotype: glycerolphosphate dehydrogenase (GPDH), fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), insulin-stimulated glucose transporter GLUT4, insulin receptor, adipocyte-specific fatty acid binding protein aP2, and many others (Spiegelman et al., 1993). Importantly, 3T3-L1 adipocytes also accumulate large amounts of triglyceride, and become insulin sensitive (Rubin et al., 1977).

Even though cultured adipocytes provide a great model for studying adipocyte functions, they are still not completely representative of fat in vivo. Adipocytes in vivo exist in conjunction with their extracellular matrix: stromovascular cells, preadipocytes and nerve cells. These other cell types can effect adipocyte function. For example, cultured fat cells produce very low levels of leptin, unlike adipocytes in vivo. Also, unlike other tissues, WAT is located in several depots around the body (perigonadal, omental, retroperitoneal, subcutaneous), and the behavior of mature adipocytes differs between various depots (Ostman et al., 1979). Therefore, it is still essential to use in vivo approaches to recapitulate the findings derived from cultured adipocytes.

Several rodent lines have been developed over the last few decades to serve as models for studying obesity, diabetes, and general adipose physiology. Most commonly used are ob/ob mice which do not produce leptin, db/db mice (and equivalent Zucker
rats) which lack the leptin receptor (OBR), and several lines of agouti (A^Y) mice with elevated levels of agouti protein which antagonizes the melanocortin (MC4-R) receptor (Ingalls et al., 1996; Zhang et al., 1994; Aubert et al., 1985; Hummel et al., 1966; Bultman et al., 1992; Gantz et al., 1993). All these animal models develop severe obesity, type 2 diabetes, and other metabolic complications. Another strain of interest is the inbred C57B1/6J strain. These mice are not obese on a standard diet, but become obese on a high-fat diet, thus providing a great model for diet-induced obesity (DIO) studies (Surwit et al., 1988). With the advances in transgenic and gene knockout technologies, more and more animal models are being developed to mimic the possible scenarios that may lead to the development of obesity and type 2 diabetes.

Recent statistics concerning the epidemic of obesity and type 2 diabetes are alarming and demand immediate attention. In the light of the vast body of knowledge already generated in the field of adipose physiology, it is obvious why further research in this arena is crucial in developing a uniform strategy to help combat this deadly epidemic.

1.2 JAK/STAT Signaling Pathway

Ligand binding to receptors results in a cascade of intracellular phosphorylation events. However, most cytokine receptors possess no intrinsic kinase activity and therefore depend on the associated cytosolic kinases to relay the signal downstream of the ligand bound receptor. The Janus kinase (JAK) family of tyrosine kinases is a group of proteins that can provide this activity. JAK kinases are used by several receptor families including IL-2, IL-3, IL-10, IFN-γ, IFN-α, gp130, and single chain receptor families, as well as some receptor tyrosine kinases (RTKs), and some G-protein-coupled receptors.
JAK/STAT signaling is initiated when cytokine binding leads to the conformational change in the cytoplasmic portion of its corresponding receptor. The conformational change brings the associated JAKs in close proximity, leading to their activation by trans-phosphorylation (Remy et al., 1999). Activated JAKs then phosphorylate specific tyrosine residues on the receptor, making them available as docking sites for SH2-domain containing proteins (Hubbard and Till, 2000). STATs are a family of SH-2 domain proteins that dock on the phosphorylated receptor. Once recruited to the receptor, they are themselves tyrosine phosphorylated by JAKs. Phosphorylated STATs then dissociate from the receptor and dimerize. Activated STAT dimers translocate to the nucleus, and bind specific and unique DNA elements (ISRE or GAS elements), thus acting as transcription factors linking extracellular signaling with gene transcription (Decker et al., 1997; Kessler et al., 1988).

The JAK family of tyrosine kinases is comprised of four members: TYK2, JAK1, JAK2, and JAK3. JAK1, JAK2, and TYK2 are expressed ubiquitously, whereas JAK3 is expressed exclusively in the cells of myeloid and lymphoid lineage (Krolewski et al., 1990; Wilks et al., 1991; Harpur et al., 1992; Rane and Reddy, 1994; Kawamura et al., 1994; Witthuhn et al., 1994). JAK1 is involved in signaling by the members of the IL-2, IL-4, and gp130 receptor families, as well as the members of class II cytokine receptor family (Schindler and Strehlow, 2000). Consistent with the pleiotropic use of JAK1, JAK1 knockout animals exhibited a lethal phenotype, similar to that of LIFR knockout animals (Rodig et al., 1998). JAK2 is used by the members of the single chain receptor family, as well as the members of gp130 family (Schindler and Strehlow, 2000). JAK2 knockout mice have an embryonic lethal phenotype due to failure in erythropoiesis (Wu
et al., 1995; Neubauer et al., 1998; Parganas et al., 1998). JAK3, due to its limited
expression, is used by receptors employing the common gamma chain (Schindler and
Strehlow, 2000). JAK3 knockout animals exhibit defects in lymphopoiesis, but live
normally in a pathogen free environment (Nosaka et al., 1995; Cao et al., 1995). JAK3
deficiency in humans leads to severe combined immunodeficiency (SCID) (Russell et al.,
1994). TYK2, the first identified JAK, is involved in signaling by IFNα, IL-6, IL-10, and
IL-12 (Schindler and Strehlow, 2000; Velazquez et al., 1992). Even though TYK2 null
animals have very subtle defects (Karaghiosoff et al., 2000; Shimoda et al., 2000), TYK2
seems to be important in mediating responses to IL-12 and lipopolysaccharide (LPS)
(Bogdan et al., 2000).

JAKs are constitutively associated with a proline-rich, membrane proximal,
cytosolic domain of cytokine receptors (often referred to as box 1 and box 2 regions)
(Ihle, 2001). There is also evidence that suggests that JAKs can also associate with
receptor tyrosine kinases, and some G-protein coupled receptors, enabling them to signal
via STATs (Schindler and Strehlow, 2000). Each of the JAK proteins are over 1000
amino acids long and range in size from 120 to 130 kDa. Sequence analysis of JAKs
shows seven regions of high homology, termed JH1-JH7. Even though their activities
remain unclear, JH1 exhibits classical features of a kinase domain (Hubbard and Till,
2000), while JH2 represents a kinase-like (KL) pseudokinase domain (Ihle, 2001; Yeh et
al., 2000). This pseudokinase domain is a unique feature of JAK kinases (Ihle, 2001). It
has all the structural features of a tyrosine kinase, except the catalytic activity. It most
likely serves as a regulator of kinase activity, and possibly as a mediator of the
JAK/STAT interaction (Yeh et al., 2000; Velazquez et al., 1995; Flores-Morales et al.,
1998). Domains JH3-JH7 (amino-terminal domains) are thought to be involved in receptor association. Specifically, JH6-7 are required for JAK2 and JAK3 (Chen et al., 1997; Kohlhuber et al., 1997), TYK2 requires additional JH elements (Yan et al., 1998), while the entire N-terminus (including the FERM domain) is required for JAK1 association with the receptor (Kohlhuber et al., 1997).

The STAT family of proteins in mammals has seven identified members: STATs 1-4, STAT 5A and 5B, and STAT 6 (Fu et al., 1992; Zhong et al., 1994b; Akira et al., 1994; Zhong et al., 1994a; Mui et al., 1995; Liu et al., 1995). It has been demonstrated that the STAT family of proteins arose from the same primitive gene, evolving to accommodate increasingly complex cell-to-cell communication needs in evolving organisms (Aubry and Firtel, 1999; Barillas-Mury et al., 1999; Oates et al., 1999; Zeidler et al., 2000; Pascal et al., 2001). In mammals, STATs mostly mediate host responses to different stress circumstances (Ihle, 2001).

STAT 1 was first discovered through its involvement in signaling pathways induced by various interferons (IFNs), and is best characterized for its actions in the regulation of the immune system (Durbin et al., 1996; Meraz et al., 1996; Shankaran et al., 2001). STAT 2 has been characterized along side STAT 1 for its functions in interferon signaling. Unlike other STATs, it has not been shown to bind GAS elements (Park et al., 1999). STAT 2 is thought to mediate not only IFN-induced signals, but also the actions of STAT 1 in response to these signals (Park et al., 2000). To date, STAT 2 is the least well understood of all STAT proteins. STAT 3 was first identified, and is best known, for its involvement in the signaling of IL-6 family of cytokines (Akira et al., 1994). More recent studies also point to its actions in cancer development (Wang et al.,
2004), immune functions (Takeda *et al.*, 1998; Takeda *et al.*, 1999), acute phase response (Alonzi *et al.*, 2001), and skin and hair development (Sano *et al.*, 1999). Complete ablation of STAT 3 is embryonic lethal (Takeda *et al.*, 1997). STAT 4 was identified in screens for STAT homologues, but has since been shown to be important in mediating IL-12 signaling, crucial for proper T-cell function (Kaplan *et al.*, 1996; Thierfelder *et al.*, 1996). In humans, STAT 4 is also activated by type I IFNs (Rogge *et al.*, 1998), and this specificity is attributed to the divergence of human STAT 2 which interacts with STAT 4 (Park *et al.*, 1999; Farrar *et al.*, 2000). Two STAT 5 proteins, STAT 5A and STAT 5B have very closely related sequences and are encoded by two linked genes with high identity (Azam *et al.*, 1995; Mui *et al.*, 1995). Even though they were first identified as prolactin (PRL) induced transcription factors, they have been shown to be activated by cytokines including IL-3, IL-2, and single-chain families (Arnould *et al.*, 1999; Asao *et al.*, 2001). STAT 5 proteins exhibit functional redundancy, but can have distinct functions in the cell (Liu *et al.*, 1997; Moriggl *et al.*, 1996; Teglund *et al.*, 1998; Ormandy *et al.*, 1997; Floyd and Stephens, 2003). STAT 6 is known for its roles in the acquired immunity via its involvement in IL-4 and IL-13 signaling (Lin *et al.*, 1995), often through interaction with IRS-2 adapter protein (Ryan *et al.*, 1996).

All STATs share several conserved structural domains. The N-terminal domain is well conserved among the STATs. Several studies have implicated it to function in GAS element binding, transcriptional coactivator and repressor interactions, receptor binding, and nuclear translocation (Vinkemeier *et al.*, 1996; Horvath, 2000; Shuai, 2000; Leung *et al.*, 1996; Strehlow *et al.*, 1998). The coiled-coil domain has a helical structure that creates large hydrophilic surfaces available for interactions with other helical proteins,
receptor binding, tyrosine phosphorylation, and nuclear export (Horvath, 2000; Begitt et al., 2000; Zhang et al., 2000). The DNA-binding domain has a β-barrel structure with an immunoglobulin fold. The conformation of this domain changes with STAT activation, and interacts with the DNA within the proximal half of the GAS element (Decker et al., 1997; McBride et al., 2000). The linker domain connects the DNA-binding and the SH2 domains (Chen et al., 1998). It has been speculated that this allows the regulation of DNA binding by the conformational changes in SH2 domain (following the binding of phosphorylated tyrosine) and therefore regulates transcription (Yang et al., 1999). The SH2 domain is the most highly conserved STAT domain. Its well conserved structure, including a conserved arginine residue allows the SH2 domain to interact with phosphorylated tyrosine residues (Kawata et al., 1997). This interaction is crucial for STAT recruitment to the activated receptors, JAK association, and STAT dimerization (Gupta et al., 1996; Darnell, 1997). The transcriptional activation (TAD) domain is not well conserved among the STATs, consistent with its function in regulating unique transcriptional activities (Schindler and Strehlow, 2000). Serine phosphorylation within this region can also modulate the transcriptional activity of some genes (Decker and Kovarik, 2000).

JAK/STAT signaling mediates numerous important physiological functions. Thus, it is not surprising to find several regulatory mechanisms whose function is to modulate the rate of STAT signal transduction. Negative regulation of STAT signaling is achieved at several points in the signal cascade, including activated receptor endocytosis (Dittrich et al., 1996), targeted degradation of both JAKs and STAT by the ubiquitin-proteasome system (Haspel et al., 1996; Callus and Mathey-Prevot, 2000), and
dephosphorylation of JAKs by SH2 domain-containing tyrosine phosphatases such as SHP1 and SHP2 (Yi et al., 1993). Generation of carboxy-terminally truncated STAT isoforms (at RNA or protein level) generates proteins with dominant-negative function that successfully attenuate STAT activity (Caldenhoven et al., 1996). STAT methylation may also play a role in the regulation of signaling (Mowen et al., 2001). Activation of JAK/STAT signaling pathways also leads to the initiation of negative feedback loops. Suppressors of cytokine signaling (SOCS) are a group of proteins whose expression is induced in this manner. Members of this family include CIS and SOCS-1, -2, and -3. They inhibit STAT signaling by blocking the STAT recruitment to the activated receptor (CIS), impairing STAT interaction with JAK (SOCS-1), or via other, not yet elucidated mechanisms (Yoshimura et al., 1995; Yasukawa et al., 1999). Another group of STAT-interacting proteins are the PIAS (Protein Inhibitor of Activated STATs) proteins. They bind activated STAT dimers in the nucleus, thus interfering with, or blocking their ability to successfully bind DNA (Liao et al., 2000; Chung et al., 1997). STAT signaling can also be enhanced. Serine phosphorylation of STATs has been shown to enhance their transcriptional activity (Decker and Kovarik, 2000; Wen et al., 1995), and affect stability (Beuvink et al., 2000). Most importantly, STAT dimers have been shown to interact with other proteins/transcription factors, and these interactions can serve to enhance their transcriptional activity. Some of these proteins include the glucocorticoid receptor (Aittomaki et al., 2000), C/EBPβ (Wyszomierski and Rosen, 2001), c-Jun (Zhang et al., 1999), NF-κB (Wang et al., 2003), as well as chromatin modifying proteins (CBP/p300, YY1, BRAC1) (Bhattacharya et al., 1996), and various cytosolic proteins (STAM, WD40, SH2-B) (Kim et al., 2001).
Recent work in the field of JAK/STAT signaling has led to many important discoveries, while also leaving many new questions for further investigation. Adipocytes *in vivo* and *in vitro* express JAKs 1 and 2, TYK2, and STATs 1, 3, 5A, 5B, and 6 (Stephens *et al.*, 1996). Adipose STATs are activated in highly specific patterns by different agonists, and these activations lead to very specific transcriptional effects (Balhoff and Stephens, 1998). Therefore, studying JAK/STAT pathway in adipocytes is a systematic approach to further elucidate the intricacy of fat physiology, especially in response to altered metabolic states of the organism.

### 1.3 Biology of gp130 Cytokines

The interleukin 6 (IL-6) cytokine family, a member of the cytokine superfamily, is a group of functionally and structurally related proteins including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF) and cardiotrophin-1 (CT-1) (Hirano *et al.*, 1994; Mackiewicz *et al.*, 1995). All these cytokines possess a similar helical structure and signal via structurally related receptors (Bazan, 1990a; Bazan, 1990b; Taga, 1996; Pennica *et al.*, 1996c; Kishimoto *et al.*, 1995). All of the IL-6 family cytokines were originally identified as factors with distinct and unique properties: immunoglobulin production in activated B cells for IL-6 (Lee *et al.*, 1988), growth promotion of myeloma and plasmacytoma for IL-11 (Paul *et al.*, 1990), growth inhibition of myeloid leukemia cells for LIF (Gearing *et al.*, 1987), growth inhibition of melanoma cells for OSM (Zarling *et al.*, 1986), promotion of ciliary neuron survival for CNTF (Stockli *et al.*, 1989; Lin *et al.*, 1989), and induction of cardiac hypertrophy for CT-1 (Pennica *et al.*, 1995a). However, we now know these cytokines can function in a pleiotropic and redundant manner (Paul, 1989; Kishimoto *et al.*, 1992).
In fact, members of the IL-6 cytokine family play pivotal roles in the immune, hemotopoietic, nervous, cardiovascular, and endocrine systems, as well as in bone metabolism, inflammation, and acute phase responses (Sehgal et al., 1988; Hirano, 1998; Metcalf, 1991; Patterson and Nawa, 1993; Pennica et al., 1996c; Kishimoto et al., 1995; Hirano, 1992; Hilton, 1992; Yang, 1993; Ip and Yancopoulos, 1996; Gearing, 1993).

Molecular cloning and structural analyses of the receptors for the cytokine superfamily proteins have established two distinct and distantly related cytokine receptor families. IL-6 family cytokine receptors belong to the class I cytokine receptor family (Taga and Kishimoto, 1990; Bazan, 1990b; Taga and Kishimoto, 1992; Miyajima et al., 1992). Members of this receptor family share a common domain of approximately 200 amino acid residues. Within this domain are four positionally conserved cysteine residues (near the amino-terminal) and a WSXWS motif in the carboxy-terminal (Bazan, 1990b). The domain itself is comprised of two fibronectin type III modules which are essential for ligand binding because they form a barrel-shaped ligand binding pocket (Yawata et al., 1993; Patthy, 1990). Aside from the IL-6 family cytokine receptors, the members of this family include glycoprotein 130 (gp130), leptin receptor (OBR), and other similar proteins such as G-CSFR and IL-12R (Fukunaga et al., 1990a; Fukunaga et al., 1990b; Tartaglia et al., 1995; Lee et al., 1996; Chen et al., 1996; Chua et al., 1994; Chua et al., 1995). Class I cytokine receptors are of great interest due to the findings that their functional receptor forms are actually comprised of multiple membrane proteins, and that these receptor complexes share common signal transducing subunits (Miyajima et al., 1992; Taniguchi and Minami, 1993; Kishimoto et al., 1994). This shared usage of signal transducers in part explains the functional redundancy of cytokines.
Functional receptor complexes for the IL-6 cytokine family members all share gp130 as a component required for both ligand binding and signal transduction (Taga, 1996; Pennica et al., 1996c; Wollert et al., 1996; Taga et al., 1992). Hence, IL-6 family cytokines are often referred to as gp130 cytokines. Molecular cloning of gp130 cDNA has demonstrated the receptor to be comprised of six fibronectin type III modules, two of which show features typical of the class I cytokine receptor family (Hibi et al., 1990). Several gp130 cytokines (LIF, OSM, CNTF, CT-1) also require gp130-related protein LIFR (LIF receptor) as a part of their functional receptor complexes (Gearing et al., 1991; Gearing et al., 1992; Ip et al., 1992; Davis et al., 1993b; Pennica et al., 1995b). In fact, most of them have very low affinity to gp130 until it becomes a part of the LIFR/gp130 heterodimer.

Apart from the shared functional receptor components, IL-6, IL-11, OSM, and CNTF also have cytokine-specific receptors, often referred to as the α-receptors (Murakami et al., 1993; Yin et al., 1993; Hilton et al., 1994). IL-6 first binds to its α-receptor, IL-6R, and then two of these IL-6/IL-6R complexes associate with gp130 each, allowing the formation of a gp130 homodimer (Ward et al., 1994; Paonessa et al., 1995). A similar pattern has also been proposed for the formation of IL-11 functional receptor complex (Yin et al., 1993). OSM can either signal through the LIFR/gp130 heterodimer (Gearing et al., 1992; Liu et al., 1992), or through its α-receptor OSMR associated with a gp130 subunit (Thoma et al., 1994; Murakami-Mori et al., 1995). Structurally, OSMR is closely related to both LIFR and gp130. However, neither OSMR nor LIFR can bind OSM in the absence of gp130 subunit. The α-receptor for CNTF, CNTFRα, is structurally closely related to extracellular region of IL-6R, but is anchored to the cell
membrane via a glycosyl-phosphatidyl-inositol (GPI) linkage. CNTF binds to CNTFRα, followed by the recruitment of gp130 and LIFR (Ip et al., 1992; Davis et al., 1993b). This complex is actually hexameric, containing two CNTF and two CNTFRα proteins along with a LIFR/gp130 heterodimer (De Serio et al., 1995).

The α-receptors for IL-6, IL-11, and CNTF can also be found in a soluble form, and these soluble receptors can bind their ligands, just like their membrane-associated forms (Baumann et al., 1996b; Taga et al., 1989). Soluble receptors for IL-6 are found circulating in serum, while the ones for CNTF are found in cerebrospinal fluid and are released from skeletal muscle in response to peripheral nerve injury. This gives them the potential to confer cytokine responsiveness to cells expressing gp130 and LIFR that do not express membrane-associated forms of the receptor (Hibi et al., 1990; Davis et al., 1993a). Interestingly, the combination of IL-6 and soluble IL-6R (sIL-6R) can mimic not only the actions of IL-6, but also the actions of all other gp130 cytokines, on the cells expressing gp130. This is evident from the IL-6/sIL-6R ability to promote pluriopotentiality of ES cells, induce osteoclast formation in osteoblast/osteoclast progenitor cocultures, or cardiomyocyte hypertrophy (Yoshida et al., 1994; Wolf et al., 1994; Pennica et al., 1995a; Hirota et al., 1995; Tamura et al., 1993). These functions are normally observed with LIF, OSM, and CNTF in the ES cells, LIF, OSM, and IL-11 in osteoclast progenitor cells, or CT-1 in cardiomyocytes.

Functionally, it is the formation of the gp130/gp130 homodimer or the LIFR/gp130 heterodimer that is essential for the downstream signal transduction of gp130 cytokines. The function of the α-receptor is to recruit the ligand and aid in the
formation of signal-transducing dimers in response to the ligands that are themselves incapable of doing so.

The ubiquitous expression of gp130 in every cell type examined explains the pleiotropic nature of gp130 cytokine action, while shared usage of gp130 by all these cytokines in part explains the redundancy of their actions. Unlike gp130, LIFR and the specific α-receptors exhibit a more tissue-specific expression (Saito et al., 1992) and therefore contribute to the specificity of gp130 cytokine actions. Spatial and temporal expression of the individual cytokines is another factor that contributes to the specificity of their actions. For instance, following the hypoglossal nerve injury, IL-6 becomes expressed in neighboring Schwann cells, while IL-6R becomes detectable in the nerve cell body (Hirota et al., 1996).

Animals lacking IL-6, LIF, or CNTF do not exhibit the phenotypic abnormalities expected with the ablation of cytokines with such pleiotropic actions, probably due to the compensatory effects of other family members (Kopf et al., 1994; Stewart et al., 1992; Masu et al., 1993). However, some complications developing later in life were observed. Moreover, complete ablation of gp130 or LIFR is lethal (Yoshida et al., 1996; Ware et al., 1995). Mice lacking gp130 die during development, 12 days postcoitum, while LIFR deficient animals die within a day of birth. Histological examinations also reveal that these animals suffered from hypoplastic myocardium, severely decreased hemotopoietic cell numbers, loss of neurons, and other abnormalities, giving insight into the in vivo functions of gp130 and LIFR.

Following stimulation by gp130 cytokines, target cells undergo a wide variety of fates: growth promotion, growth arrest, differentiation, or the expression of specific
genes. The specificity of cytokine actions in a given cell type arises not only from the differences in the receptor and cytokine expression, but also from the differences in downstream signaling pathways activated by the cytokine.

Upon stimulation by gp130 cytokines, gp130 receptor itself undergoes homo- or heterodimerization governed by the phosphorylated tyrosine residues on the cytoplasmic region of the protein. This dimerization triggers the activation of cytoplasmic protein kinases associated with gp130 receptor. In the case of gp130, those kinases are JAK1, JAK2, and TYK2 (Ihle and Kerr, 1995; Darnell et al., 1994; Lutticken et al., 1994; Stahl et al., 1994; Stahl et al., 1995; Narazaki et al., 1994). Activated JAKs phosphorylate tyrosine residues in the distal part of gp130 and LIFR, and provide docking sites for SH2 domain-containing proteins such as STATs (Ihle, 1996). Even though the STAT family consists of 7 members, it is generally recognized that gp130 cytokines activate STAT 3 and, to a lesser extent, STAT 1 (Akira et al., 1994; Zhong et al., 1994b). Once recruited to the receptor, STATs can now initiate the JAK/STAT pathway, ultimately ending up in the nucleus, where STAT dimers bind DNA and modulate transcription.

Stimulation by gp130 cytokines can also lead to the activation of RAS/MAPK pathway (Satoh et al., 1992). In this case, phosphorylated tyrosine residues on gp130 serve as docking sites for SH2 domain-containing adapter protein Shc, which is then phosphorylated by JAKs (Kumar et al., 1994). Shc then forms a complex with Grb2 and SOS that is capable of activating RAS (Gurney et al., 1995). The activation of RAS leads to phosphorylation and activation of the Raf/ Mek/ ERK MAPK cascade, and its downstream effectors (Boulton et al., 1994; Daeipour et al., 1993).
1.4 Ciliary Neurotrophic Factor

Ciliary Neurotrophic Factor (CNTF) is a naturally occurring protein with a molecular mass of approximately 22 kDa. It was originally characterized as a trophic factor that supports the survival of embryonic chick ciliary ganglion neurons in vitro (Adler et al., 1979; Lin et al., 1989). However, subsequent cloning and sequencing of CNTF revealed that it is unrelated to neurotrophins, but is rather a member of the IL-6 cytokine family including IL-6, IL-11, LIF, OSM and CT-1 (Kishimoto et al., 1994; Stahl and Yancopoulos, 1994; Pennica et al., 1995b). Its amino acid sequence reveals it is a cytosolic protein with no signal peptide, and only one free cysteine residue (Sendtner et al., 1992; Rende et al., 1992; Friedman et al., 1992; Sleeman et al., 2000). Mammalian CNTF lacks a N-terminal secretion sequence and is thus not secreted. Instead, it is considered to be a lesion factor because it is known to be released from the ruptured glial cells to restore neuronal function following injury (Adler, 1993). CNTF shows a very low sequence similarity to other known proteins, including related cytokines. LIF, one of the most similar cytokines to CNTF, is only 15% homologous, while different mammalian CNTF proteins share about 80% homology with one another (McDonald et al., 1995; Stockli et al., 1989; Masiakowski et al., 1991; Negro et al., 1991). Chicken CNTF, also known as GPA, shares only about 50% homology with mammalian CNTF, and is known to be secreted (Leung et al., 1992; Reiness et al., 2001). As a member of the IL-6 cytokine family, CNTF was predicted to have a four $\alpha$-helix bundle topology similar to that of GH, G-CSF, PRL, and EPO, as well as that of other IL-6 family cytokines. This notion was proven to be true once the tertiary structure of CNTF had been solved by x-ray crystallography (McDonald et al., 1995) and NMR (Panayotatos et
Structurally, CNTF consists of four anti-parallel \( \alpha \)-helices (A, B, C, D) connected by two cross-over loops between A-B and C-D, and one short loop between B-C (Kallen et al., 1999). This structure confers both stability and receptor-binding specificity onto the CNTF molecule. Specifically, the residues R25 and R28 in helix A, Q63 and W64 in the A-B loop, Q74 in helix B, and D175 and R177 in helix D, have been shown to form an epitope for the CNTF-receptor recognition (Panayotatos et al., 1995; Inoue et al., 1995). Under physiological conditions, 40\( \mu \)M or below, CNTF is believed to exist in a monomeric form, but it does dimerize at concentrations above 40\( \mu \)M, and its crystal structure reveals a dimer (McDonald et al., 1995). Most cytokines function as dimers or trimers \textit{in vivo}. Residue A177 is believed to be important for the cytokine dimer formation, because the A117R CNTF mutant does not form dimers at any concentration (Panayotatos et al., 1995).

CNTF-specific receptor, CNTFR\( \alpha \), has been molecularly cloned as a 75 kDa protein, showing homology with the IL-6R (Davis et al., 1991). Upon translation, the C-terminus of CNTFR\( \alpha \) is cleaved. This region contains hydrophobic residues found in other transmembrane proteins. Hence, CNTFR\( \alpha \) has no transmembrane or cytosolic domains and is found only on the outer surface of the cell membrane. CNTFR\( \alpha \) is attached to the membrane by a GPI linkage, sensitive to PI-PLC treatment (Davis et al., 1991). It has been demonstrated that new protein synthesis is required to restore CNTF signaling upon the removal of CNTFR\( \alpha \) by PI-PLC (Huber et al., 1993). Initially CNTFR\( \alpha \) has been described as being distributed predominantly within neural tissues (Davis et al., 1991), but has since been reported in skeletal muscle, adrenal gland, sciatic nerve, skin, kidney, and testes (Ip et al., 1992). CNTFR\( \alpha \) cleaved from the cell surface
can exist and act in a soluble form. The soluble CNTFRα has been detected in the serum and the cerebrospinal fluid, and has been shown to initiate signaling in cells not responsive to CNTF alone (Davis et al., 1993a; Helgren et al., 1994). Few mutagenesis studies have also shown that mice lacking CNTF develop normally and appear to have no visible defects well into adulthood, when they develop minor loss of motor neurons (Masu et al., 1993). However, mice lacking CNTFRα tend to have severe motor neuron defects and die perinatally because they fail to initiate feeding behaviors (DeChiara et al., 1995). These results imply that CNTFRα plays an important role in the development of the nervous system, not only as a receptor for CNTF, but also as a receptor for another unknown CNTF-like factor. The finding that CNTF expression is undetectable in the feeding-relevant brain sites that express high levels of CNTFRα further supports this notion (Gloaguen et al., 1997).

CNTF signaling is initiated when CNTF binds CNTFRα, either in its soluble or membrane-bound form (Stahl and Yancopoulos, 1994). Once a CNTF/CNTFRα complex is formed, two of these heterodimers come together and recruit gp130 transducer protein, followed by a subsequent recruitment of LIFR protein. The resulting receptor complex is a hexamer of CNTF, CNTFRα, gp130, and LIFR in a 2:2:1:1 ratio, respectively (De Serio et al., 1995). Within this complex, CNTF and CNTFRα make direct contacts with all the complex components (Davis et al., 1991). Mutagenesis data from several sources now suggest specific sites and residues on CNTF involved in receptor complex interactions (Savino et al., 1994; Inoue et al., 1995; Bazan, 1991). Binding to CNTF also induces the formation of disulfide links between gp130 and LIFR within the complex. Aside from this hexameric, high-affinity binding complex, CNTF
can bind its receptors in a low-affinity manner (Huber et al., 1993). CNTF/CNTFRα is considered to be a low-affinity binding complex until further bound to gp130 and LIFR. This is especially evident in cases where CNTF can induce signaling in the absence of CNTFRα, solely by binding of the gp130/LIFR dimeric receptor (Gearing et al., 1994; Monville et al., 2001). Once the CNTF receptor complex is fully assembled and the signal is propagated, all the components of the receptor complex are believed to be endocytosed into a non-recycling intracellular pool (Alderson et al., 1999).

Unlike CNTFRα, both gp130 and LIFR contain membrane-spanning hydrophobic domain and a cytosolic domain (Stahl and Yancopoulos, 1994). Upon receptor complex assembly, gp130/LIFR dimerization occurs, leading to the activation of the associated JAKs. JAK 1, JAK 2, and JAK 3 kinases have been shown to be pre-associated with gp130/LIFR within the CNTF receptor complex. The activation of JAKs leads to the subsequent phosphorylation of the tyrosine residues on gp130 and LIFR, which can now act as docking sites for SH2 domain-containing proteins. Once these proteins dock, they themselves are phosphorylated by the JAKs and are capable of propagating the signal (Stahl et al., 1994). The STAT family of transcription factors, primarily STAT 3 and, to a lesser degree, STAT 1, are the main targets of JAKs following CNTF activation (Bonni et al., 1993). STAT 3 is phosphorylated at residue Y705, while STAT 1 is phosphorylated at Y701. Activated STAT proteins then form homo or hetero dimers and subsequently translocate to the nucleus (Bonni et al., 1993; Wegenka et al., 1993), where they initiate transcription of genes such as c-fos (Halvorsen et al., 1996; Larkfors et al., 1994) and tis-11 (Ip et al., 1992). Also, specific members of the SOCS protein family, especially SOCS-3, are induced in response to CNTF (Bjorbaek et al., 1999). The
RAS/MAPK pathway can also be engaged by CNTF via Shc, Grb2, Raf, and Mek cascade (Boulton et al., 1994; Kumar et al., 1994). Shc is a SH2 domain-containing adapter protein that can dock on the phosphorylated gp130 within the receptor complex (Inoue et al., 1995). Apart from the JAK/STAT and RAS/MAPK pathways, CNTF can lead to the activation of PLCγ and PI3K-p110 via direct associations with LIFR and indirect associations with gp130 (Bjorbaek et al., 1999). PTP-1D, and pp120 src substrate, can also be activated via the CNTF receptor complex (Bonni et al., 1993; Boulton et al., 1994).

As previously mentioned, CNTF was first identified as a trophic factor for the motor neurons in the ciliary ganglion, and was later found to act on other motor neuron populations (Sendtner et al., 1991). Thus, it was readily evaluated as a therapeutical tool in patients suffering from motor neuron diseases such as amyotrophic lateral sclerosis (Miller et al., 1996). However, during these trials, CNTF administration resulted in unexpected weight loss (ALS CNTF Treatment Study Group, 1996). This finding implied that CNTF might act in a manner similar to cachectic cytokines, but subsequent studies showed that it is more likely to act via a leptin-like mechanism. Cloning of the leptin receptor OBR showed that it was closely related to the components of the CNTF receptor complex (Davis et al., 1993b). Further studies showed that, like leptin, CNTF can activate same signaling molecules, and that CNTFRα is co-localized with OBR in the hypothalamic nuclei involved in feeding (Baumann et al., 1996a; Carpenter et al., 1998; Ghilardi et al., 1996; Vaisse et al., 1996). Even though it is not secreted, CNTF can readily cross the blood-brain barrier in a manner similar to leptin (Pan et al., 1999). CNTF treatment of leptin-deficient ob/ob mice was found to reduce adiposity,
hyperphagia, and hyperinsulinemia associated with this genotype. Leptin administration has the same effect in these animals. However, unlike leptin, CNTF also corrected obesity-related phenotypes in leptin-resistant, OBR-deficient, db/db mice, and in DIO mice partially resistant to leptin (Gloaguen et al., 1997; Lambert et al., 2001). CNTF and its synthetic analog Axokine® have also been found to suppress NPY gene expression (Xu et al., 1998), and pCREB in the feeding-relevant brain sites (Lambert et al., 2001). The weight loss caused by CNTF administration is due to the preferential loss of fat (Henderson et al., 1996). It is believed to occur by resetting the hypothalamic weight set point, such that cessation of CNTF treatment does not result in overeating and rebound weight gain (Lambert et al., 2001). Unlike cachectic cytokines, the appetite diminution during CNTF treatment is not due to stress, inflammatory responses, nausea, or conditioned taste aversion, but rather due to the modification of NPYergic signaling (Lambert et al., 2001; Pu et al., 2000). This is obvious when we consider that central infusions of NPY can completely reverse weight-reducing effects of CNTF (Pu et al., 2000).

Considering its potency and specificity in all experimental forms of obesity, it is clear why CNTF, as well as other gp130 cytokines, should be further investigated as an effective treatment for obesity and type 2 diabetes.

1.5 Cardiotrophin

Cardiotrophin (CT-1) was first identified in a screen of a cDNA library derived from the mouse embryoid bodies that had previously been shown to display spontaneous contractile activity and express cardiac-specific gene markers (Pennica et al., 1995a). Conditioned media from these bodies has been shown to support in vitro cardiomyocyte
survival and hypertrophy. CT-1 is a naturally occurring protein, 200 amino acids long, with a molecular mass of approximately 21.5 kDa (Pennica et al., 1996b). Rat and human CT-1 sequences display 94% and 79% amino acid homology with the murine CT-1, respectively (Ishikawa et al., 1996). Further sequence analyses and structural considerations have shown that CT-1 is a member of the IL-6 cytokine family (Pennica et al., 1995a). The members of this family do not share a great deal of primary amino acid sequence homology (14-24%) (Rose and Bruce, 1991), but they do share a common four \( \alpha \)-helix bundle topology (Robinson et al., 1994) and utilize gp130 signal transducer protein in their receptor complexes (Kishimoto et al., 1995).

CT-1 lacks the conventional hydrophobic amino terminal secretion sequence, but is found in the media of transfected cells. The coding regions of human and murine CT-1 are contained on three separate exons spanning 6-7 kbp of genomic DNA. Human CT-1 was localized to chromosome 16p11.1 p11.2. (Pennica et al., 1996b). CT-1 mRNA expression has been detected at high levels in the heart, skeletal muscle, prostate, ovaries, and liver, as well as fetal heart, lung, and kidney. Lower amounts were detected in the thymus, small intestine, lung, kidney, pancreas, testes and the brain, while no expression was found in the spleen (Pennica et al., 1995a). Both functional and receptor binding studies in cultured cardiomyocytes have shown that CT-1 signals through the gp130/LIFR heterodimer without the further requirement for the \( \alpha \)-subunit. (Wollert et al., 1996; Pennica et al., 1995b; Pennica et al., 1996a) However, CT-1 signaling in neuronal cells may require an additional \( \alpha \)-subunit. Cross-linking of iodinated CT-1 to the cell surface of neuronal cell lines has identified this \( \alpha \)-subunit as an 80 kDa (45 kDa after N-linked deglycosylation) protein present in the receptor complexes along side
gp130 and LIFR (Wollert et al., 1996; Robledo et al., 1997). These studies have also shown that CT-1 binds the LIFR with the equivalent affinity as LIF, but fails to bind gp130 alone. However, the addition of gp130 enhances its binding to LIFR. From these observations, it has been deduced that CT-1 initially binds LIFR with a low affinity, followed by the recruitment of gp130 into a high affinity binding complex (Pennica et al., 1995b). The formation of gp130/LIFR complex triggers the activation and auto/trans-phosphorylation of receptor associated JAK1, JAK2, and TYK2 kinases (Narazaki et al., 1994; Lutticken et al., 1994; Stahl et al., 1994), as well as other non-receptor tyrosine kinases (Hck, Btk, Tec, Fes) (Ernst et al., 1994; Matsuda et al., 1995b; Matsuda et al., 1995a), and SH-2 domain signaling molecules (Zhong et al., 1994b; Guschin et al., 1995; Gerhartz et al., 1996) resulting in the subsequent activation of associated signaling pathways. The pattern of kinase activation appears to be cell type specific (Stahl et al., 1994).

The activation of JAK family kinases leads to the phosphorylation of the receptor subunits, which can then recruit STAT 1 and STAT 3, leading to their phosphorylation and nuclear translocation. There, the STAT dimers bind the cognate DNA response elements. Phosphorylated receptor subunits can also recruit SH-2 domain adapter Shc, leading to the complex formation with Grb2 and SOS (Kumar et al., 1994). This complex can then activate p21ras, leading to the subsequent, cell type specific, activation of raf-1, Mek, and ERK 1/2 MAP kinases (Nakafuku et al., 1992; Boulton et al., 1994).

As previously noted CT-1 was first identified as a factor promoting cardiac myocyte hypertrophy in vitro with activity at concentrations (0.1 nM) much lower than other IL-6 family cytokines. Later studies have revealed that this type of hypertrophy is
distinct from that induced by α-adrenergic stimulation in both cell morphology and gene expression pattern. Namely, the increase in cell size is caused by an increase in length, not width, while the sarcomeric unit assembly occurs in series rather than in parallel (Wollert et al., 1996). CT-1 hypertrophy also results in the increased atrial natriuretic peptide (ANP) but, unlike α-adrenergic stimulation, it does not induce skeletal α-actin and myosin light chain-2 synthesis. This type of hypertrophy is closely related to the volume-overload hypertrophy during cardiac valve insufficiency.

Similarly, an in vivo chronic administration of CT-1 to rodents resulted in a dose dependent increases in both heart weight and ventricular weight, while the total body weight was unaffected (Jin et al., 1996). These results mimicked those of chronic in vivo stimulation of gp130 receptor (Hirota et al., 1995). Thus, CT-1 probably plays a role in cardiac development ensuring that the myocardium develops to the correct thickness (Yoshida et al., 1996). It has been suggested that this function of CT-1 is governed through its activation of the JAK/STAT pathway (Sheng et al., 1997).

Apart from its hypertrophic effects, CT-1 also has a cardioprotective effect in cardiomyocytes. CT-1 can enhance the survival of neonatal myocytes in serum free medium, as well as protect the cells against heat shock and simulated ischemia/hypoxia (Sheng et al., 1996; Stephanou et al., 1998). This effect was associated with the ability of CT-1 to induce the activation of C/EBPβ via the MAPK pathway, leading to the increased levels of hsp70 and hsp90, and increased phosphorylation of hsp27 (Heads et al., 1994; Heads et al., 1995; Cumming et al., 1996). Another aspect of the cardioprotective effect is that CT-1 minimizes the degree of apoptosis (Stephanou et al., 1998). This aspect of its function is currently under investigation.
Other functions of CT-1 have recently been elucidated. CT-1 was shown to elevate cardiac output and heart rate, while lowering mean arterial pressure and systemic vascular resistance (Jin et al., 1996). It has also been shown to reduce the expression of TNF-α in LPS treated animals (Benigni et al., 1996). Elevated TNF-α levels are correlated with myocardial infarction and chronic heart failure (Latini et al., 1994). Like other IL-6 cytokine family members, CT-1 can induce liver acute phase response (Peters et al., 1995). In vivo administration of CT-1 has also resulted in hypertrophy of the liver, kidneys, and spleen whilst causing atrophy of the thymus and increasing platelet and red blood cell counts (Jin et al., 1996).

Similarly to induction of ANP, CT-1 also induces the expression of the brain natriuretic peptide (BNP) (Kuwahara et al., 1998). CT-1 has also been shown to induce a switch in the transmitter phenotype of cultured sympathetic neurons to a cholinergic phenotype (Habecker et al., 1995). Most interestingly, CT-1 supports the long-term survival of spinal motor neurons, as well as dopaminergic neurons lost in Parkinson's disease, and ciliary ganglion neurons (Pennica et al., 1995b). CNTF can only mimic the effect on ciliary ganglions. However, all of these functions require the presence of the 80 kDa receptor subunit (Robledo et al., 1997).

Because of its ability to potently activate JAK/STAT pathway through the gp130/LIFR dimer, CT-1 can potentially affect adipocytes. Hence, these could possibly prove important in adipose function and metabolic regulation. Recently, it has been shown that the circulating levels of CT-1 are increased in the serum of patients with ischemic heart disease and valvular heart disease (Freed et al., 2003). Since the onset of cardiovascular disease (CVD) is associated with obesity/type 2 diabetes (Sowers and
Frohlich, 2004; Reaven *et al.*, 2004), the actions of CT-1 in fat may prove to be a link between the clinical aspects of these two deadly diseases, and should therefore be extensively studied.
CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 Materials

Dulbecco's Modified Eagle's Media (DMEM) was purchased from Life Technologies. Bovine and fetal bovine (FBS) sera were purchased from Sigma and Invitrogen, respectively. Rat recombinant CNTF and human recombinant CT-1 were purchased from Calbiochem. Recombinant mouse IL-6 and recombinant human OSM were purchased from Bio Source International. Mouse recombinant LIF was purchased from Chemicon International. Insulin, human recombinant GH, leupeptin, chloroquine, cycloheximide, cytochalasin B, and collagenase were all purchased from Sigma. Epoxomicin and MG132 were purchased from Boston Biochem. U0126 was purchased from Promega, DNase I and Trizol were purchased from Invitrogen, and PNGaseF was purchased from New England BioLabs. All STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories or polyclonal IgGs purchased from Santa Cruz. The highly phospho-specific polyclonal antibodies for STAT 1 (Y\textsuperscript{701}), STAT 3 (Y\textsuperscript{705}), and STAT 5 (Y\textsuperscript{694}) were IgGs purchased from BD Transduction Laboratories and Upstate Biotechnology, Inc. LIFR, gp130, and SREBP-1 antibodies were rabbit polyclonal IgGs purchased from Santa Cruz. CNTFRα antibody was a mouse monoclonal IgG purchased from BD Transduction Laboratories. PPARγ antibody was a mouse monoclonal IgG purchased from Santa Cruz. ERK1/ERK2 antibody was a rabbit polyclonal IgG purchased from Santa Cruz. Active ERK and active Akt (S\textsuperscript{473}) antibodies were rabbit polyclonal IgGs purchased from Cell Signaling Technology. Akt and FAS antibodies were rabbit polyclonal IgGs purchased from BD Transduction Laboratories.
IRS-1 antibody was a polyclonal IgG purchased from Upstate Biotechnology, Inc. The phospho-specific IRS-1 (Y^{896}) antibody was a polyclonal IgG purchased from Biosource International. HRP-conjugated streptavidin used for detection of ACC was purchased from Pierce. LPL antibody was a monoclonal IgG purchased from R & D Systems. Acrp30 antibody was a polyclonal IgG purchased from Affinity Bioreagents. C/EBPα antibody was a rabbit polyclonal IgG provided by the lab of Dr. Ormond MacDougald (Ann Arbor, MI), and GLUT 4 antibody was a rabbit polyclonal IgG provided by the lab of Dr. Paul Pilch (Boston, MA). HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch. Enhanced chemiluminescence (ECL) kit was purchased from Pierce. Nitrocellulose and Zeta Probe-GT membranes were purchased from BioRad.

2.2 Cell Culture

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

2.3 Preparation of Whole Cell Extracts

Monolayers of 3T3-L1 preadipocytes or adipocytes were rinsed with phosphate-buffered saline (PBS) and then 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 (Nonidet P-40), 1 µM PMSF, 1 µM pepstatin, 50 trypsin inhibitory milliunits of
aprotinin, and 10 µM leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 minutes on ice and centrifuged at 15,000 rpm at 4°C for 15 minutes. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions.

2.4 Preparation of Nuclear and Cytosolic Extracts

Cell monolayers were rinsed with PBS and then harvested in a nuclear homogenization buffer (NHB) containing 20 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM MgCl₂. Igepal CA-630 (Nonidet P-40) 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 1500 rpm for 5 minutes. Supernatants were saved as cytosolic extract, and the nuclear pellets were resuspended in half the volume of NHB and were centrifuged as before. The pellet of intact nuclei was resuspended again in half of the original volume of NHB and centrifuged again. A small portion of the nuclei was used for Trypan Blue staining to examine the integrity of the nuclei. The majority of the pellet (intact nuclei) was 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. Nuclei were extracted for 30 minutes on ice and then placed at room temperature for 10 minutes. Two hundred units of DNase I was added to each sample, tubes were inverted and incubated an additional 10 minutes at room temperature. Finally, the sample was subjected to centrifugation at 15,000 rpm at 4°C for 30 minutes. Supernatants containing nuclear extracts were analyzed for protein content, using a BCA protein assay kit (Pierce).
2.5 SDS Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Proteins were separated in 5%, 7.5%, 10% or 12% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (Laemmli, 1970) and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% fat-free milk for 1 hour at room temperature. Results were visualized with HRP-conjugated secondary antibodies and enhanced chemiluminescence.

2.6 RNA Analysis

Total RNA was isolated from cell monolayers with Trizol according to 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 (Stephens and Pekala, 1991). Probes were labeled by random priming using the Klenow fragment and [α³²P] dATP.

2.7 Determination of [³H] 2-Deoxyglucose Uptake

The assay of [³H] 2-deoxyglucose uptake was performed as previously described (Stephens and Pekala, 1991). 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 [³H] 2-deoxyglucose uptake in the presence of 5 µM cytochalasin B. Nonspecific uptake and absorption was always less than 10% of the total uptake.
2.8 Rodent 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(Nonidet P-40), 1 µM PMSF, 1 µM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, and 10 µM leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 minutes at 5,000 rpm to remove any debris and insoluble material and then analyzed for protein content. Adipocyte and stromovascular fractions were isolated from the epididymal fat pads of male C57Bl/6J mice by collagenase digestion. Seven week old ob/+ and ob/ob mice were purchased from Jackson Laboratories. Eight week old fa/+ and fa/fa rats were purchased from Harlan. For DIO experiments, 3-5 week old C57Bl/6J mice were placed on a high fat/high sucrose diet (Research Diets # 12331 - Surwit diet) or low fat/high glucose sucrose diet (Research Diets # 12329). Twelve week-old transgenic mice expressing agouti protein under the control of the β-actin promoter, and all other C57Bl/6J mice were obtained from a colony at the Pennington Biomedical Research Center. All animal studies were carried out with protocols which were reviewed and approved by institutional IACUCs.
CHAPTER 3: EFFECTS OF CNTF ON ADIPOCYTES*

3.1 Results

The sensitivity of 3T3-L1 cells to cytokine treatment was examined by treating undifferentiated preadipocytes and fully differentiated 3T3-L1 adipocytes with an acute treatment of 0.8 nM CNTF or 0.8 nM LIF. As shown in Figure 3.1, immunoblotting of

![Immunoblot Image]

**Figure 3.1** The effects of acute CNTF or LIF treatment on 3T3-L1 preadipocytes and adipocytes. Whole cell extracts were prepared from confluent undifferentiated preadipocytes, and from fully differentiated 3T3-L1 adipocytes following a 15 min. treatment with CNTF (0.8 nM) or LIF (0.8 nM). Whole cell extracts were prepared and 75 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

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whole cell extracts demonstrated that both preadipocytes and adipocytes express STAT 3. Treatment of both preadipocytes and adipocytes with LIF or CNTF resulted in the rapid activation of STAT 3, as evident by increased tyrosine phosphorylation. However, treatment of preadipocytes resulted in a greater stimulation of STAT 3 activation, relative to adipocytes, despite equivalent expression of STAT 3 protein. In addition, CNTF and LIF treatment caused a robust activation of Akt in preadipocytes, whereas the same treatment of adipocytes did not result in a detectable activation of Akt. The expression of two CNTF receptor complex proteins, LIFR and gp130, was also examined. LIFR was expressed at a substantially higher level in preadipocytes than in adipocytes, whereas the expression of gp130 protein was not different in these two cell types.

The expression of CNTF receptor complex proteins was also examined during a time course of adipocyte differentiation. As shown in Figure 3.2A, the expression of CNTFRα protein decreases notably after 15 minutes of induction of differentiation and this lower level of expression is maintained for 48 hours. However, there were no detectable levels of CNTFRα 72 hours after the initiation of differentiation in whole cell extracts. Yet, we did observe the presence of CNTFRα at lower levels in the media up to 72 hours (Figure 3.2B). As indicated in Figure 3.1, the expression of LIFR decreased during adipogenesis and there was a slight modulation of gp130 expression. The expression of STAT 5A is known to be induced during adipocyte differentiation and is shown as a positive control for adipogenesis.

Since the expression of two CNTF receptor complex proteins was reduced during adipogenesis, we wanted to determine whether these proteins were expressed in adipose tissue, and to compare the expression levels to other tissues. Whole cell extracts were
Figure 3.2. The expression of CNTF receptor complex proteins during adipocyte differentiation. Whole cell extracts and media samples were prepared from 3T3-L1 cells at various times following the induction of differentiation. Cells were induced to differentiate at 2 days post confluence with the addition of a differentiation cocktail containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxantine, 1.0 µM dexamethasone, and 1.7 µM insulin. After 48 hours this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this medium until utilized for experimentation. In each panel, 100 µg of corresponding extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

isolated from the various tissues indicated in Figure 3.3. Western blot analysis revealed lung, stomach, epididymal fat, spleen, heart, brain, testes, and skeletal muscle as tissues expressing both CNTFRα and LIFR. All of these tissues had comparable receptor expression levels, except for the brain, which had significantly higher levels of CNTFRα.
expression. Also, the molecular weight of CNTFRα in stomach and brain was greater than in other tissues. In agreement with our earlier observations (Figure 3.2), the

Figure 3.3  Tissue distribution of CNTF receptor complex components in rodents. Tissue extracts were prepared from eight-week old Sprague-Dawley rats. Seventy five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Whole cell extracts from 3T3-L1 preadipocytes and adipocytes, as well as tissue extracts from the epididymal fat pads from lean and obese Zucker rats, were also examined by Western blot analysis as positive controls.
expression of CNTFRα was abundant in preadipocytes and undetectable in 3T3-L1 adipocytes. We also observed that the expression of CNTFRα was upregulated in the epididymal fat pad of an obese Zucker rat as compared to a lean littermate.

To determine if the altered mobility of CNTFRα was due to glycosylation, tissue extracts were incubated with PNGaseF. As shown in Figure 3.4, treatment with PNGaseF resulted in deglycosylation of CNTFRα and LIFR. In particular, the CNTFRα bands of larger molecular weights (brain and stomach) co-migrated with the CNTFRα from other tissues following digestion, indicating that the size difference between CNTFRα in these tissues was due to different glycosylation patterns. Also, all LIFR bands migrated at the same molecular weight following PNGaseF treatment.

![Figure 3.4 Glycosylation of CNTF receptor complex components in rat tissues.](image)

Tissue extracts were prepared from eight-week old Sprague-Dawley rats. Eighty µg of each extract was incubated with 4 µL of PNGaseF (5,000 U/µl) as directed by the manufacturer’s instruction and then separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
Although our results demonstrate that fully differentiated 3T3-L1 adipocytes do not express CNTFRα, it has previously been demonstrated that CNTF can signal via gp130 and LIFR in the absence of CNTFRα (Gearing et al., 1994; Monville et al., 2001). Therefore, we examined the ability of CNTF to activate STATs in a time dependent manner in 3T3-L1 adipocytes. Serum-deprived fully differentiated 3T3-L1 adipocytes were harvested at the times indicated in Figure 3.5 and fractionated into cytosolic and nuclear extracts. As shown in Figure 3.5A, CNTF administration to 3T3-L1 adipocytes resulted in the nuclear translocation of STAT 3. STAT 3 was present in the nucleus after a 10 or 30 minute treatment with CNTF and the amount of STAT 3 nuclear protein was decreased after a 1 hour treatment. After 2 hours, there was little STAT 3 present in the nucleus. CNTF treatment did not result in the activation/nuclear translocation of STAT 1 or STAT 5B, indicating the specificity of the response. Also, CNTF did not effect the distribution of STAT 5A. Unlike other adipocyte expressed STATs, some STAT 5A is always present in the adipocyte nucleus (Balhoff and Stephens, 1998). The dose dependent effects of CNTF on 3T3-L1 adipocytes were also examined by treating adipocytes for 10 minutes with varying concentrations of CNTF. As shown in Figure 3.5B, CNTF had no effect on STATs 1 or 5A, but resulted in the tyrosine phosphorylation and nuclear translocation of STAT 3. In addition, CNTF treatment resulted in a dose dependent activation of MAPK (ERKs 1 and 2). To assess the dose effects of CNTF on STAT 3 activation, we compared STAT 3 activation in preadipocytes and adipocytes. As shown in Figure 3.5C, CNTF results in a dose dependent effect on STAT 3 activation in preadipocytes, but not in adipocytes.
Figure 3.5  Time and dose dependent effects of CNTF administration on the phosphorylation and nuclear translocation of STAT proteins in 3T3-L1 cells.
Cytosolic and nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes following a treatment with (A) 0.8 nM CNTF for the times indicated, or after (B) 10 min. CNTF treatment at the doses indicated. (C) Whole cell extracts were prepared from both preadipocytes and from fully differentiated 3T3-L1 adipocytes following a 10 min. treatment with CNTF at the doses shown in the figure. Seventy five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
A

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- 91 kDa
- 92 kDa
- 95 kDa

B

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<td>0 0.1 0.4 0.8 1.6 3.2</td>
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- 91 kDa
- 92 kDa
- 95 kDa
- 42/44 kDa
- Active MAPK
- MAPK

C

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- 92 kDa
- 92 kDa
- 60 kDa
- CNTFRα
To further characterize the effects of CNTF, fully differentiated 3T3-L1 adipocytes were treated for a 12 hour period. As shown in Figure 3.6, acute CNTF treatment resulted in a time dependent activation of STAT 3 and MAPK, but was unable to activate Akt. A positive control for Akt activation (10 min. treatment of 3T3-L1 adipocytes with 50 nM insulin) is shown in the bottom panel of the figure. Acute CNTF treatment did not affect the expression levels of STATs 1, 3, and 5A, or the 67 kDa active form of SREBP-1.

Next, we examined the effects of chronic CNTF administration on the expression of adipocyte transcription factors and other adipocyte proteins. Fully differentiated 3T3-L1 adipocytes were exposed to CNTF over a 96 hour period. A fresh bolus of CNTF was added to the cells every 24 hours. Whole cell extracts were isolated at the times indicated in Figure 3.7 and subjected to Western blot analysis. Chronic administration of CNTF did not alter the expression of adipocyte expressed STATs, PPARγ, or C/EBPα. Also, there were no notable differences in the levels of gp130 and LIFR expression, and chronic CNTF treatment was insufficient to induce the expression of CNTFRα. A positive control of confluent preadipocytes is shown for CNTFRα expression. We also observed that CNTF treatment did not alter the expression of Acrp 30 in 3T3-L1 adipocytes. Moreover, CNTF had no effect on the expression or secretion of leptin from 3T3-L1 adipocytes (data not shown). Interestingly, CNTF treatment resulted in a decrease of fatty acid synthase (FAS) expression and a substantial increase in the expression of IRS-1. Also, the levels of the 67 kDa SREBP-1 protein were slightly decreased by CNTF treatment after 72 hours.
Figure 3.6 The effects of acute CNTF administration on the expression of adipocyte proteins. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes treated with 0.8 nM CNTF for the times shown. Seventy five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

Although we did not observe substantial levels of CNTFRα in cultured 3T3-L1 adipocytes (Figures 3.2, 3.3, and 3.5), we were able to detect the expression of CNTFRα in rodent adipose tissue from an obese Zucker rat and the levels of this receptor appeared to be up regulated in conditions of obesity (Figure 3.3). Hence, we examined the
Figure 3.7 The effects of chronic CNTF administration on the expression of adipocyte proteins. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes treated with 0.8 nM CNTF for the times indicated at the top of the figure. Seventy five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
expression of CNTF receptors in adipose tissue of additional rodent models of obesity/type 2 diabetes. Whole cell extracts were prepared from epididymal fat pads of five ob/ob and five ob/+ lean littermates. As shown in Figure 3.8A, we observed very little expression of CNTFRα in the adipose tissue of lean mice, but observed a substantial increase in the expression of this receptor in three of the five obese insulin resistant ob/ob littermates. In the other two ob/ob mice, there was a modest increase in CNTFRα expression. In addition, we observed increased LIFR expression in all five ob/ob mice compared to lean littermates, but did not observe any substantial changes in gp130 expression. We also examined the expression of these proteins in the epididymal fat pads of fa/+ and fa/fa rats. As shown in Figure 3.8B, the expression of CNTFRα was also substantially up regulated in this rodent model of obesity/type 2 diabetes. However, we did not observe an increase in LIFR levels in the fa/fa rats as compared to their lean littermates, although there was a modest increase in gp130 levels in adipose tissue from fa/fa rats. We also examined the expression of these receptors in transgenic mice that over express agouti under the control of the β-actin promoter, a condition which causes obesity and type 2 diabetes (Klebig et al., 1995). As shown in Figure 3.8C we observed a substantial increase in CNTFRα levels in the epididymal fat pads of three obese transgenic mice (Tg/+ ) compared to wild type lean (+/+ ) mice. There was also a modest decrease in LIFR and gp130 in the fat pads of mice with agouti-induced obesity. Finally, we examined the expression of CNTF receptors after low fat or high fat feeding in C57B1/6J mice. Seven mice from each condition were analyzed for CNTF receptor expression. The results in Figure 3.8D only include three animals per condition.

However, this pattern of regulation was observed for all seven animals examined
Figure 3.8 The expression of CNTF receptor components in the adipose tissue of lean and obese rodents. Whole cell extracts were isolated from the epididymal fat pads of (A) seven-week old ob/+ (lean) and ob/ob (obese) littermate mice, (B) eight-week old fa/+ (lean) and fa/fa (obese) littermate rats, (C) twelve-week old lean mice or obese agouti (Tg+/+) littermates, and (D) seventeen-week old C57B1/6J mice fed a low or high fat diet for twelve weeks. In each panel, 75 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
for each condition (data not shown). In C57B1/6J mice, we observed an increase in CNTFRα levels with high fat feeding after twelve weeks. A similar pattern was also observed after seven weeks (data not shown). Overall, there was no modulation of LIFR or gp130 with high fat feeding in the C57B1/6J mice.

We have shown that cultured adipocytes do not express CNTFRα, but rodent adipose tissue expresses detectable levels of the receptor. Therefore, we examined the ability of CNTF to activate STAT 3 in vivo. C57B1/6J mice were given an intraperitoneal injection of CNTF (33.3 µg/kg) or vehicle (saline) control. Fifteen minutes after the injection, the mice were sacrificed and epididymal adipose tissue, brains, and skeletal muscle were immediately removed and frozen in liquid nitrogen. Whole cell extracts were prepared from these tissues and analyzed for STAT 3 phosphorylation by Western blot analysis. As shown in Figure 3.9A, acute CNTF treatment resulted in the activation of STAT 3 in epididymal adipose tissue. We were unable to detect STAT 3 phosphorylation in the adipose tissue of five saline injected mice, but four of the five CNTF injected mice had readily detectable levels of phosphorylated STAT 3. The increase in STAT 3 phosphorylation was not due to increased STAT 3 expression. Also, the expression of LIFR was not changed and the levels of CNTFRα were variable in the ten mice. The results in Figure 3.9B demonstrate constitutive STAT 3 phosphorylation in brain, which was unresponsive to exogenous CNTF. Moreover, we observed an increase in STAT 3 phosphorylation in the skeletal muscle of CNTF treated animals, as compared to saline controls. As previously indicated (Figure 3.3), the levels of CNTFRα in the brain are substantially greater than the levels in skeletal muscle.
Figure 3.9 *In vivo* effect of acute CNTF administration in rodents. Six-week old male C57B1/6J mice were given an intraperitoneal injection of CNTF (33.3 µg/kg) or vehicle (saline) control. Fifteen minutes after the injection the mice were sacrificed and epididymal fat pads, brains, and skeletal muscle were immediately removed and frozen in liquid nitrogen. Tissue extracts were analyzed from (A) epididymal fat pads and (B) brain and skeletal muscle. In each panel, 75 µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
Figure 3.10 In vivo expression of CNTFRα in epididymal fat pads. Epididymal fat pads were extracted from six-week old lean C57B1/6J mice and fractionated into adipocyte and stromovascular fractions. Seventy five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

Our results demonstrate that CNTFRα receptor expression was decreased during the adipogenesis of 3T3-L1 cells, but expressed in the fat pads of rodents. Therefore, we fractionated epididymal fat pads from C57B1/6J mice to determine if the CNTFRα receptor was expressed in the stromovascular fraction or in the adipocytes. As shown in Figure 3.10, our results clearly demonstrate that CNTFRα is expressed highly in the adipocytes whereas STAT 3 is expressed at higher levels in the stromovascular portion. We hypothesize that the loss of CNTFRα that occurs during differentiation in vitro (Figure 3.2A) could be an artifact of cell culture since this receptor is expressed in native adipocytes (Figure 3.10) and in media obtained from cultured 3T3-L1 adipocytes (Figure 3.2B).
Figure 3.11 CNTF does not cause insulin resistance, but increases GLUT 4 expression. (A) Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes treated with 0.8 nM CNTF for the times shown. Seventy five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. (B) Fully differentiated 3T3-L1 adipocytes were treated with CNTF for 72 hours. A fresh bolus of CNTF was added to the cells very 24 hours. Monolayers of adipocytes were used to examine glucose uptake as indicated in the Experimental Procedures.
Figure 3.12 The effects of acute CNTF treatment on IRS-1 and Akt activation in 3T3-L1 adipocytes. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes treated with 0.8 nM CNTF or 50 nM insulin for the times indicated in the figure. Seventy five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

In light of the reports that CNTF administration to ob/ob, db/db, and DIO mice has been shown to improve insulin sensitivity in vivo, we examined the ability of CNTF to regulate insulin sensitive glucose uptake in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated for 24 hours with CNTF. As shown in Figure 3.11A,
CNTF treatment resulted in a notable increase (25-50%) in GLUT 4 levels. However, additional treatments of CNTF did not result in a further increase in GLUT 4 levels as chronic CNTF treatment did not substantially increase GLUT 4 mRNA or protein levels (data not shown). Therefore, we examined the ability of CNTF to affect glucose uptake. Fully differentiated adipocytes were treated for 72 hours with CNTF. Every 24 hours, cells were treated with a fresh bolus of CNTF. Acute insulin treatment (50 nM, 7 min.) resulted in a five-fold increase in insulin-stimulated glucose uptake and was relatively unaffected by chronic CNTF treatment (Figure 3.11B). In addition, CNTF had no effect on basal glucose uptake.

Since CNTF treatment resulted in an increase in IRS-1 expression levels (Figure 3.7), we examined the ability of this cytokine to induce IRS-1 activation, as judged by tyrosine phosphorylation at residue 896. As shown in Figure 3.12, acute insulin treatment (15 min.) results in the activation of IRS-1 and Akt, whereas acute CNTF treatment does not. However, CNTF pretreatment (30 min.) prior to insulin stimulation resulted in an increased IRS-1 activation (>20%) and increased Akt phosphorylation (>25%). The efficacy of the CNTF is demonstrated by the activation of STAT 3.

3.2 Discussion

In the light of recent findings demonstrating that CNTF administration results in weight loss and correction of many other obesity/type 2 diabetes related symptoms (Gloaguen et al., 1997; Henderson et al., 1996; Lambert et al., 2001; ALS CNTF Treatment Study Group, 1996), we hypothesized that the effects of this cytokine may not be limited to the CNS, and that CNTF may also have effects on peripheral tissues such as adipose tissue. Our in vitro studies using 3T3-L1 preadipocytes and adipocytes have
shown that CNTF indeed has significant, yet different, effects on these two cell types. In 3T3-L1 adipocytes we observed that CNTF was a potent activator of the Jak/STAT pathway, in particular STAT 3, as well as an activator of a MAPK signaling cascade that resulted in activation of ERKs 1 and 2. In preadipocytes CNTF elicited similar effects but also resulted in the activation of Akt. Our studies revealed that two of the three CNTF receptor components, LIFR and CNTFRα, were down regulated during the adipogenesis of 3T3-L1 cells. Our study clearly demonstrates that the expression of CNTFRα is substantially decreased during the course of adipocyte differentiation. Other studies have shown that CNTFRα is down regulated during astrocyte differentiation (Monville et al., 2001). A previous investigation had also indicated a decrease in LIFR during adipogenesis (Aubert et al., 1999b), but this is the first investigation to demonstrate a decrease in CNTFRα during adipogenesis. We hypothesize that decreased expression of CNTF receptors upon differentiation accounts for cultured adipocytes being less sensitive to CNTF treatment than preadipocytes, as judged by STAT 3 or Akt activation.

Although LIFR and CNTFRα protein levels are reduced in cultured adipocytes, as compared to preadipocytes, we observed that adipocytes were still responsive to CNTF. It has been previously demonstrated that CNTF can induce signaling in the absence of CNTFRα, solely by binding to a gp130:LIFR dimeric receptor (Gearing et al., 1994; Monville et al., 2001). Acute treatment of CNTF did not alter the expression levels of any STATs or any other adipocyte transcription factors in 3T3-L1 adipocytes. Hence, we examined the chronic effects of CNTF on 3T3-L1 adipocytes and observed that this cytokine affected the expression of several adipocyte-enriched proteins; including SREBP-1, FAS, GLUT4, and IRS-1. The reduction in the levels of SREBP-1 and FAS is
indicative of decreased biosynthesis of fatty acids that may account for some portion of weight loss and decreased fat mass observed in patients treated with CNTF (Axokine). In agreement with previous finding that this CNTF-induced weight loss was not due to cachexia or inflammation (Lambert et al., 2001; Pu et al., 2000), we did not observe any effect of CNTF on PPARγ or C/EBPα, two transcription factors known to be down regulated by inflammatory cytokines such as TNFα and IFNγ (Ganoth et al., 2001; Tanaka et al., 1999; Waite et al., 2001). Also, unlike the in vitro effects of other cytokines (Stephens et al., 1992; Stephens et al., 1993; Bastard et al., 2002; Waite et al., 2001), CNTF treatment of 3T3-L1 adipocytes did not result in the onset of insulin resistance (Figure 3.11). Moreover, chronic CNTF treatment of these cells actually resulted in an increase in both GLUT4 and IRS-1 protein levels. However, we did not observe any effects of CNTF on basal or insulin-stimulated glucose uptake. Clearly, additional experiments are required to determine if CNTF can act as an insulin sensitizer. Nonetheless, we have shown that CNTF appears to act synergistically with insulin to increase the level of IRS-1 and Akt phosphorylation in 3T3-L1 adipocytes.

Our results strongly suggest that CNTF affects adipose tissue and skeletal muscle in vivo since an acute intraperitoneal injection of CNTF resulted in STAT 3 activation in both tissues. We also observed that CNTFRα is expressed not only in brain and skeletal muscle, but also in adipose tissue, spleen, heart, testes, lungs and stomach. The receptor expression levels, as well as the protein size, vary among these tissues, but our deglycosylation studies clearly demonstrate that they all express CNTFRα. One of the most important findings we observed was that, in vivo, CNTFRα expression was significantly increased in four different rodent models of obesity/type 2 diabetes,
including both genetic and diet-induced obesity. Moreover, we have shown that CNTFRα is expressed at higher levels in the adipocytes as compared to the stromovascular portion of the fat pad (Figure 3.10). Although we observed an increase in the expression of the LIFR in the ob/ob mice, as compared to lean littermates, the expression of this receptor was not altered in the fa/fa rats or in C57Bl/6J mice with diet-induced obesity.

The results of our study suggest that CNTF and CNTFRα may play a role in the regulation of adipocyte metabolism and, perhaps, the control of adipose tissue mass. Our results have led us to hypothesize that CNTF can act as an insulin sensitizer in adipocytes. Therefore, the up regulation of CNTFRα in adipose tissue of obese/type II diabetic rodents could be an adaptive response attempting to increase insulin sensitivity. Interestingly, some studies suggest that CNTFRα may not only act as receptor for CNTF, but also as a receptor for another unknown CNTF-like factor. For example, mice lacking CNTF develop normally and appear to have no visible defects well into adulthood, when they develop minor loss of motor neurons (Masu et al., 1993). Yet, mice lacking CNTFRα tend to have severe motor neuron defects and die perinatally because they fail to initiate feeding behaviors (DeChiara et al., 1995). Also, the finding that CNTF expression is undetectable in the feeding-relevant brain sites, that express high levels of CNTFRα (Gloaguen et al., 1997), further supports the notion that CNTFRα may have additional ligands and/or functions.

In summary, we observed that native as well as cultured adipocytes are responsive to CNTF treatment. Interestingly, CNTFRα is not highly expressed in cultured adipocytes, but readily detectable in rodent adipose tissue and furthermore highly up
regulated in multiple rodent models of obesity/type 2 diabetes. This is the first demonstration that this receptor is expressed in adipose tissue and that it is highly regulated in obesity/type 2 diabetes. Current studies are underway to determine the role of CNTFRα in adipose tissue function and examine the ability of CNTF to act as insulin sensitizer in fat and muscle.
CHAPTER 4: EFFECTS OF CT-1 ON ADIPOCYTES

4.1 Results

In order to examine the sensitivity of 3T3-L1 cells to CT-1 administration, confluent 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipocytes were treated with CT-1 (0.20nM) for the times indicated in Figure 4.1. Western blot analysis of cell extracts revealed that both undifferentiated and differentiated 3T3-L1 cells responded to CT-1 treatment in a time dependent manner.

Figure 4.1  The effects of acute CT-1 treatment on 3T3-L1 preadipocytes and adipocytes. Whole cell extracts were prepared from confluent undifferentiated preadipocytes, and from fully differentiated 3T3-L1 adipocytes following a treatment with 0.2 nM CT-1 for the times indicated. One hundred and twenty five µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

Exposure to CT-1 resulted in a time dependent activation and tyrosine phosphorylation of STATs 1 and 3, as well as activation of MAPK (ERKs 1 and 2). The magnitudes of the responses were undistinguishable in undifferentiated and differentiated 3T3-L1 cells.
However, the tyrosine phosphorylation of STAT 3 was sustained for a longer period of time in preadipocytes. The total levels of MAPK are shown as a control for even loading.

The subcellular distribution of STAT proteins following CT-1 treatment was assessed by treating fully differentiated 3T3-L1 adipocytes with CT-1 (0.20nM) for various periods of time, followed by isolation of cytosolic and nuclear extracts. Western blot analysis of these extracts, shown in Figure 4.2, clearly demonstrated that CT-1 treatment results in the nuclear translocation of STATs 1, 3, 5A, and 5B, as well as the activation of MAPK.

Figure 4.2 The effects of acute CT-1 treatment on the activation and nuclear translocation of STAT proteins in 3T3-L1 adipocytes. Cytosolic and nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes following a treatment with 0.2 nM CT-1 for the times indicated. One hundred µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
For each STAT protein examined, nuclear translocation occurred within 15 minutes and returned to basal level within 2 hours following the cytokine treatment. This pattern is consistent with STAT activation by CNTF (Zvonic et al., 2003a) and other gp130 cytokines in 3T3-L1 adipocytes.

To further examine the ability of CT-1 to activate STATs in 3T3-L1 adipocytes in comparison to other gp130 cytokines, we exposed fully differentiated adipocytes to various doses of CT-1, CNTF, and LIF for 15 minutes.

![Figure 4.3: Dose dependent effects of CT-1 on 3T3-L1 adipocytes](image)

**Figure 4.3 Dose dependent effects of CT-1 on 3T3-L1 adipocytes.** Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes following a 15 min. treatment with CT-1, CNTF, or LIF, with the doses indicated in the figure. One hundred and fifty µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
Western blot analysis of whole cell extracts, shown in Figure 4.3, indicated that the activation of STATs 1, 3, and 5, and MAPK (ERKs 1 and 2) by CT-1 is dose dependent. Moreover, the STAT 3 activation was achieved at much lower doses of CT-1 than those required for activation of STAT 1 and STAT 5. As previously shown CNTF does not activate STAT 3 in a dose dependent manner (Zvonic et al., 2003a). CNTF treatment also did not result in either STAT 1 or STAT 5 activation. Treatment with 2.0 nM CNTF resulted in substantially less STAT 3 and MAPK activation when compared to 2.0 nM CT-1. LIF treatment also resulted in a dose dependent activation of STATs 1, 3, and 5, and MAPK, in a manner and dose comparable to that of CT-1. The total levels of MAPK are shown as a control for even loading.

To characterize in vivo effects of CT-1 on native adipocytes, seven-week old C57B1/6J mice were given an intraperitoneal injection of CT-1 (0.5µg/animal) or vehicle (saline) control and sacrificed after 15 minutes. Western blot analysis of whole cell extracts isolated from the epididymal fat pads of the animals, shown in Figure 4.4, demonstrated that the four mice injected with CT-1 had a significant increase in levels of active MAPK and tyrosine phosphorylated STATs 1 and 3. There was no detectable STAT activation in the four saline injected mice, although some active MAPK was observed. Interestingly, the activation of STAT 5 by CT-1 was not observed in this experiment. The total levels of MAPK are shown as a control for even loading, while an extract from 3T3-L1 adipocytes treated with GH was used as a positive control for STAT 5 activation (Zvonic et al., 2003b).

To assess the effect of chronic CT-1 treatment on 3T3-L1 adipocytes, fully differentiated adipocytes were exposed to CT-1 over a time course of 96 hours. A fresh
Figure 4.4  \textit{In vivo} effect of acute CT-1 administration in rodents. Seven-week old male C57B1/6J mice were given an intraperitoneal injection of 0.1 nM CT-1 (0.5 µg/animal) or vehicle (saline) control. Fifteen minutes after the injection the mice were sacrificed and epididymal fat pads were immediately removed and frozen in liquid nitrogen. One hundred and fifty µg of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

A bolus of CT-1 was added to the cells every 24 hours. As shown in Figure 4.5, chronic administration of CT-1 resulted in decreased protein levels of FAS and IRS-1 after 72 and 96 hours of treatment. The levels of ACC, the 67 kDa form of SREBP-1, and Akt were unchanged. In addition, there were no significant effects on the levels of STATS 1, 3, and 5A. Interestingly, CT-1 administration resulted in a transient downregulation of PPAR\textgreek{g} protein following a 24 hour CT-1 treatment. However, after 48 hours, PPAR\textgreek{g} levels had returned to basal levels and additional CT-1 treatments did not result in decreased PPAR\textgreek{g} following chronic (48-96 hrs) CT-1 treatment.
Figure 4.5 The effects of chronic CT-1 administration on the expression of adipocyte proteins. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes following a treatment with 0.2 nM CT-1 for the times shown. One hundred µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

The differentiation of 3T3-L1 preadipocytes into mature adipocytes is governed by a variety of cell signals. To determine whether CT-1 signaling can affect adipocyte differentiation we exposed differentiating preadipocytes to CT-1, as well as CNTF. Either
cytokine was added to the culture at the time of the induction of differentiation, and the cells were maintained in the presence of the cytokine for the entire course of differentiation. Whole cell extracts were collected at the various time points indicated in Figure 4.6, and were analyzed by Western blotting. Our results demonstrate that the treatment with neither CT-1 nor CNTF had any profound effects on adipocyte differentiation, as evident by the unaltered expression levels of various adipocyte markers such as PPARγ, IRS-1, STAT 5A, and ACC. Interestingly, the presence of CT-1 during 3T3-L1 differentiation resulted in a notable decrease in FAS expression. CNTF also had a modest effect on FAS expression. The presence of these cytokines did not effect the expression of their shared receptor complex proteins, LIFR and gp130, nor did it affect the expression levels of their downstream kinases, such as Akt and ERKs 1 and 2. The expression of PPARγ and STAT 5A are known to be induced during adipocyte differentiation and serve as obvious markers of successful adipogenesis.

Since CT-1 did not affect the adipogenesis of 3T3-L1 cells, we examined the effects of this cytokine on the expression of genes in fully differentiated 3T3-L1 adipocytes. Since this cytokine is a potent STAT activator, both in vitro and in vivo (Figure 4.2 and Figure 4.4), we hypothesized that activated STATs would induce a transcriptional modulation of their target genes. Serum deprived, fully differentiated 3T3-L1 adipocytes were treated with CT-1 (0.20nM) or CNTF (0.45nM) for various times, as indicated in Figure 4.7. Total RNA and whole cell extracts were collected following the treatment. As shown in Figure 4.7A, both CT-1 and CNTF administration resulted in a rapid induction of SOCS-3 mRNA levels. However, this upregulation is very transient following CNTF treatment, while CT-1 treatment, at a dose lower than that of CNTF,
Figure 4.6 The effects of CT-1 and CNTF treatment on the differentiation of 3T3-L1 adipocytes. Whole cell extracts were prepared from 3T3-L1 cells at various times following the induction of differentiation. Cells were induced to differentiate as described in Figure 3.2. A fresh bolus of CT-1 (0.2 nM) or CNTF (0.45 nM) was added to the cells every 24 hours during the course of differentiation. One hundred µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
Figure 4.7  The effects of acute CT-1 and CNTF administration on the expression of adipocyte proteins. Total RNA (A and C) or whole cell extracts (B) were isolated from fully differentiated 3T3-L1 adipocytes following a treatment with CT-1 (0.2 nM), CNTF (0.45 nM), or U0126 (5.0 µM) for the times shown. Twenty µg of each total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis. One hundred µg of each whole cell extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
resulted in a more sustained induction of SOCS-3 mRNA. Both cytokines also resulted in a transient downregulation of PPARγ mRNA, which was more evident following a 2 hour CT-1 treatment. The levels of aP2 mRNA were unchanged. The efficacy of the cytokines in this experiment was demonstrated by their ability to induce STAT 3 phosphorylation (Figure 4.7B). In order to determine whether the repression of PPARγ mRNA or induction of SOCS-3 mRNA induced by CT-1 was mediated through the ERK MAP kinase pathway, 3T3-L1 adipocytes were pretreated with U0126, a MEK inhibitor. The Northern blot analysis in Figure 4.7C clearly demonstrates that both SOCS-3 mRNA upregulation and PPARγ mRNA downregulation are not dependent on the activity of ERKs 1 and 2, because the addition of U0126 failed to prevent modulation of mRNA levels by CT-1.

4.2 Discussion

Cardiotrophin (CT-1) is a newly identified member of the gp130 cytokine family that signals via a receptor complex comprised of gp130 and LIFR signal transducer proteins. Recent work from our laboratory has shown that another member of the gp130 cytokine family, CNTF, can activate several signaling pathways and regulate protein expression levels in 3T3-L1 adipocytes (Zvonic et al., 2003a). These effects are likely to be induced by utilizing only gp130 and LIFR as signal transducers, since the specific CNTF receptor, CNTFRα, is not expressed in fully differentiated 3T3-L1 adipocytes (Figure 3.2A). Since, both IL-6 and CNTF have effects on body weight regulation (Henderson et al., 1996; Bastard et al., 2002), we have investigated the actions of CT-1 in vitro and in vivo to determine if this gp130 cytokine can induce similar signaling effects in fat cells, and regulate adipocyte gene expression.
Our results demonstrated that administration of CT-1 to both preadipocytes and 3T3-L1 adipocytes resulted in similar downstream responses. Interestingly, and in contrast to CNTF, CT-1 activated STATs 1 and 3, as well as ERKs 1 and 2 MAP kinases in both cell types, and the responses were of identical magnitudes, but slightly more sustained in preadipocytes. Since the levels of gp130 remain fairly unaffected by differentiation, and LIFR is expressed by mature adipocytes, both cell types have a significant population of these receptors on their cell surfaces. Therefore, both are likely activated by CT-1, and these cells are responsive to the administration of this cytokine. The attenuation of the response duration in adipocytes, as compared to preadipocytes, could be attributed to the slight loss of LIFR expression that occurs during adipocyte differentiation (Aubert et al., 1999a).

Further investigation of CT-1 actions in adipocytes showed that CT-1 was also capable of activating STATs 5A and 5B, in addition to STATs 1 and 3. STATs 5A and 5B in 3T3-L1 adipocytes were not activated by CNTF or other gp130 cytokines. Hence, their activation by CT-1 presents a rather novel finding and may, upon further investigation, prove to be a mechanism for the specificity of this cytokine's action. The activation of these proteins was confirmed through their detection in the nuclear fraction from the cells following cytokine treatment. STAT proteins translocated to the nucleus within 15 min after the treatment, and were mostly gone within 2 h. These are parameters consistent with STAT activation by other cytokines in adipocytes (Stephens et al., 1998). These data also indicate that STATs activated by CT-1 are not only tyrosine phosphorylated, but also move into the nucleus where they can act as transcription factors.
Since CNTF has not been shown to activate adipocyte STAT 3 and ERKs 1 and 2 in a dose dependent manner, we treated adipocytes with a range of CT-1 doses. Unlike CNTF, CT-1 was capable of activating STATs 1, 3, and 5, as well as ERKs 1 and 2, in a dose dependent manner. Activation of these proteins by CT-1 also occurred at much lower doses, and with higher magnitude of activation, if compared to that of CNTF. Once again, we hypothesize that this response is due to the abundance of gp130 and LIFR in adipocytes. Unlike CNTF, which has a limited affinity for gp130/LIFR dimer in the absence of CNTFRα, CT-1 is not limited by the absence of the cytokine-specific receptor component and can bind gp130/LIFR dimer with a very high affinity (Pennica et al., 1995b). LIF, a gp130 cytokine which also utilizes the gp130/LIFR dimer to transduce its signals, is capable of activating an identical profile of adipocyte STATs as CT-1, and also does so in a dose dependent manner. Thus, taking into account the results discussed so far, one can hypothesize that the availability of receptor component proteins can significantly affect the magnitude, duration, variety, specificity, and dose dependency of a cytokine response in a given cell type.

Since native adipocytes also express both gp130 and LIFR proteins, we investigated the in vivo effect of CT-1 administration on adipose tissue of C57B1/6J mice. Shortly after injection, CT-1 was able to activate STATs 1 and 3, as well as ERKs 1 and 2, in the epididymal fat pads of these animals, mimicking the in vitro effect in 3T3-L1 adipocytes. However, the dose administered (0.1 nM) was not sufficient to induce the activation of STAT 5. This is consistent with the dose dependency results from the in vitro studies. Even though further studies will be necessary to determine if CT-1 can activate STAT 5 at higher doses in vivo, it is still quite exciting to discover that this
cytokine directly affects fat tissue, and may potentially have biological roles in native adipocyte function.

In light of our results from the acute CT-1 administration, we also examined the effects of chronic CT-1 treatment in 3T3-L1 adipocytes. Upon 96 hours of treatment, CT-1 resulted in a decrease of FAS and IRS-1 protein levels. CNTF had an identical effect on FAS but an opposite effect on IRS-1 (Figure 3.7). The levels of STATs, Akt, and SREBP-1 remained constant throughout the treatment, unlike that of PPARγ. CT-1 first induced a transient decrease in the protein levels, consistent with the decrease in mRNA levels we observed in the acute treatment. However, PPARγ protein expression was then transiently upregulated before returning to the basal level. The potential ability of CT-1 to regulate PPARγ could prove to be interesting in relation to its effects on adipocytes. More importantly, the downregulation of IRS-1 expression by CT-1 could be a possible marker of impaired insulin sensitivity of these cells. Therefore, further work should be done to address this finding. Even though there are no reports of upregulated circulating levels of CT-1 in conditions of insulin resistance, this modulation of insulin resistance by CT-1 can serve as a model to study the effect of elevated circulating levels of CT-1 in the serum of patients with ischemic heart disease and valvular heart disease (Freed et al., 2003). Clinical aspects of these diseases are tightly linked to obesity/type 2 diabetes (Reaven et al., 2004; Sowers and Frohlich, 2004), while the principal cause of diabetes mortality is cardiovascular disease (CVD) (Nesto, 2003). Therefore, CT-1 may act as a link between obesity-related complications and CVD.

Adipocyte differentiation is marked by changes in the expression of several adipocyte-specific proteins. Since CT-1 and CNTF both activate the JAK/STAT pathway
in 3T3-L1 cells, we examined the ability of these cytokines to regulate adipogenesis. Unlike TNFα or IFNγ, the presence of CT-1 or CNTF did not inhibit adipocyte differentiation. These studies suggest that the anti-obesity effects of CNTF are not mediated by inhibiting fat cell differentiation. Interestingly, both cytokines induced a decrease in the expression of FAS, which we have achieved previously by a chronic CNTF treatment in adipocytes (Figure 3.7). However, the expression of ACC, a rate limiting enzyme in fatty acid synthesis was not affected by the treatment. Since CT-1 administration did not affect lipid accumulation (data not shown), we can not be certain whether CT-1 could potentially affect lipid biosynthesis in adipocytes. There was no evident change in the expression levels of LIFR, gp130, STATs, MAPK (ERKs 1 and 2), IRS-1, or Akt, indicating that neither of these cytokines regulate the expression of proteins involved in their own signaling and their further downstream actions. Most importantly, the expression of PPARγ, a key marker of adipogenesis, increased during the course of differentiation, and was not affected by either cytokine.

Although CT-1 and CNTF did not affect adipocyte differentiation, we further investigated the acute effects of these cytokines on fully differentiated 3T3-L1 adipocytes. Acute treatment of 3T3-L1 adipocytes with CNTF and CT-1 resulted in a significant induction of SOCS-3 mRNA levels. Within an hour, the levels returned to baseline in the CNTF treated cells, but remained elevated even after 8 hours in CT-1 treated adipocytes. However, this data was not very surprising due to the fact that SOCS-3 mRNA levels are regulated by activated STATs (Brender et al., 2001), and we have shown that the activation of STATs by CT-1, especially the activation of STAT 3, is temporally prolonged, compared to that induced by CNTF. Another interesting finding is
that both cytokines, especially CT-1, resulted in a transient decrease of PPAR\(\gamma\) mRNA levels. This result may be related to the transient decrease in the PPAR\(\gamma\) protein observed during the chronic administration (Figure 4.5). Previous studies (Tanaka et al., 1999), including one from our laboratory (Waite et al., 2001), have already shown that STATs activated by various cytokines regulate PPAR\(\gamma\) mRNA levels. Since CT-1 is predominantly known for its roles as an activator of the JAK/STAT and MAPK pathways, we were interested in determining which of these pathways was involved in the induction of SOCS-3 and the attenuation of PPAR\(\gamma\) mRNA levels. When MAPK pathway was inhibited by a specific MEK inhibitor U0126, we still observed a rise in SOCS-3 and a decrease in PPAR\(\gamma\) mRNA levels upon CT-1 treatment. Hence, our studies suggest that the MAPK pathway is not involved in CT-1 induced regulation of SOCS-3 and PPAR\(\gamma\) mRNA levels. Because the previous work from our lab has also shown that STAT 1 activated by IFN\(\gamma\) downregulates the expression of PPAR\(\gamma\) via the -221 to -207 site within the promoter (Hogan and Stephens, 2001), it would be interesting to investigate the ability of STAT 1 activated by CT-1 to bind this promoter element.

In summary, we have observed that CT-1 acts as a potent activator of both JAK/STAT and MAPK pathways in both preadipocytes and 3T3-L1 adipocytes, as well as in rodent fat pads in vivo. Unlike CNTF, CT-1 activation of these pathways is more robust, dose dependent, and sustained. This is also the first report of STAT 5 activation by a gp130 cytokine in 3T3-L1 adipocytes. Even though neither CNTF nor CT-1 had any significant effects on adipocyte differentiation, both cytokines induced transient changes in SOCS-3 and PPAR\(\gamma\) mRNA and protein levels in adipocytes, which mirrored the ability of the individual cytokine to activate the JAK/STAT pathway. Since other gp130...
cytokines have been implied as potential mediators of various aspects of obesity/type 2 diabetes, it would be interesting to further investigate the actions of CT-1 in this arena. However, the strongest focus should be placed on elucidating the actions of elevated levels of circulating CT-1 during obesity-related CVD, and the effects of this cytokine on adipose tissue in patients suffering from obesity/type 2 diabetes.
5.1 Results

One of the unifying features of all gp130 cytokines is their ability to activate JAK/STAT and MAPK (ERKs 1 and 2) signaling pathways. We therefore wanted to determine if gp130 cytokines can work synergistically to initiate these signals, or if their actions antagonize each other. In order to answer this question, we pretreated fully differentiated 3T3-L1 adipocytes with various gp130 cytokines for 2 hours, and then treated them for 15 minutes with either 0.2 nM CT-1, 0.45 nM CNTF, 0.1 nM LIF, or GH (125 ng/mL). As shown in Figure 5.1, gp130 cytokines primarily interacted in an antagonistic fashion. Results in Figure 5.1A demonstrate that the ability of CT-1 to activate either the JAK/STAT or the MAPK pathway can be impaired by pretreatment with several different gp130 cytokines. Consistent with our previous data (Figure 4.1), CT-1 treatment resulted in a robust activation of STAT 1 and STAT 3. However, a 2 hour pretreatment with LIF, OSM, or CT-1 completely blocked STAT 1 activation induced by a 15 minute CT-1 treatment. These same cytokines also interfered with the activation of STAT 3 by CT-1. In this case, LIF acted as the most potent inhibitor, while CT-1 and OSM only partially blocked the effects of acute CT-1 treatment. No appreciable STAT 5 activation occurred with CT-1 treatment, while GH pretreatment caused a robust STAT 5 activation detectable even at the end of experiment. The ability of LIF, OSM, and CT-1 to block further actions of CT-1 was also evident through their
Figure 5.1  gp130 cytokine administration affects cell signaling of their family members. Fully differentiated 3T3-L1 adipocytes were preincubated with either IL-6 (2 ng/mL), LIF (0.1 nM), OSM (0.5 ng/mL), GH (125 ng/mL), CNTF (0.45 nM), or CT-1 (0.2 nM) for 2 hours. The cells were then treated for 15 min. with either (A) CT-1, (B) CNTF, (C) LIF, or (D) GH. Following 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.
A

91 kDa

92 kDa

95 kDa

42/44 kDa

Active MAPK

STAT 1 Tyr$^{701}$

STAT 3 Tyr$^{705}$

STAT 5 Tyr$^{694}$

STAT 5A

B

91 kDa

92 kDa

95 kDa

42/44 kDa

Active MAPK

STAT 1 Tyr$^{701}$

STAT 3 Tyr$^{705}$

STAT 5 Tyr$^{694}$

STAT 5A

2 h pretreatment

15 min treatment
(Figure 5.1 cont.)

C

91 kDa  STAT 1 Tyr^{701}
92 kDa  STAT 3 Tyr^{705}
95 kDa  STAT 5 Tyr^{694}
95 kDa  STAT 5A
42/44 kDa  Active MAPK

D

91 kDa  STAT 1 Tyr^{701}
92 kDa  STAT 3 Tyr^{705}
95 kDa  STAT 5 Tyr^{694}
95 kDa  STAT 5A
42/44 kDa  Active MAPK

2 h pretreatment
15 min treatment

CTL  IL-6  LIF  OSM  GH  CNTF  CT-1
LIF

2 h pretreatment
15 min treatment

GH
effects on MAPK (ERKs 1 and 2) signaling, since the activation of this pathway by chronic CT-1 administration was attenuated by pretreatment with these cytokines.

Even though CNTF is not a potent activator of JAK/STAT or MAPK signaling in adipocytes (Figure 3.5), its ability to activate STAT 3 (Figure 5.1B) was affected by the pretreatment with other gp130 cytokines. As was the case with the chronic CT-1 treatment, 2 hour pretreatment of adipocytes with either LIF, OSM, or CT-1 significantly decreased the magnitude of STAT 3 activation by CNTF. These effects were not observed in relation to MAPK due to the fact that, in this experiment, CNTF failed to activate this pathway.

Data in Figure 5.1C recapitulates the fact that, in adipocytes, LIF acts as a potent activator of JAK/STAT and MAPK signaling. Still, its actions as a STAT 1 and STAT 5 activator were completely abolished by the 2 hour preincubation of adipocytes with LIF, OSM, or CT-1. An identical pattern of inhibition was also observed with the activation of MAPK (ERKs 1 and 2) by a 15 minute LIF treatment. In this experiment, acute LIF administration resulted in an extremely robust STAT 3 activation, and only the 2 hour pretreatment with LIF resulted in its slight attenuation.

Acute treatment with GH, shown in Figure 5.1D, also lead to a robust induction of JAK/STAT and MAPK signaling, as evident by the activation of STATs 1, 3, and 5, and ERKs 1 and 2. However, unlike the gp130 cytokines, the ability of GH to activate these pathways was not affected by a 2 hour preincubation with any of the gp130 cytokines. In fact, LIF, OSM, and CT-1 seemed to work synergistically with GH when activating STAT 3. The only inhibitory effect observed was the inhibition of GH by a 2 hour
preincubation with GH itself. STAT 5A levels were shown in all panels of Figure 5.1 as controls for even loading.

In order to confirm these findings, we used a similar experimental approach in an \textit{in vivo} model. C57B1/6J mice were injected with 0.05 nM CT-1 or the appropriate vehicle control, then injected again 4 hours later, and finally sacrificed 15 minutes following the second round of injections. As shown in Figure 5.2, intraperitoneal administration of CT-1 lead to the activation of STAT 3 in the mouse epididymal fat pads. Interestingly, MAPK (ERKs 1 and 2) appeared to be active even in animals injected with vehicle (saline) control. As expected, phosphorylated proteins were present in the cell extracts 15 minutes after the injection. We were also able to detect active STAT 3 and MAPK even 4 hours following CT-1 administration, at a level comparable to that of a 15 minute treatment. However, the animals injected with CT-1 for 4 hours, and then again for 15 minutes, showed no change in STAT 3 or MAPK activation, as compared to animals injected with CT-1 for either length of treatment. Total MAPK levels are shown as an even loading control.

Another common feature of gp130 cytokines is their shared use of gp130 and LIFR proteins as signal transducers within their receptor complexes. Therefore, we examined the effect of gp130 cytokine signaling on these proteins. As shown in Figure 5.3A, the expression level of LIFR in 3T3-L1 adipocytes dramatically decreases within 6 hours in the absence of \textit{de novo} protein synthesis (achieved through incubation with cycloheximide). Addition of LIF, a ligand for LIFR, further accelerates this degradation process, to where a significant decrease in LIFR levels is apparent within 1 hour of the treatment, and no protein is detectable after 4 hours. Addition of a proteasome inhibitor,
Figure 5.2 CT-1 pretreatment *in vivo* blocks acute CT-1 signaling in rodent fat pads. Six-week old male C57B1/6J mice were given an intraperitoneal injection of 0.05 nM CT-1 (0.25 µg/animal) or vehicle (saline) control. Four hours later the animals were injected again. Fifteen minutes after the second injection the mice were sacrificed and epididymal fat pads were immediately removed and frozen in liquid nitrogen. One hundred and fifty µg of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

MG132, failed to prevent the loss of LIFR protein, nor did it have an effect on accelerated protein loss following LIF treatment. However, the addition of leupeptin and chloroquine, which together act as a lysosome inhibitor, prevented the degradation of LIFR under basal conditions, or after LIF treatment.

These findings were recapitulated by the experiment shown in Figure 5.3B. In this case, we used a highly specific proteasome inhibitor, epoxomicin, in lieu of MG132. The data shown clearly indicate that the loss of LIFR induced by 6 hour incubation with
Figure 5.3 Administration of LIF alters the half-life of LIFR protein in adipocytes. (A) Fully differentiated 3T3-L1 adipocytes were treated with either 0.1 nM LIF, 20 μM MG132, a mixture of 200 μM chloroquine and 10 μM leupeptin (L/C), or a combination of LIF and the inhibitors. The treatments were done in the presence of 5 μM cycloheximide (CH). Whole cell extracts were prepared from treated adipocytes at the times indicated. (B) Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes treated for 6 hours with either 0.1 nM LIF, 100 nM epoxomicin (EPX), L/C, or a combination of LIF and the inhibitors, in the presence of CH. In each panel, 150 μg of extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.
**Figure 5.4 Half-life of LIFR is altered by gp130 cytokine administration.** Whole cell extracts were prepared from 3T3-L1 adipocytes treated with either LIF (0.1 nM), CT-1 (0.2 nM), CNTF (0.45 nM), OSM (0.5 ng/mL), IL-6 (2 ng/mL), or GH (125 ng/mL) for 4 hours in the presence of 5 µM cycloheximide (CH), or a mixture of CH and 200 µM chloroquine and 10 µM leupeptin (L/C). One hundred µg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

cycloheximide was not affected by epoxomicin, but was prevented with a combination of leupeptin and chloroquine. Furthermore, these effects were mirrored by the ability of leupeptin and chloroquine to prevent the complete loss of LIFR induced by LIF treatment. Epoxomicin was unable to generate the same effect. Total MAPK (ERKs 1 and 2) levels are also shown as a control for even loading.

In order to explain why LIF, CT-1 and OSM seemed to have prominent inhibitory effects on gp130 cytokine signaling, we examined the ability of all gp130 cytokines to affect the expression levels of LIFR protein in 3T3-L1 adipocytes. As shown in Figure 5.4, 4 hour incubation with cycloheximide induced a notable decrease in LIFR levels. However, addition of LIF, CT-1, or OSM to these cells resulted in a complete or, in case of OSM, nearly complete loss of LIFR expression. Interestingly enough, the addition of CNTF, IL-6, or GH did not induce a decrease in LIFR levels greater than the decrease
induced by cycloheximide. Consistent with the data from Figure 5.3, the loss of LIFR expression through the action of cycloheximide alone, or with LIF, CT-1, and OSM, was blocked by the addition of leupeptin and chloroquine to the cell media. Total MAPK (ERKs 1 and 2) levels are shown as controls for even loading.

5.2 Discussion

Synchronous functional redundancy and specificity is likely the most interesting attribute of gp130 cytokine biology (Paul, 1989; Kishimoto et al., 1992). Even though the specific mechanisms governing the actions of these cytokines are still unknown, there is a general consensus regarding several aspects of gp130 cytokine signaling. As their name suggests, all members of the gp130 cytokine family use gp130 protein as a transmembrane signal transducer within their functional receptor complexes (Taga et al., 1992; Taga, 1996). The presence of gp130 is crucial for the recruitment of other receptor complex components (Savino et al., 1994), and for the propagation of signal to the downstream targets, such as the components of the JAK/STAT or MAPK pathways (Narazaki et al., 1994; Lutticken et al., 1994). Several gp130 cytokines (LIF, OSM, CNTF, CT-1) also use LIFR as a part of their functional receptor complexes (Gearing et al., 1991; Gearing et al., 1992; Ip et al., 1992; Davis et al., 1993b; Pennica et al., 1995b). Both gp130 and LIFR are expressed in vivo as well as in cultured adipocytes (Aubert et al., 1999c; Zvonic et al., 2003a), and several studies have shown that various gp130 cytokines have the ability to induce intracellular signaling in these cell types (Path et al., 2001; Metcalf, 2003; Zvonic et al., 2003a).

The question posed by the findings in the above mentioned studies is whether gp130 cytokines can act synergistically to activate their downstream targets, or whether
their actions antagonize the individual cytokine’s ability to induce signaling. The results from our studies are a strong argument for the latter scenario. CT-1, LIF, and OSM demonstrated the ability to attenuate or, in some cases, completely abolish subsequent signaling by their family members. CNTF or IL-6 did not seem to have the same effect. However, the inhibitory effects of CT-1, LIF, and OSM were not observed with GH treatment, while GH was only able to interfere with its own signaling. This finding suggests that the attenuation of gp130 cytokine signaling does not occur at the level of intracellular signal transducers. Is this were the case, then GH signaling would have been affected as well, since this growth factor propagates its signal through the JAK/STAT and MAPK pathways in a manner similar to gp130 cytokines (Dinerstein-Cali et al., 2000). Interestingly, the results from our in vivo study were quite consistent with our findings. In this case, CT-1 was able to block subsequent CT-1 induction of STAT 3 activation. However, unlike in 3T3-L1 adipocytes, STAT 3 activation by CT-1 was sustained throughout the pretreatment period. However, no additional STAT 3 activation occurred after subsequent CT-1 injections, demonstrating the inhibitory effect of the pretreatment. Nonetheless, due to the limited scope of this study, we were not able to demonstrate whether the inhibition occurred at the receptor or at the cytoplasmic signaling level.

Since the experiments in cultured adipocytes pointed at a possible inhibition of signaling at the level of the receptor, we examined the effects of gp130 cytokines on the stability and availability of LIFR. Previous studies (Blanchard et al., 2001) have shown that gp130 cytokine signaling affects the turnover and degradation of LIFR while having no effect on gp130. Consistent with these findings, we have shown that the half-life of LIFR is dramatically shortened by LIF administration. We have also demonstrated that,
in 3T3-L1 adipocytes, basal or ligand-induced degradation of LIFR can be prevented by
the addition of lysosomal inhibitors leupeptin and chloroquine. Proteasome inhibitors
MG132 or epoxomicin did not alter the half-life of LIFR under basal conditions or
following LIF administration.

To further investigate the effects of gp130 cytokines on LIFR stability, we treated
3T3-L1 adipocytes with these cytokines and analyzed the levels of LIFR following the
treatment. Aside from LIF, both CT-1 and OSM also had profound effects on the
degradation of LIFR. All of these cytokines use LIFR as a component of their receptor
complexes, and therefore induce its endocytosis and degradation (Blanchard et al., 2000).
The effect of CNTF was marginal, while IL-6 and GH had no effect on LIFR levels. IL-6
signals via the complex of gp130 and IL-6Rα, and therefore does not affect LIFR, while
the receptor for GH shares no common components with gp130 cytokine receptor
complexes. CNTF signals through the receptor complex of gp130, LIFR, and CNTFRα.
However, differentiated 3T3-L1 adipocytes do not express CNTFRα (Zvonic et al.,
2003a), so the ability of this cytokine to form functional receptor complexes, and induce
LIFR degradation, is severely impaired. In agreement with the previous experiment,
inhibition of the lysosome with leupeptin and chloroquine completely prevented the loss
of LIFR.

In summary, a striking parallel has emerged during this study, linking the ability
of a given cytokine to block subsequent cytokine signaling and its ability to induce LIFR
degradation. Namely CT-1, LIF, and OSM, three cytokines with the strongest inhibitory
effects on signaling, were also the most potent inducers of LIFR degradation. Therefore,
we hypothesize that the ability to block the signaling of gp130 cytokines is closely tied to
their ability to induce LIFR degradation, making it unavailable for the formation of receptor complexes to propagate further signaling. Conversely, CNTF, IL-6, and GH did not affect the turnover of the LIFR and therefore did not block the signaling abilities of other cytokines. Needless to say, several other studies will be necessary to elucidate the exact fate of LIFR following cytokine treatment, namely whether lysosomal inhibition prevents endocytosis of LIFR, as well as its degradation. In this case, lysosomal inhibitors could potentially reverse the ability of CT-1, LIF, and OSM to inhibit cytokine signaling, because LIFR would remain available for further signaling events.

However, these findings do serve as a base for future studies of cytokine interactions, which could be used to mimic various disease states marked by simultaneous expression of several cytokines (Kamimura et al., 2003), or to create novel approaches in inducing the differentiation of various progenitor cell lines (Gimble et al., 1994). Most importantly, this system may be used to describe the signaling events in adipocytes under the influence of macrophages. Two recent studies (Weisberg et al., 2003; Xu et al., 2003) have demonstrated that macrophage accumulation in adipose tissue, and subsequent secretion of cytokines by these macrophages, could possibly be some of the initial events leading to the onset of obesity and type 2 diabetes.
CHAPTER 6: SUMMARY AND CONCLUSIONS

6.1 Summary

Signaling of gp130 cytokines in various cell types has been extensively studied during the last decade. Studies of this system have led to numerous important discoveries that impact our understanding of cell biology and physiology. Previous work on gp130 cytokine actions in adipocytes has shown that certain members of this family, such as IL-6 (Bastard et al., 2002) or LIF (Tanaka et al., 1999), have the ability to dramatically alter the physiology of fat, and consequently impact the overall function of the body.

Our studies with CNTF have demonstrated several interesting functions of this cytokine in adipocytes. Apart from being able to activate JAK/STAT (STAT 3) and MAPK (ERKs 1 and 2) signaling, both in vitro and in vivo, CNTF was capable of modulating the expression of SREBP-1, FAS, GLUT4, and IRS-1 protein levels. Administration of CNTF to 3T3-L1 adipocytes also increased the ability of insulin to activate its downstream targets IRS-1 and Akt. However, this cytokine did not alter the ability of insulin to stimulate the uptake of glucose by adipocytes in vitro.

Another novel finding of our study is that the expression of the cytokine-specific receptor for CNTF, CNTFRα, is lost during the differentiation of 3T3-L1 adipocytes. However, we have shown that CNTFRα is expressed in rodent fat pads in vivo. Our study has also demonstrated the expression of CNTFRα in other rodent tissues such as lung, stomach, skeletal muscle, spleen, heart, pancreas, brain, testes, and kidneys. Interestingly, these tissues expressed not only different quantities of CNTFRα, but also
different forms of the protein. Subsequently, we were able to show that the variation in CNTFRα size was due to different glycosylation patterns of the protein. However, the most interesting finding in this study was that the expression of CNTFRα in rodent fat pads is substantially increased under the conditions of obesity/type 2 diabetes. This was the case in all three genetic and one diet-induced models of obesity we examined. Therefore, we hypothesize that CNTF may act as an insulin sensitizer in insulin-resistant fat pads in vivo, under the conditions of obesity/type 2 diabetes.

We followed up our CNTF studies by testing the ability of CT-1 to affect 3T3-L1 adipocytes in a similar fashion. CT-1 treatment was able to initiate JAK/STAT and MAPK (ERKs 1 and 2) signaling, both in vitro and in vivo. However, the activation pattern induced by CT-1 occurred in a dose-dependent manner, and was much more robust and sustained, closely resembling LIF signaling. Also, unlike CNTF which activated only STAT 3, CT-1 administration lead to the activation and nuclear translocation of STATs 1, 3, 5A, and 5B. Chronic treatment of 3T3-L1 adipocytes with CT-1 resulted in downregulation of FAS and IRS-1 protein levels, as well as a transient decrease in PPARγ protein expression. Just like CNTF, CT-1 had no significant effects on the expression levels of adipocyte proteins during the time course of fat cell differentiation. However, both cytokines induced the upregulation of SOCS-3 mRNA in 3T3-L1 adipocytes shortly after their administration. Consistent with its potent activation of the JAK/STAT and MAPK pathways, CT-1 generated a much more robust and sustained increase in SOCS-3 mRNA, when compared to CNTF. Both cytokines also caused a transient decrease in PPARγ mRNA levels, resembling the transient effect on its protein levels. When administered in the presence of a MEK inhibitor U0126, CT-1 was
still capable of modulating both the SOCS-3 and PPARγ mRNA expression, indicating that MAPK pathway is not required for this cytokine-induced regulation of gene expression. Even though our study of CT-1 actions in adipocytes is still under way, we hypothesize that this cytokine may act to modulate acute PPARγ expression, and can also affect insulin signaling, due to its ability to decrease the expression of IRS-1.

We also tested the ability of gp130 cytokines to interact with signaling of their family members. The results of our study indicate that CT-1, LIF, and OSM have interfered with the ability of gp130 cytokines to induce phosphorylation of various STATs and ERKs 1 and 2. However, since the signaling of GH was not affected by gp130 cytokines, we hypothesized that the inhibition occurs at the level of the receptor, and not the cytosolic signal transducers. Therefore, we examined the ability of gp130 cytokines to alter the half-life of shared receptor proteins. The half-life of gp130 protein is not affected by cytokine binding (Blanchard et al., 2000), so we focused on LIFR. Our results demonstrate that cytokine binding dramatically shortened the half-life of LIFR protein, and that the degradation of LIFR can be prevented by the addition of lysosome inhibitors leupeptin and chloroquine, but not by proteasome inhibitors MG132 or epoxomicin. Within the gp130 cytokine family, CT-1, LIF, and OSM were capable of inducing LIFR degradation. These were the same cytokines capable of inhibiting signaling of their family members. Hence, even though strictly based on correlative data, we hypothesize the cytokine-induced loss of LIFR makes this protein unavailable for the formation of functional receptor complexes upon further cytokine stimulation, and therefore plays an important part in the mechanism of gp130 cytokine-induced signaling inhibition.
6.2 Conclusions

A serendipitous finding that CNTF may induce weight loss and improve insulin sensitivity in obese/diabetic patients (ALS CNTF Treatment Study Group, 1996) has implicated gp130 cytokines as possible therapeutic agents for the treatment of these diseases. Even though the mechanism of CNTF action in this scenario has not yet been elucidated, and the clinical trials have faced serious problems, we now know that the members of the gp130 cytokine family have the ability to substantially affect adipose physiology.

The ability of CNTF to activate JAK/STAT and MAPK signaling in adipocytes, and to regulate the expression of several proteins involved in adipocyte function (FAS, IRS-1, GLUT4, SREBP-1), suggests that the actions of this cytokine in adipose may contribute to the overall amelioration of obesity/type 2 diabetes-related complications by the CNTF treatment. This notion is further supported by our finding that, in adipocytes, CNTF acts to enhance insulin signaling at the level of IRS-1 and Akt activation. The increased expression of CNTFRα in obese/diabetic rodents suggests that a ligand for this receptor may act to reverse the symptoms of these diseases. Since recent studies (DeChiara et al., 1995) have demonstrated CNTF is not the preferred ligand for CNTFRα, the cloning of a specific (or production of a synthetic) ligand for this receptor may prove to be a much more effective obesity/type 2 diabetes treatment.

Even though CT-1 is primarily known for its roles in cardiomyocyte physiology, we have demonstrated the ability of CT-1 to activate STAT 5 and modulate the expression of PPARγ mRNA in adipocytes. Since the activity of PPARγ and STAT 5 is very important for adipocyte differentiation (Tontonoz et al., 1994; Chawla et al., 1994;
Floyd and Stephens, 2003), CT-1 may prove to be capable of regulating this process. Of most interest, however, is the downregulation of IRS-1 expression by CT-1, which can possibly lead to the onset of insulin resistance. As previously indicated (Freed et al., 2003), circulating levels of CT-1 are increased in the serum of patients with ischemic heart disease and valvular heart disease. Since the clinical aspects of these diseases are tightly linked to obesity/type 2 diabetes (Reaven et al., 2004; Sowers and Frohlich, 2004), CT-1 may prove to be a link between obesity-related complications and CVD, such as hypertension or atherosclerosis. To this day, the principal cause of diabetes mortality is cardiovascular disease (CVD) (Nesto, 2003).

The potential of cross-talk among gp130 cytokines is also of utmost interest. As we have shown in our studies, several gp130 cytokines have the ability to attenuate the actions of other family members, by altering the availability of receptor complex components. Therefore, this approach could be used to develop combination therapies, where one cytokine could be used to “prime” the cell for the action of another. Hence, some of the detrimental side-effects of a given therapeutic cytokine could be attenuated by pretreating the patient. Since various disease states are marked by the elevation in one or more cytokines (Kamimura et al., 2003), gp130 cytokine interaction system can be used to model such events for further study. The differentiation of progenitor cell lines into terminally differentiated cell types can also be governed by gp130 cytokines (Gimble et al., 1994). Hence, our understanding of the interactions among these factors may be applied toward optimizing the differentiation potential and efficiency of various progenitor cell lines. Two recent studies (Weisberg et al., 2003; Xu et al., 2003) have also demonstrated that macrophage accumulation in adipose tissue, and subsequent
simultaneous secretion of multiple cytokines by these macrophages, could possibly be the initial events leading to the onset of obesity and type 2 diabetes. Therefore, understanding how cytokines, alone and in combination with others, affect the function of adipocytes, will be crucial in elucidating the mechanism by which macrophage accumulation in fat leads to obesity and type 2 diabetes symptoms.

Understanding the physiology of adipose tissue, as well as its functions in the onset of various disease states, is becoming increasingly important in our quest to develop a strategy to combat the deadly epidemic of obesity and type 2 diabetes. The results from our studies clearly indicate that gp130 cytokines exhibit a wide spectrum of actions in adipocytes, both *in vitro* and *in vivo*. Therefore, we believe that continued research of gp130 cytokine actions in adipocytes will prove useful in the development of novel approaches to study adipose physiology and obesity-associated diseases.
LITERATURE CITED


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Sincerely,
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VITA

Sanjin Zvonić was born on August 4, 1979, in Split, Croatia, where he attended elementary and high school. In August of 1995 he traveled to Columbia, South Carolina, as one of six Croatian high school students chosen to spend a year attending high school in the United States as a part of an international student exchange program, sponsored by the United States Information Agency. A year later he enrolled in Louisiana State University, where he received a Bachelor of Science degree in biochemistry in May of 2000. He continued his graduate education at Louisiana State University in the Department of Biological Sciences, where he earned the degree of Doctor of Philosophy in biological sciences in May of 2004, while working in the laboratory of Dr. Jacqueline M. Stephens. The title of his doctoral dissertation was "Effects of gp130 Cytokines on Adipocytes". He will continue his scientific career as a post-doctoral researcher at Pennington Biomedical Research Center, working with Dr. Randall L. Mynatt and Dr. Jeffrey M. Gimble.