The expression of cardiotrophin-1 is differentially regulated in murine and human obesity type II diabetes

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THE EXPRESSION OF CARDIOTROPHIN-1 IS DIFFERENTIALLY REGULATED IN MURINE AND HUMAN OBESITY TYPE II DIABETES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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in

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ABSTRACT

Cardiovascular disease is the leading cause of mortality in all developed nations. Several independent studies have shown that cardiotrophin-1 (CT-1) serum levels are modulated in patients with various types of cardiovascular disease including ischemic heart disease, valvular heart disease, and accelerated artherosclerosis. CT-1 is a member of the Interleukin-6 family, or gp130 family of cytokines. It is also known to induce cardiomyocyte hypertrophy in vitro and in vivo, and is a critical component for cardiomyocyte survival. CT-1 is a naturally occurring protein with a molecular mass of approximately 21.5 kD and a 200 amino acid long sequence, it was discovered in a cDNA screen of murine stem cells and was originally identified in cardiomyocytes. Since then, CT-1 expression has been reported in several other tissues including skeletal muscle, liver, ovary, kidney and lung.

Interestingly, our studies reveal that CT-1 protein expression is significantly modulated in adipose tissue following high fat feeding in C57BL/6 mice. Of note, we did not observe regulation of CT-1 expression in white adipose tissue in human obesity and Type II Diabetes Mellitus (T2DM). We can readily detect CT-1 in the media of cultured adipocytes. This study suggests that CT-1 secretion from murine adipose tissue may be an important contributor to the levels of circulating CT-1. In summary, our studies demonstrate that CT-1 is an adipokine that is modulated in murine obesity and T2DM.
CHAPTER 1
INTRODUCTION

1.1 Adipose Tissue

Studies over the last two decades have established adipose tissue as a dynamic organ that carries out several important physiological processes. There are two distinct classifications of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose tissue accounts for the majority of fat present in adult humans and is a critical site for energy homeostasis, insulin signaling and endocrine action. Brown adipose tissue is predominantly responsible for non-shivering thermogenesis via the mitochondrial protein uncoupling protein-1 (UCP-1) proton transport during lipid oxidation (1). Brown fat has been well established as an important fat depot in postnatal babies and in a variety of mammals. To much surprise to the scientific community, several recent studies using $^{18}$F-fluorodeoxyglucose-positron emission tomography and computed tomography suggest that BAT depots are also present in the thoracic and supraclavicular regions of adult humans (2;3). Additional studies suggest that this adipose tissue may be metabolically active BAT due to the presence UCP-1, an important component in BAT classification (4). Studies conducted by the Cinti group in 2010 revealed surprising evidence suggesting that brown adipocytes are detectable in subcutaneous WAT in mice (5). These observations have increased interest in the potential use of brown adipocytes in correcting WAT pathologies.

Adipose tissue is comprised of a variety of cell types including endothelial cells, blood cells, fibroblasts, pericytes, preadipocytes, macrophages and other immune cells (6). However, the predominant cells present in adipose tissue are mature adipocytes. Brown and white adipocytes require key transcription factors that are necessary to promote preadipocyte
differentiation into mature adipocytes. As shown in Figure 1, these transcriptional regulators include critical factors such as peroxisome proliferator-activated receptorγ (PPARγ) and other transcription factor families including CCAAT/enhancer binding proteins, STATs, and KLF proteins.

1.2 White Adipocytes

White adipocytes are generally spherical cells with an average diameter in a young animal of less than 10µm, however this size can increase to as much as 100µm particularly in the epididymal fat depots (7). The organelles of white adipocytes are typically subjected to a confined arrangement due to the presence of a unilocular lipid droplet that occupies the majority of the cytosol. As a result of the imposing mass of this fat deposit, the nucleus is usually compressed between the fat and plasma membrane. There is a sparse distribution of mitochondria in white adipocytes and other classical cellular organelles such as the Golgi and smooth and rough endoplasmic reticulum are present.

White adipose tissue is a key organ that carries out insulin stimulated glucose uptake, an important physiological process that is mediated by a signal pathway where insulin activates a cascade that results in glucose transporter-4 (GLUT-4) translocation to the plasma membrane to facilitate an increase in the influx of glucose. The details of this mechanism are well studied worldwide by numerous groups. Brown adipocytes do not take up significant amounts of glucose upon insulin stimulation, however they are responsive to the sympathetic nervous system and norepinephrine can stimulate the uptake of glucose independent of insulin (8).

1.3 Molecular Regulation of Adipogenesis

The development of fully differentiated mature adipocytes from precursor cells is an elegant progression of the sequential activation of a battery of transcription factors. In white
adipocytes, this sequence commences with the activation of members of the AP-1 family of transcription factors, and continues with the induction and expression of PPARγ a critical pro-adipogenic transcription factor (see Figure 1). Other transcription factors facilitate adipocyte maturation including STATs, members of the KLF family of proteins, SREBP-1, and members C/EBP family. There are also potent negative repressors of adipocyte differentiation including Pref-1 and members of the GATA and Wnt families. In addition to these factors, the adipogenic program can subjected to humoral regulation. Peptide and steroid hormones can exert paracrine and endocrine actions that modulate adipocyte differentiation.

1.3.1 Positive Effectors of Adipocyte Development

Activating Protein-1 (AP-1): Activating protein-1 (AP-1) is a large group of transcription factors that include v-Jun, c-Jun, JunB, JunD, v-Fos, c-Fos, FosB, Fra1, Fra2 and activating transcription factors (ATF2, ATF3/LRF1, B-ATF). These proteins are bZIP (basic region leucine zipper) transcription factors. Upon activation, the AP-1 proteins form homodimers or hetrodimers before interacting with DNA to regulate genes involved in the differentiation and proliferation of various cell types. In addition, studies have suggested that AP-1 can also be a part of cellular pro-apoptotic machinery (9;10). In regards to adipocyte development, in vitro studies primarily performed with 3T3-L1 and 3T3-F442A preadipocytes have established a strong association with AP-1 transcription factors and adipogenesis. In fact, the mRNA expression of c-Fos, c-Jun, Fos-B, and Fra-1 are all induced at the onset of adipocyte differentiation (11;12). In 1987 the Spiegelman group demonstrated that AP-1 modulated the expression of aP2 particularly via interaction between c-Fos and the FSE2 regulatory sequence of the aP2 promoter (13). Additionally, in vivo work was conducted with mice that were engineered to have a fat specific expression of A-ZIP/F using the aP2 promoter. In these studies,
investigators engineered A-ZIP/F, a dominant negative protein that hinders bZIP-DNA interaction and disrupts C/EBP and Jun transcription activity. These mice had impaired WAT development and significantly less BAT (14).

**Krüppel-like Factors (KLFs):** The nomenclature for this family of transcription factors was derived from the strong homology, particularly at the zinc finger DNA interaction domains, with the *Drosophila* embryonic pattern regulator protein Krüppel (15). To date, there are seventeen members of the C2H2 zinc finger Krüppel-like family of transcription factors and they are implicated in development, cellular differentiation, and proliferation (reviewed in (16)). It is interesting to note that members of this family can assume opposing transcriptional roles: some being activators of gene transcription, while others act as repressors of transcription. This section will focus on KLFs 4, 5, 6, and 15 that have been shown to be positive effectors of adipogenesis.

KLF5 expression is induced during the early stages of adipocyte differentiation and has been shown to be necessary for adipocyte differentiation *in vitro* and *in vivo* (17). KLF5-siRNA expression in 3T3-L1 preadipocytes results in impaired adipocyte differentiation and a substantial decrease in lipid accumulation. In addition, KLF5 *+/−* heterozygote mice have a reduction in fat pad size (17). Around the same time in 2005, it was revealed that KLF15 is also essential for adipogenesis (18). KLF15 is highly induced during the differentiation of 3T3-L1 cells and dominant negative mutations and RNAi knockdowns results in attenuated adipocyte differentiation (18). These studies also showed that ectopic expression of KLF15 can confer adipogenesis by inducing PPARγ activation in NIH-3T3 cells. KLF15 plays another key role in adipocyte physiology as studies have demonstrated an interaction of KLF15 with the GLUT-4 promoter, indicating a link between adipocyte insulin sensitivity and KLF15 (19). KLF4 is also highly expressed in white adipose tissue but it is expressed at much lower levels *in vitro* in
mature 3T3-L1 adipocytes. Further analysis revealed that the enrichment of KLF4 in adipose tissue can be attributed to its high expression in preadipocytes of the stromal-vascular fraction of white adipose tissue (20). These findings lead to the hypothesis that KLF4 may also be a key regulator of adipogenesis. In 2008, studies demonstrated that KLF4 mRNA was induced early in 3T3-L1 differentiation and siRNA knockdown resulted in marked decreases in lipid accumulation (21). KLF6 has also been identified as a positive effector of adipogenesis. KLF6 is induced during 3T3-L1 adipogenesis (22), and studies suggest that KLF6 promotes adipocyte differentiation by repressing of DLK-1 gene expression in 3T3-L1 preadipocytes (23). Taken together, these studies demonstrate that KLF4, KLF5, KLF6 and KLF15 are transcription factors that promote adipocyte development.

CCAAT/Enhancer Binding Proteins (C/EBPs): The C/EBPs is another family of basic leucine zipper transcription factors. There are six C/EBP isoforms, all of which possess a highly conserved bZIP domain that serves as a point of interaction for homodimerization or hetrodimerization with other family members (24). The most common nomenclature for the members is: C/EBPα, β, δ, γ, ε, and ζ. Both in vivo and in vitro experiments have demonstrated that C/EBPα, β, δ promote adipocyte differentiation. McKnight and colleagues presented the first set of data to demonstrate the orchestrated induction of these family members during adipogenesis. C/EBPβ is primarily induced by methylisobutylxanthine (MIX) and C/EBPδ is highly induced by dexamethasone (DEX) in the very early stages of adipogenesis. C/EBPβ and C/EBPδ work in concert to trans-activate C/EBPα (25). Loss of function experiments in 3T3-L1 and NIH-3T3 cell lines confirmed the importance of C/EBPβ and C/EBPδ as inhibition of either of these C/EBPs resulted in the attenuation of adipogenesis (26). Interestingly, ectopic expression studies with C/EBPα demonstrated that it was sufficient to induce adipogenesis in
precursor cell lines that are susceptible to adipogenesis, and in non-precursor fibroblasts (26;27). In vivo studies investigating the relevance of the C/EBPs to the adipogenic program have been conducted by numerous labs. C/EBPβ and/or δ-deficient mice exhibit defective adipose tissue development (28). Studies with C/EBPα-deficient mice revealed a phenotype of substantially low lipid accumulation (29;30).

**Sterol Regulatory Element Binding Protein-1 (SREBP-1):** This basic helix-loop-helix regulatory molecule was originally termed the adipocyte determination and differentiation factor-1 (ADD-1) by the Spiegelman laboratory in 1993 (31). ADD-1 was identified in a rat adipocyte cDNA expression library screen as a factor that binds to an E-box domain, a DNA recognition site for basic helix-loop-helix proteins. Northern blot analysis demonstrated a substantial increase in ADD-1 mRNA expression during adipocyte differentiation in three well established models of adipocyte development: 3T3-F442A, 3T3-L1, and 10T½ cells. A mere two months later, the Brown and Goldstein laboratory isolated the human homologue of the same protein for its ability to bind to the sterol regulatory element in the promoter region of the low-density lipoprotein receptor, thus labeling it sterol regulatory element binding protein-1 (SREBP-1) (32). To date there are three isoforms of SREBP: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c originate from the same gene through utilization of different promoters (33). SREBP-1a is the more potent transcription factor due to a longer NH3-terminal DNA binding domain (34). SREBP-1c is the predominantly expressed isoform in white adipose tissue (35) and a chief regulator of lipogenic gene transcription. SREBP-2 is transcribed from an alternate gene and primarily regulates the expression of genes involved in cholesterol biosynthesis (36). In vitro evidence suggested that ADD-1/SREBP-1 is a critical factor in adipocyte development. Ectopic expression of ADD-1/SREBP-1 in NIH-3T3 fibroblasts in the presence of hormonal
induction cocktail resulted in differentiation that was accompanied by increased expression of adipocyte specific genes and lipid accumulation (37). In this study, investigators also performed experiments to ectopically express a dominant-negative ADD-1 that contained a point mutation within the DNA binding domain. This mutation resulted in a profound inhibition of differentiation and repression of adipocyte marker genes.

On the contrary, in vivo studies exploring the involvement of SREBP-1 in adipogenesis are less persuasive. Transgenic mice engineered with adipose tissue specific knockout of SREBP-1 had minimal inhibition of adipose tissue development with no overt effects on the expression of adipocyte marker genes (38). Ectopic expression of a constitutively active SREBP-1c surprisingly conferred lipodystrophy in mice (39). Other in vivo studies by Yahagi and colleagues engineered a double mutant by crossing leptin-deficient ob/ob mice and SREBP-1/+ mice. These studies also suggested that SREBP-1 was not needed for adipose tissue expansion (40). Overall, the data suggest that while there is in vitro evidence to show SREBP-1 is required for adipocyte differentiation, the in vivo studies consistently demonstrate that SREBP-1 expression is not critical for adipose tissue development and/or expansion.

Signal Transducers and Activators of Transcriptions (STATs): There are seven signal transducers and activators of transcription (STAT) proteins designated STATs 1, 2, 3, 4, 5A, 5B, and 6 that exhibit unique tissue distributions and regulate the expression of tissue specific genes (41). STAT expression and modulation of gene expression can be cell specific and transgenic knockout studies have shown critical roles for every member of the STAT family (41). The STATs are primarily activated by cytokines and hormones. Ligand binding initiates a cascade that results in STAT tyrosine phosphorylation, dimerization and translocation to the nucleus where STATs modulate transcription (41;42). Several groups have studied STAT
biology in fat cells and it is known that STATs 1, 3, 5A, 5B and 6 are expressed in adipocytes. The first study of STAT expression in adipocytes demonstrated that STATs 1, 5A and 5B are substantially induced during 3T3-L1 adipocyte differentiation (43). Subsequently, Harp and colleagues observed similar induction patterns of STATs 5A and 5B in human subcutaneous preadipocytes (44). The studies in mouse and human cells revealed similar induction of STATs 3, 5A, and 5B, but there was a difference in STAT1 induction in mouse and human cells. However, it is unlikely that STAT1 plays a critical role in adipocyte development because STAT1 knockout mice do not have any apparent body weight abnormalities (45).

Although the relevance for STAT1 expression during the adipogenic program remains unclear, there are several studies that demonstrate a role of STATs 3, 5A and 5B in human and murine adipogenesis in a variety of well characterized models. STAT3 expression has been shown to increase during the proliferative phase of 3T3-L1 adipogenesis (46), and its expression is tightly regulated by protein inhibitor of activated STAT3 (PIAS3) (47). Investigators observed a disruption in adipogenesis using AG490, a JAK2 inhibitor and STAT3 siRNAs (48). Additionally, ectopic expression of a dominant negative STAT3 suppresses adipocyte differentiation (49). Mice lacking STAT3 in adipose tissue were generated using the aP2 promoter and exhibited higher body weights and increased adipocyte size compared to wildtype littermates (50). Taken together, these studies suggest a possible role for STAT3 in adipogenesis and body weight homeostasis. However, additional studies are necessary to further clarify the contribution of STAT3 in adipocyte development and physiology.

The involvement of STAT5 in adipogenesis has been widely studied. Ectopic expression of C/EBPβ and δ in non-precursor cells has been shown to induce adipogenesis (51) in a manner that promotes an increase in STAT5A and STAT5B expression (52). PPARγ has also been
shown to upregulate the expression of both STAT5 proteins during adipogenesis (53). In 3T3-F442A preadipocytes, the modulating effect of growth hormone on adipogenesis is attenuated by STAT5 antisense oligonucleotides (54) and constitutively active STAT5 can drive adipogenesis in this model system (55). Ectopic expression studies and transgenic knockout experiments have confirmed the physiological relevance of STAT5 proteins in adipogenesis. Ectopic expression of STAT5A induces adipogenesis in 3T3-L1 preadipocytes (56), and in two non-precursor fibroblast cell lines: BALB/c and NIH-3T3 cells (57). Of note, STAT5B was unable to display similar proadipogenic properties in these non-precursor cells (57). Studies have shown that the growth hormone activated STAT5 proteins can induce PPARγ expression in 3T3-L1 cells and C3H10T1/2 cells (58), suggesting a mechanism by which STAT5 proteins are able to promote adipocyte differentiation. Interestingly, transgenic knockout experiments have shown that disruption in either STAT5A or STAT5B or both genes resulted in abnormal adipose tissue and mice lacking both STAT5 proteins had fat pads one-fifth the normal size (59). To date, there are no studies on tissue specific knockout of STAT5 genes in adipocytes and the phenotype of the STAT5 null mice could be attributed to developmental effects of STAT5 that are independent of direct effects on preadipocyte differentiation. However, recent observations have demonstrated that ectopic STAT5A expression can confer the adipogenic capabilities of Swiss 3T3 fibroblasts in athymic mice (60). In addition to demonstrating a direct role of STAT5A in preadipocyte differentiation in vivo, these studies also demonstrate the usefulness of athymic mice in studying the role of transcription factors in adipose tissue development. In summary, the importance of the STAT5 proteins in adipogenesis has been demonstrated in vitro and in the whole animal.

**Peroxisome Proliferator-activated Receptorγ (PPARγ):** There are many proadipogenic transcription factors that have been characterized, but none are as critical as
PPARγ, the master regulator of adipocyte differentiation and gene expression. Of note, the majority of identified repressors and activators of adipogenesis have been shown to modulate PPARγ expression and/or activity. Studies using in vitro models of adipogenesis have consistently shown that PPARγ mRNA is induced by several transcription factors including C/EBPβ, C/EBPδ, EBF1, and KLF5. Repressors of adipogenesis such as GATA2, KLF2, and CHOP have been shown to attenuate PPARγ expression (reviewed in (61)). PPARγ is a member of a super family of hormone nuclear receptors, and has been shown to be crucial and sufficient for adipocyte development in vitro and in vivo (62-64). In 1995, the anti-diabetic thiazolidinediones (TZDs) drugs were shown to be high affinity PPARγ ligands (65). This study was the first to indicate that PPARγ was a target for this anti-diabetic class of drugs. PPARγ proteins are expressed in two forms, PPARγ1 and PPARγ2 that are produced by a combination of alternative promoter usage and alternative splicing (66). PPARγ1 is expressed at low levels in multiple tissues while PPARγ2 is highly expressed in fat cells and differs from PPARγ1 by an N-terminal extension of 30 amino acids (64). A key regulatory role of PPARγ in fat cell differentiation was observed by gain of function experiments that revealed that ectopic expression and activation of PPARγ in fibroblasts or myocytes promoted adipogenesis (62;64). Recent studies have shown that PPARγ2, but not PPARγ1, profoundly affects adipogenesis (67) and that only increases of PPARγ2 were observed in adipocytes of morbidly obese individuals (68). Although these studies suggest differential roles of the PPARγ isoforms, a study in PPARγ null cells demonstrated that both PPARγ1 and PPARγ2 can stimulate robust adipogenesis (69). However, PPARγ2 exhibits an enhanced ability to confer adipogenesis due to its increased sensitivity to ligands and enhanced ability to bind components of the DRIP/TRAP complex (69).
In vivo studies have shown that PPARγ deletion in mice results in placental dysfunction and embryonic lethality (70). To overcome this developmental limitation, fat specific knockout mice and mice in which placental dysfunction was averted were engineered in order to study the proadipogenic capabilities of PPARγ. The use of these mouse model systems has demonstrated that PPARγ is critical for fat cell development (63) and mice with adipose tissue specific loss of PPARγ display decreased fat pad size and insulin resistance in adipose tissue and liver (71). Although PPARγ is required for adipogenesis, there is evidence to suggest that this nuclear receptor may not be needed to maintain the differentiated state of the cell after adipogenesis (72). Of note, PPARγ heterozygote mice exhibit enhanced insulin sensitivity (73), suggesting the amount of protein present is highly important. The importance of PPARγ in human adipose tissue has also been established and subjects with mutations in the PPARγ gene can develop severe insulin resistance and lipodystrophy (74-76). Taken together, these studies demonstrate the requirement for PPARγ in adipocyte differentiation and whole body insulin sensitivity.

The expression and activity of PPARγ can be modulated by various pathways, and several studies in the last decade have focused on the post-translational regulation of this nuclear receptor. In particular, the ubiquitin-proteasome system has emerged as an important regulator of PPARγ (77-79). Both the AF-1 and ligand-binding domains (LBDs) of PPARγ are targeted to the proteasome for degradation, but only the LBD is conjugated to ubiquitin (80). Of note, a fully functional ubiquitin system is required for PPARγ activation (80). Overall, these studies indicate that the ubiquitin-proteasome pathway is an integral determinant of PPARγ activity and that this nuclear receptor is targeted to the proteasome for degradation via ubiquitin independent and ubiquitin dependent mechanisms. Small ubiquitin-like modifier (SUMO), a protein structurally homologous to ubiquitin, is another important regulator of PPARγ. SUMOylation can occur in
the AF-1 domain at Lys\textsuperscript{107} of PPARγ\textsubscript{2}, or Lys\textsuperscript{77} of PPARγ\textsubscript{1} to control stability and activity (81-84). PPARγ can also be SUMOylated in a ligand-dependent manner in the LBD at Lys\textsuperscript{365} (83). Another regulatory covalent modification of PPARγ is phosphorylation at Ser\textsuperscript{112} by mitogen-activated protein kinases (MAPK) such as p44/p42 (ERKs 1 and 2) and c-Jun amino-terminal kinase (JNK), which results in transcriptional inactivation of PPARγ (85). Although it is widely accepted that phosphorylation of PPARγ\textsubscript{2} inhibits its activity, a point mutation at Ser\textsuperscript{112} does not disrupt the ability of PPARγ\textsubscript{2} to confer adipogenesis (86). However, \textit{in vivo} studies have shown that PPARγ\textsubscript{2} phosphorylation at this residue modulates insulin sensitivity in the setting of diet-induced obesity (86). Very recent studies have revealed CDK5 phosphorylates PPARγ at Ser\textsuperscript{273} to attenuate PPARγ trans-activation of important adipocyte genes including adiponectin (87). The tightly controlled regulation of PPARγ by ubiquitylation, SUMOylation, and phosphorylation indicates the importance of modulating the expression and activity of this master regulator of adipogenesis.

1.3.2 Negative Effectors of Adipocyte Development

\textbf{Wnt Signaling:} The Wnt family is a group of more than sixteen secreted glycoproteins that has pleiotropic effects on cell fate specification, proliferation, and differentiation (reviewed in (88)). Wnts signal in an autocrine or paracrine manner through the Frizzled receptors, or low density lipoprotein receptor-related protein (reviewed in (89)). Upon binding its Frizzled receptor, Wnt activates the canonical pathway that inhibits glycogen synthase kinase 3 (GSK3) and results in an increase of cytosolic β-catenin which then translocates to the nucleus where it binds to the T-cell factor/lymphoid-enhancing factor (TCF/LEF) transcription factors to regulate the expression of Wnt target genes (reviewed (88)). Within the past decade, microarray analyses revealed that Wnts 10b and 5a are repressed during adipogenesis (90;91).
Figure 1 Positive and negative effectors that regulate adipogenesis. Several transcription factors can promote adipocyte differentiation. This sequence begins with the induction of AP-1 transcription factors, followed by the activation of other proadipogenic transcription factors. Adipogenesis can be inhibited by negative effectors of adipogenesis including members of the Wnt family.
As shown in Figure 1, Wnt signaling inhibits fat cell differentiation by reducing the expression of PPARγ and C/EBPα in 3T3-L1 and 3T3-F442A cells (90;92;93). Of note, activation of the Wnt pathway attenuates the expression of brown adipocyte marker genes UCP-1 and PGC-1α while the expression of other adipocyte genes are maintained (94). To further examine the effects of Wnts on adipogenesis, researchers engineered transgenic mice that express ectopic Wnt 10b using the aP2 promoter. These mice exhibited a 50% reduction in white adipose tissue and severely reduced brown adipose tissue. Interestingly, this inhibition of adipose tissue development did not result in lipodystrophic diabetes and these mice exhibited enhanced insulin sensitivity (95).

**GATA Factors:** There are six members of this zinc finger domain family of transcription factors, designated GATA 1 – 6. These transcription factors bind to specific (A/T)GATA(A/G) DNA consensus sequences to regulate cell differentiation and proliferation (96). Of the six factors, only GATA-2 and -3 are expressed in preadipocytes that are present in WAT but not in BAT (97). The expression of these transcription factors is repressed during adipocyte differentiation which suggests that GATAs 2 and 3 exert antiadipogenic effects on preadipocytes (see Figure 1). In support of this hypothesis, GATA 2-deficient embryonic stem cells exhibit an enhanced differentiation potential and ectopic expression of GATA-2 partially reduces fat cell development by directly binding to a PPARγ promoter region to repress basal activity (97). GATA-2 and 3 can also associate with C/EBPs α and β to disrupt their transcriptional activity (98), suggesting GATA can attenuate adipogenesis via multiple pathways. GATA proteins can be regulated post-translationally by acetylation, SUMOylation, and phosphorylation (99-101). Akt phosphorylation of GATA results in cytosolic sequestering and inhibition of nuclear translocation (101). Recent studies have suggested that friend of GATA (FOG) and C-terminal
binding proteins (CTBPs) act together with GATA-2 as co-regulators of adipocyte proliferation and differentiation (102).

**Krüppel-like Factors (KLFs):** As indicated above, KLFs 4, 5, 6, and 15 promote adipogenesis. However other members of the KLF family can inhibit fat cell differentiation. Studies have shown that KLF2 and KLF7 are negative regulators of fat cell differentiation. KLF2 mRNA is highly expressed in preadipocytes present in adipose tissue and its expression is decreased during adipogenesis. Ectopic expression of KLF2 in 3T3-L1 preadipocytes significantly disrupts lipid accumulation by repressing PPARγ2 gene expression (103) and partially restoring preadipocyte factor-1 (PREF-1) expression (104). The effect of KLF2 on adipocyte development is also evident in mouse embryonic fibroblasts derived from KLF2−/− mouse embryos that exhibit enhanced lipid accumulation (104). Ectopic expression of KLF7, another negative regulator of adipogenesis in human preadipocytes significantly inhibits adipocyte development and expression of PPARγ and C/EBPα (105).

**Preadipocyte Factor-1 (Pref-1):** Pref-1 was identified in a 3T3-L1 cDNA library for its enriched expression in preadipocytes (106). Delta-like protein-1 (DLK-1) is the human homologue of Pref-1 and was originally identified by purification from fetal circulation as fetal antigen-1 (107). Pref-1 is a transmembrane protein that belongs to a family of EGF-repeat containing proteins and shares structural homology with the Notch/Delta/Serrate proteins (reviewed in (108)). Pref-1 is activated by proteolytic cleavage to regulate cell fate specification (109). This protein is abundantly expressed in preadipocytes but levels substantially diminish during adipocyte development (110). Constitutive expression of Pref-1 reduces PPARγ and C/EBPα expression and inhibits the differentiation of 3T3-L1 preadipocytes (106). Pref-1 deficient mice exhibit growth retardation and accelerated adiposity (111). Transgenic mice with
aP2 mediated ectopic expression of Pref-1 in adipose tissue results have severe lipoatrophy, impaired glucose tolerance, and hypertriglyceridemia (112).

1.3.3 Endocrine Regulation of Adipogenesis

**Thyroid Hormone:** Thyroid hormones, triiodothyronine (T3) and thyroxine (T4) are controlled by a well studied classic negative feedback loop. T3, the biologically active form of the hormone, has pleiotrophic effects on various physiological processes and is a key regulator of metabolism and development. Thyroid hormone action is mediated through a family of nuclear thyroid hormone receptors that can repress transcription in a ligand-independent manner. An association between thyroid hormone and adipose tissue development has been established since 1888 when a study on myxedema suggested that obesity was a prerequisite for a diagnosis of hypothyroidism (reviewed in (113)). Rodent hyperthyroidism induces adipocyte hyperplasia, whereas hypothyroidism impedes adipose tissue development (114).

It is well known that T3 induces brown adipocyte differentiation. T3 supplemented media is commonly employed to stimulate differentiation of cultured brown adipocytes (115). The effect of thyroid hormone on lipogenesis has been observed in Sprague-Dawley rats treated with T3 (116), suggesting that thyroid hormone can induce the expression of lipogenic genes. Studies investigating the involvement of thyroid hormones in adipose tissue development are controversial. To date, there remains no concrete mechanism of thyroid hormone action in the adipogenic program. Nonetheless, it is clear that thyroid hormones are capable of regulating adipocyte development and modulating the expression of genes required for lipogenesis.

**Steroid Hormones:** Steroid hormones regulate the distribution and development of fat in mammals. Hence it is not surprising that adipocytes express abundant levels of steroid hormone receptors. The actions of steroid receptors are mediated by genomic responses and events that are
independent of transcription. This section will focus on the effects of estrogen (E2), androgens, and glucocorticoids on adipocyte differentiation. The receptor mediated effects of estrogen occur via two receptors, ER-α and ER-β to regulate metabolism and adipose tissue distribution (117;118). Potential gene targets of E2 can be estimated by the expression and distribution of its receptors. Studies investigating the expression patterns of ER-α and ER-β demonstrated that they are expressed in rat and human preadipocytes, mature adipocytes and other cell types present in adipose tissue including macrophages (119-121). Various studies have shown that E2 regulates adipocyte differentiation, however, the results are contradictory. Estrogen has been reported to induce the differentiation of 3T3-L1, female rat subcutaneous, and human preadipocytes (122;123). However, another study showed that estrogen inhibits 3T3-L1 preadipocyte differentiation (124). In vivo studies have yielded more consistent observations on the effects of estrogen on adipocyte development. Both male and female ER-α deficient mice have increased WAT accumulation, and insulin resistance (125). Studies using E2 deficient rodent models also supported the inhibitory effect this hormone on adiposity. Aromatase deficient mice (ArKO) develop increased fat pad mass, hyperinsulinemia, hyperlipidemia and liver steatosis (126). Mice deficient of follicle-stimulating hormone receptor exhibit increased development of visceral fat (127). Collectively, the majority of studies suggest that estrogen attenuates adipocyte development.

Androgens also regulate fat cell metabolism and adipose tissue distribution (117;118). Androgens can be found in adipocytes (128), and there is substantial evidence to demonstrate that the androgen receptor (AR) is expressed in rat (129;130) and human (131) preadipocytes and adipocytes. Together, these observations indicate that fat cells are targets of androgen action. As observed with estrogen experiments, in vitro studies have shown opposing effects of androgens
on the differentiation of preadipocytes. One study concluded that androgens induced rat fat cell development (132), whereas other studies have reported that androgens inhibit adipocyte differentiation (124;133;134). It was proposed that the two potent androgens, testosterone and dihydrotestosterone (DHT) decrease C/EBPα and PPARγ2 expression to inhibit differentiation of 3T3-L1 cells (135). Additionally, transgenic mice with targeted AR overexpression in mesenchymal stem cells have substantial reductions in both WAT and BAT depots (136). In summary, the majority of studies on androgens and adipocyte development suggest that these steroid hormones have inhibitory effects.

Glucocorticoids (GCs) are another class of steroid hormones that regulate adipocyte differentiation. The glucocorticoid receptor (GR) is expressed in most cell types and GCs bind to GR to mediate effects on metabolism and immune response (137). It is well established that GCs promote adipogenesis. Dexamethasone (DEX), a synthetic GC, is a standard component of the hormonal induction cocktail used to induce 3T3-L1 preadipocyte differentiation (138). Today, there is ubiquitous laboratory use of DEX to promote adipogenesis in a variety of cell lines including 10 T1/2 (139) and Ob1771 (140) cells. Mechanistic studies indicate that DEX induces C/EBPδ (25), which plays a role in the induction of the master adipogenic regulator PPARγ (141).

**Peptide Hormones:** A large variety of peptide hormones can modulate adipogenesis, including growth hormone (GH) and insulin. Both preadipocytes and adipocytes are responsive to GH. GH primarily signals via the JAK/STAT signaling pathway. As indicated earlier in this review, STAT5 proteins promote adipogenesis *in vitro* and *in vivo* (43;56;59;142). Since GH is a potent activator of STAT5, it is not surprising that most studies show that GH promotes adipocyte development. Recent studies have indicated that the GH/STAT5 pathway enhances
both the expression and the activity of C/EBPs β and δ and PPARγ (58), thus proposing multiple pathways through which GH promotes adipogenesis. Earlier studies demonstrated that GH exerts a dual effect on 3T3-F442A preadipocytes where there is an initial direct effect on preadipocytes to initiate the adipogenic program that is followed by an indirect effect of GH to induce insulin-like growth factor-1 (IGF-1) to stimulate growth of the committed cells (143). While it has been clearly established that GH promotes adipocyte development in clonal cell lines, several studies show an opposing effect of GH action on differentiation of primary cultured preadipocytes. In primary rat and human preadipocytes, GH inhibits differentiation (144;145).

Many investigators who use adipocytes assume that insulin is a positive regulator of fat cell development. However, the use of insulin in the standard hormonal cocktail is at supraphysiological hormone levels that are capable of activating IGF-1 receptor signaling (146). Nonetheless, there is evidence that insulin signaling can modulate the expression of genes required for adipocyte development and physiology in the whole animal. Mice that lack the insulin receptor in adipose tissue exhibit reduced fat mass and decreased levels of SREBP-1 and C/EBPα in white adipocytes. Additionally, these mice exhibit abnormally high leptin serum levels (147). In summary, these studies suggest that insulin can promote adipogenesis, however more studies are necessary to clarify the mechanism(s) of action.

1.4 Adipokines

The term adipokine refers to any growth factor, hormone, or cytokine that is synthesized and secreted from adipose tissue. Adipokines regulate key physiological processes such as satiety, energy homeostasis, insulin sensitivity, blood pressure, and inflammation. In 1994, Zhang and colleagues revealed that the satiety hormone leptin is produced in adipose tissue (148). This was the first study to show that adipose tissue is a bonafide endocrine organ.
<table>
<thead>
<tr>
<th>Hormone</th>
<th>Effect</th>
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<tr>
<td>Steroid Hormones</td>
<td></td>
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<tr>
<td>T3</td>
<td>Pro-adipogenic</td>
<td>Pro-adipogenic (115;116)</td>
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<tr>
<td>Estrogen</td>
<td>Controversial</td>
<td>Pro-adipogenic (122;123)</td>
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<td>Anti-adipogenic (124-127)</td>
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<td>Androgens</td>
<td>Controversial</td>
<td>Pro-adipogenic (132)</td>
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<td></td>
<td></td>
<td>Anti-adipogenic (124;133-136)</td>
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<td>Glucocorticoids</td>
<td>Pro-adipogenic</td>
<td>(25;138-140)</td>
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<td>Peptide Hormones</td>
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<tr>
<td>GH</td>
<td>Controversial</td>
<td>Pro-adipogenic (58;143)</td>
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<tr>
<td></td>
<td></td>
<td>Anti-adipogenic (144;145)</td>
</tr>
<tr>
<td>Insulin/IGF-1</td>
<td>Pro-adipogenic</td>
<td>(143;146)</td>
</tr>
<tr>
<td>gp130 Cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNTF</td>
<td>No effect</td>
<td>(149)</td>
</tr>
<tr>
<td>CT-1</td>
<td>No effect</td>
<td>(150)</td>
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<tr>
<td>IL-11</td>
<td>Inhibitory</td>
<td>(151-153)</td>
</tr>
<tr>
<td>LIF</td>
<td>Controversial</td>
<td>No effect (154;155)</td>
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<td></td>
<td></td>
<td>Pro-adipogenic (156)</td>
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<td></td>
<td></td>
<td>Anti-adipogenic (157)</td>
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<tr>
<td>NP</td>
<td>Inhibitory</td>
<td>(155)</td>
</tr>
<tr>
<td>OSM</td>
<td>Inhibitory</td>
<td>(155;158)</td>
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Following this ground breaking discovery, Scherer and colleagues identified a second hormone exclusively secreted from adipocytes in 1995. This hormone was characterized as adipocyte complement-related protein of 30 kD (Acrp30) (159) and is also referred to as AdipoQ (160), adipose most abundant gene transcript-1 (apM1) (161), gelatin-binding protein of 28 kD (GBP28) (162). Now, this adipocyte specific hormone is commonly known as adiponectin. Adiponectin is a pleiotropic hormone that regulates several processes. However the most clearly understood are glucose and lipid metabolism (163;164). Also, adiponectin has also been shown to be cardio-protective in mice and humans (165;166) and more recent studies indicate adiponectin can act on the brain to regulate satiety (167).

In 2000, another adipocyte specific hormone was identified as a member of the “found in inflammatory zone” (FIZZ) family, and was labeled FIZZ3 (168). Immunoneutralization studies suggested that this hormone, now widely referred to as resistin, might be involved in the pathogenesis of insulin resistance (169). In these studies, investigators observed improved insulin sensitivity when anti-resistin IgG was administered to rodents with obesity/Type II Diabetes Mellitus (T2DM). It is important to note that the distribution pattern of resistin in humans is different from mice. Several groups have shown that resistin is expressed at low levels in human adipocytes (170-172), but the majority of resistin expressed in humans is found in monocytes and macrophages that reside in adipose tissue at higher levels compared to amounts observed in adipocytes (172;173). A highly innovative experiment from the Lazar laboratory sought to determine if the resistin derived from human macrophages was sufficient to confer insulin resistance. In this study, researchers engineered a mouse model that was deficient of adipocyte-derived resistin and produced human resistin from a macrophage-specific promoter. These mice are referred to as the humanized resistin mice and they exhibit a phenotype of insulin
resistance and white adipose tissue inflammation (174). These studies indicate that resistin has
similar functions in mice and man despite the differences in expression.

1.5 Glycoprotein 130 (gp130) Cytokines

The family of gp130 cytokines regulate a variety of complex biological processes
including hematopoiesis, immune response, inflammation, proliferation, differentiation,
mammalian reproduction, cardiovascular action, and neuronal survival (reviewed in (175)).
These cytokines share glycoprotein 130 as a common signal transducer in their receptor complex
and typically activate STAT3, a latent transcription factor. The gp130 family is also referred to
as the Interleukin (IL)-6 family of cytokines and is a group of functionally and structurally
related proteins that includes Interleukin-6 (IL-6), Interleukin-11 (IL-11), Interleukin-27 (IL-27),
leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF),
cardiotrophin-1 (CT-1), novel neurotrophin-1/B cell stimulating factor-3 or cardiotrophin-like
cytokine (CLC), and neuropoietin (NP) (reviewed in (176)). The gp130 cytokines have become a
focus in T2DM and obesity research in the past decade. Several studies have shown that gp 130
cytokines can regulate adipocyte differentiation and function in vivo and in vitro (177-182). To
date, no gp130 cytokines have been shown to promote adipogenesis, but as shown in Table 1
several cytokines in this family have been shown to inhibit adipocyte development
(149;150;152-155;158;183;184). However, the mechanisms involved in gp 130 cytokine actions
in adipose tissue have not been fully elucidated.

This thesis highlights a potential role of CT-1 in the association between T2DM, obesity
and heart disease. It is well established that there is a link between cardiovascular disease and
T2DM, and many studies show that CT-1 serum levels are modulated in various heart diseases
including hypertension, myocardial infarction, ischemia, and cardiomyopathy (reviewed in
Further, cardiovascular disease is the leading cause of mortality in patients with T2DM. CT-1 is a 21.5kD molecule that was identified in 1995 in a cDNA screen of murine embryoid bodies for its ability to promote cardiomyocyte survival, proliferation, and hypertrophy (186;187). High levels of CT-1 mRNA is has been detected in a variety of tissues including the heart, skeletal muscle and liver (188). Interestingly, recent studies revealed that CT-1 is expressed in adipose tissue (189).

1.5.1 Molecular Regulation of Cardiotrophin-1 Expression

To date, the majority of studies investigating possible regulatory factors of CT-1 expression have largely been conducted in cultured cardiomyocytes and rodent models of various heart diseases. Several independent studies in the last decade have shown that CT-1 mRNA and protein expression can be modulated by endocrine action or by intracellular factors. Isolation and characterization of the CT-1 gene and promoter revealed several putative binding sites for various transcription factors including hypoxia-inducible factor-1 (HIF-1), C/EBPβ, and GATA1 (190;191). However, more studies are necessary to further elucidate the mechanisms involved in modulating CT-1 expression.

Studies by Hishinuma in 1999 suggested that hypoxic stress was capable of inducing CT-1 mRNA expression in primary cultured cardiac myocytes (192). It was later demonstrated that CT-1 expression was increased upon induction and stabilization of the α-subunit of HIF-1, in embryonic cardiomyocytes exposed to hypoxia or reactive oxygen species (ROS) (193). These studies demonstrate that CT-1 can be cardio-protective against ischemia-reperfusion injury. CT-1 expression can also be regulated by endocrine factors. Hormones such as aldosterone, norepinephrine and fibroblast growth factor-2 (FGF-2) are capable of modulating CT-1 expression. It is well established that substantially high aldosterone serum levels are observed in
patients with hypertension (194;195). Thus, it is not surprising that aldosterone is a potent inducer of CT-1 expression in HL-1 mature cardiomyocytes and rodent models of aldosterone over-load (196). These studies demonstrate a role for CT-1 in vascular wall re-modeling in hypertension. These studies underscore the cardio-protective functions of CT-1. It is therefore important identify other potential physiological sources of CT-1 and, to understand the mechanisms through which CT-1 levels are modulated in various pathologies including T2DM.

1.6 In vitro and In vivo Model Systems

3T3-L1 Cultured Adipocytes: A variety of cellular models systems are widely used to study the molecular pathways of adipogenesis and adipocyte function in vitro. These models can be largely placed in two classes. The first group of model systems is pluripotent fibroblasts that have the ability to differentiate into several cell types including myocytes, chondrocytes, and adipocytes. This class includes the 10T½, Balb/c 3T3, 1246, RCJ3.1 and CHEF/18 fibroblast cell lines. The second group of model systems is comprised of fibroblast-like unipotent preadipocytes that are committed to differentiating into adipocytes. This group includes 3T3-L1, 3T3-F422A, 1246, Ob1771, TA1 and 30A5 preadipocytes. This latter class represents the most frequently used cells to study adipogenesis and fat cell physiology. Howard Green and colleagues established the 3T3-L1 line in 1976. This cell line has been extensively characterized and utilized in adipocyte biology. 3T3-L1 preadipocytes were derived from disaggregated 17-19 day old Swiss 3T3 mouse embryos (197;198) and require glucocorticoid supplemented differentiation cocktail to induce adipogenesis (138;199;200). The 3T3-L1 cell line is preferred over other in vitro models because they develop into a homogenous population of mature adipocytes that are morphologically (201) and biochemically (202) similar to adipocytes in situ.
**C57BL/6 Mice:** The C57BL/6 mouse is an appropriate model system for human metabolic syndrome. Several lines of studies have demonstrated that the majority of instances of human obesity and subsequent development of T2DM occurs as a result of high-fat or western diets (203;204). The C57BL/6 mouse is an inbred model system that can develop severe obesity, hyperglycemia, hyperinsulinemia, and cardiovascular disease when fed a 55% Kcal high fat diet for approximately ten weeks (205-207). Further to this, it has been reported that C57BL/6 mice fed a fat enriched diet exhibit selective visceral distribution of white adipose tissue (206;208). Of note, there is a strong correlation between human visceral obesity and life threatening pathologies including T2DM and cardiovascular disease (reviewed in (209)). Hence, it is widely accepted that the progression of metabolic syndrome that occurs in response high fat feeding in C57BL/6 mice is similar to what is observed in humans.
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Dulbecco’s Modified Eagle’s Media (DMEM) was purchased from Sigma. Bovine and fetal bovine (FBS) sera were purchased from Atlanta Biological. Murine recombinant CT-1 was purchased from R&D Systems. Human polyclonal and murine monoclonal CT-1 antibodies were purchased from R&D Systems. STAT 5A and adipsin polyclonal antibody were purchased from Santa Cruz Biotechnology. Nitrocellulose and Zeta Probe-GT membranes were purchased from Bio-Rad. The BCA kit and the enhanced chemiluminescence kit were purchased from Pierce. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Glycoprotein deglycosylation kit was purchased from Calbiochem.

2.2 Cell Culture

Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in DMEM containing 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). After 48 h, this medium was replaced with DMEM supplemented with 10% FBS and cells were maintained in this medium until utilized for experimentation. In some experiments, cells were induced to differentiate in the presence of NP and treated at various times after the addition of the differentiation cocktail.
2.3 Preparation of Whole Cell Extracts and Media

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

2.4 Rodent Adipose Tissue Isolation

12 week old C57BL/6 mice were euthanized with CO₂, and tissues were immediately removed and frozen in liquid nitrogen. Frozen tissues were homogenized in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1mM EGTA, 1mM EDTA, 1% Triton-X 100, 0.5% Igepal CA-630, 1 μM PMSF, 1 μM pepstatin, and trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 minutes at 1000 x g to remove any debris and insoluble material and then analyzed for protein content. All animal studies were carried out with protocols which were reviewed and approved by institutional animal care and use committees.

2.5 Gel Electrophoresis and Western Blot Analysis

Proteins were separated in 7.5% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (210) and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Following
transfer, the membrane was blocked overnight in 4% milk at 4°C and subsequently exposed to the appropriate antibodies. Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence.

2.6 RNA Analysis

Total RNA was isolated from cell monolayers with Trizol according to the manufacturer’s instructions with minor modifications. For Northern blot analysis, 20 μg of total RNA was denatured in formamide and electrophoresed through a formaldehyde/agarose gel. The RNA was transferred to Zeta Probe-GT, cross-linked, hybridized, and washed as previously described (211). Probes were labeled by random priming using Klenow fragment and [α ^32P] dATP.

2.7 Immunoprecipitation (IP) of Cardiotrophin-1

Cells were harvested under non-denaturing conditions, and the protein content of the whole-cell extracts was analyzed as described above. After a single freeze-thaw cycle, the protein extracts were preincubated with protein A agarose, and the resulting supernatant was then incubated with 2 μg of human polyclonal CT-1 antibodies for 1 hour at 4 °C. Protein-A agarose (Repligen Corporation, Waltham, MA) was added to the mixture, and the sample was rotated for an additional hour. Bound CT-1 was isolated by pelleting this mixture. The pellets were rinsed three times with phosphate-buffered saline, and bound proteins were eluted from the agarose by incubation at 100 °C for 10 minutes after the addition of Laemmli sample buffer. These samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and analyzed by Western blotting with mouse monoclonal CT-1 antibodies.
CHAPTER 3

CARDIOTROPHIN-1 EXPRESSION IS DIFFERENTIALLY REGULATED IN MURINE AND HUMAN TYPE II DIABETES AND OBESITY

The white adipose tissue, heart, spleen, liver, skeletal muscle, kidney, and brain of an 8-week-old male Sprague Dawley rat was examined for CT-1 mRNA expression. Additionally, total RNA of 3T3-L1 mature adipocytes was examined for CT-1 mRNA. We were able to detect CT-1 mRNA in 3T3-L1 cultured adipocytes and rodent white adipose tissue.

![Figure 3.1 CT-1 mRNA is highly expressed in adipose tissue and 3T3-L1 adipocytes. Total RNA was isolated from tissue obtained from an 8-week-old Sprague Dawley male rat and from mature 3T3-L1 adipocytes using Trizol reagent. 20 μg of total RNA was electrophoresed, transferred to nylon and subjected to Northern blot analysis.

As shown in Figure 3.1, CT-1 mRNA is highly expressed in the rat adipose tissue sample that was a mixture of both epididymal and retroperitoneal fat. CT-1 mRNA was also expressed at high levels in fully differentiated 3T3-L1 adipocytes. As predicted, CT-1 mRNA was also detectable in the heart. We observed lower mRNA levels in liver, skeletal muscle, and kidney. 

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aP2 is a lipid-binding protein that is abundantly expressed in adipocytes, and was used as a positive control. β-actin was used as a loading control.

CT-1 is readily detected in both rodent and human serum (212-214). We hypothesized that adipocytes were capable of secreting CT-1. 3T3-L1 preadipocytes were induced to differentiate using media containing 3-isobutyl-1-methylxanthine, dexamethasone, and high doses of insulin (MDI). This process prompts 3T3-L1 cells to undergo postconfluent mitotic clonal expansion, exit the cell cycle, and begin to express adipocyte-specific genes (138). Media was then obtained at 10 and 20 days post MDI in the presence of 1μL PMSF and examined for CT-1 protein. As shown in Figure 3.2, we observed that the CT-1 dimer was detectable in the media of 3T3-L1 adipocytes. Adiponectin, an adipocyte-specific hormone was used as a positive control for this experiment.

![Figure 3.2 CT-1 is secreted from 3T3-L1 adipocytes. 3T3-L1 adipocytes were induced to differentiate and media was obtained at 10 and 20 days post MDI in the presence of 1μL PMSF. The samples were then separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis.](image)
Our results in rodent adipose tissue, cultured murine adipocytes, and murine adipocyte media indicate that CT-1 is expressed in, and secreted from adipocytes.

The association between T2DM and cardiovascular disease has been well acknowledged. Since CT-1 is expressed in and secreted from adipocytes, we hypothesized that CT-1 expression is modulated in T2DM/obesity. We examined the epididymal and retroperitoneal adipose tissue of seven 12-week-old C57BL/6 mice that were fed a 60% high fat diet (HFD) for six weeks and seven littermates that were fed a 10% low fat diet (LFD) diet for the same period. Total RNA was isolated from the tissues and subjected to Northern blot analysis. As shown in Figure 3.3, CT-1 mRNA is decreased in the mice that developed diet induced obesity (DIO) as a result of being fed a diet high in fat. It is well established that adipsin expression is decreased in murine T2DM/obesity and we see that levels of adipsin mRNA is also decreased in mice that were placed on a HFD.

![Figure 3.3 CT-1 mRNA is decreased in WAT in DIO.](image)

**Figure 3.3 CT-1 mRNA is decreased in WAT in DIO.** Seven mice were placed on a 60% HFD for 6 weeks and seven were fed a 10% LFD for the same period. Total RNA was isolated from epididymal fat pads of 14 male 12 week old C57BL/6 mice. Total RNA was electrophoresed, transferred to Nylon and subjected to Northern blot analysis.
MCP-1 and TNFα are used as positive controls for this experiment because it is well known that the expression of these proteins is upregulated in adipose tissue in T2DM/obesity due to macrophage infiltration. Additionally, tissues of seven 12-week-old C57BL/6 mice that were fed a 60% HFD for six weeks and seven littermates that were fed a 10% LFD for the same period were homogenized using a non-denaturing buffer and subjected to Western blot analysis. As shown in Figure 3.4, we observed a substantial decrease in CT-1 protein expression in C57BL/6 mice that were subjected to a HFD. Data for three animals of each condition is shown. Adipsin is used as a control for this experiment and STAT5A is used as a loading control, because it is not regulated in T2DM/obesity. Next, we sought to determine whether CT-1 protein expression was modulated in other tissues of mice with T2DM/obesity. For this experiment, we analyzed the heart, skeletal muscle and liver of the same mice that were used in the previous experiment.

**Figure 3.4 CT-1 protein expression is substantially decreased in WAT of DIO.** Epididymal and retroperitoneal fat pads were obtained from male C57BL/6 mice after 12 week feeding of a 10% LFD or 60% HFD. Tissues were homogenized using a non-denaturing buffer and separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis. Data for three mice of each condition is shown.
Figure 3.5 CT-1 protein expression is substantially decreased in tissues of DIO. Heart, skeletal muscle, and liver samples were obtained from male C57BL/6 mice after 12 week feeding of 10% LFD or 60% HFD. Tissues were homogenized using a non-denaturing buffer and separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis. Data for three mice of each condition is shown.

Surprisingly, we found that CT-1 protein expression was substantially decreased in these tissues as well (Figure 3.5). Taken together, our data present novel findings that CT-1 mRNA and protein expression is decreased in murine T2DM/obesity.

To determine whether CT-1 protein is expressed in human adipose tissue, we analyzed the adipose tissue derived from human lipoaspirate. Human adipose tissue was homogenized using a non-denaturing buffer, the adipocyte and stromal-vascular fractions were then separated by SDS-PAGE and subjected to Western blot analysis. Interestingly, CT-1 protein was detectable in human adipose tissue in both the monomeric and dimeric form (Figure 3.6). Additionally, it was observed that CT-1 was present in the stromal-vascular fraction of human adipose tissue. Adiponectin is abundantly expressed in mature adipocytes and is used as a positive control for the adipocyte fraction. STAT5A was used as a loading control.
Figure 3.6 CT-1 is expressed in human adipose tissue. Human adipose tissue was homogenized using a non-denaturing buffer. The adipocyte and stromal vascular fractions were separated by SDS-PAGE and subjected to Western blot analysis.

To determine whether CT-1 protein expression is modulated in human obesity, we examined the white adipose tissue of male and female obese patients. The lipoapirate of six lean, and six obese male and female patients was homogenized using a non-denaturing buffer, the adipocyte and stromal-vascular fractions were then separated by SDS-PAGE and subjected to Western blot analysis. Of note, no regulation of CT-1 protein expression was observed among the lean and obese male and the lean and obese female patients (Figure 3.7). The lipoaspirate of three lean and three obese non-diabetic patients, and three lean and three obese diabetic patients was examined to determine if CT-1 protein expression was modulated in human non-insulin dependent diabetes mellitus (NIDDM). Interestingly, no noticeable modulation in CT-1 expression was observed among the diabetic and non-diabetic patients (Figure 3.8).
Figure 3.7 CT-1 expression is not regulated in human obesity. White adipose tissue from 6 lean and 6 obese female patients and 6 lean and 6 obese male patients was homogenized using a non-denaturing buffer. Samples were then separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis.

Figure 3.8 CT-1 is expression is not regulated in human T2DM. White adipose tissue from 3 lean and 3 obese non-diabetic patients and 3 lean and 3 obese diabetic patients was homogenized using a non-denaturing buffer. Samples were then separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis.

In most of our experiments, we observed that CT-1 consistently migrated to a higher molecular weight than expected. In order to confirm that the band observed corresponded with CT-1, CT-1 from 3T3-L1 whole cell extract and media, and murine epididymal fat was immunoprecipitated using CT-1 human polyclonal antibody, and Western blot of CT-1 was observed using mouse monoclonal CT-1 antibody. This experiment demonstrated the specificity of the antibodies that were used for all experiments that were performed (Figure 3.9).
Figure 3.9 Confirmation of CT-1 detection. Whole cell extracts and media were isolated from fully differentiated 3T3-L1 adipocytes. Epididymal fat pads were obtained from male C57BL/6 mice after 12 week feeding of a 10% LFD. Each sample was subjected to immunoprecipitation (IP) with a human polyclonal antibody directed against CT-1 and Western blotting analysis was conducted using mouse monoclonal CT-1 antibody. 300 μg of protein was used for IP, and 90 μg of protein was used for Western blotting. One IP contained recombinant CT-1 protein (Ctrl) and one contained buffer, but not extract (mock).

Previous studies have reported that CT-1 contained putative sites for glycosylation (186). To determine whether the glycosylation is the reason for the observed migration pattern of CT-1, murine epididymal fat was incubated with N-Glycosidase F, Endo-α-N-acetylgalactosaminidase and α2-3,6,8,9-Neuraminidase to remove possible N-linked glycans. Additionally, murine epididymal fat was incubated with β1,4-Galactosidase and β-N-Acetylgalcosaminidase to disrupt possible O-linked glycans. Another sample was incubated with all endoglycosidases in order to remove both N-linked and O-linked glycans. As shown in Figure 3.10, glycosidase treatment did not alter the mobility of CT-1. The results of this experiment were repeated three times. A parallel experiment using bovine fetuin that was provided with the glycoprotein deglycosylation kit was used as a positive control (Figure 3.11). The control experiment was conducted using the same conditions that were used in Figure 3.10, and visualized using coomassie blue staining.
Figure 3.10 Deglycosylation analysis of CT-1. Epididymal fat pads were prepared from male C57BL/6 mice after 12 week feeding of a 10% LFD. Samples were incubated with N or O endoglycosidases as directed by the manufacturer’s instructions and then separated SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis. C1 and C2, no endoglycosidases; N, treatment with a mix of N-glycosidases(N-Glycosidase F, Endo-α-N-acetylgalactosaminidase and α2-3,6,8,9-Neuraminidase); O, treatment with a mix of O-glycosidases (β1,4-Galactosidase and β-N-Acetylglucosaminidase); N + O, treatment with both N- and O-glycosidases.

Figure 3.11 Deglycosylation analysis of purified bovine fetuin. Purified bovine fetuin was incubated with N or O endoglycosidases as directed by the manufacturer’s instructions, separated SDS-PAGE, and subjected to coomassie blue staining.

For the majority of experiments in this project, we made an interesting observation about the CT-1 dimer interaction. The CT-1 dimer was detectable even after undergoing SDS-PAGE electrophoresis, a process that typically denatures and separates proteins according to size and no other physical property. These observations lead to the hypothesis that the CT-1 monomers can form strong dimer interactions. To examine the stability of the CT-1 dimer, media was collected from fully differentiated 3T3-L1 adipocytes in the presence of 1μL
PMSF. The samples were then prepared with 8% β-mercaptoethanol (β-ME), this is twice the concentration that is normally used, and subjected to boil at 100°C for 15 minutes, a time period that is three times a greater than the standard time. Following this process, the samples were then separated by SDS-PAGE electrophoresis and subjected to western blot analysis. Surprisingly, as shown in Figure 3.12, the CT-1 dimer was detectable after being subjected to these rigorous conditions. These results are representative of three independent experiments. Adiponectin, an adipocyte-specific hormone was used as a positive control for this experiment.

**Figure 3.12 Analysis of CT-1 dimer.** Media was obtained from fully differentiated 3T3-L1 adipocytes. The samples were then prepared with 4% β-ME and heated to 100°C for 5 minutes, or with 8% β-ME and heated to 100°C for 15 minutes. The samples were then separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis.
CHAPTER 4
DISCUSSION

Obesity has emerged as a serious international epidemic and the association between obesity and T2DM is well established and accepted. Additionally, the risk of developing heart disease as a result of T2DM/obesity has become a focus in recent decades. T2DM can adversely affect several metabolic processes that lead to various interconnected endothelial and vascular dysfunctions and result in several cardiovascular pathologies including ischemia and myocardial infarction. Further, cardiovascular disease is the main cause of mortality in patients with diabetes. However the underlying mechanisms that connect these diseases are not well understood (215). Thus, the purpose of this work is to present a potential link between these pathologies.

Our data has shown that CT-1 is expressed in both murine and human adipose tissue. We were also able to show that the CT-1 dimer is detectable in the media of cultured 3T3-L1 mature adipocytes. To our knowledge, this is the first study to show that CT-1 is secreted from adipocytes. These experiments clearly demonstrate that CT-1 is produced in adipose tissue and our novel findings show that 3T3-L1 adipocytes can secrete the dimeric form of CT-1. It is well established that CT-1 is critical for cardiomyocyte survival (186), and there is a potential link between T2DM/obesity and cardiovascular disease. We therefore hypothesized that CT-1 expression would be modulated T2DM/obesity. Our results showed that CT-1 protein expression was substantially decreased in white adipose tissue, heart, skeletal muscle and liver in murine T2DM/obesity. Interestingly, we did not observe regulation of CT-1 expression in human white adipose tissue. This surprising finding demonstrates that the mechanism(s) governing CT-1
regulation may be different in mice and in humans. More studies will be necessary to elucidate the physiological conditions that can regulate CT-1 expression in human adipose tissue.

In the majority of our experiments in this study, the CT-1 protein dimer migrated to approximately 52 kD rather than the predicted 42 kD molecular weight. To address this, we performed immunoprecipitation experiments to confirm that the detected protein is CT-1. Upon this confirmation, we then sought to determine whether CT-1 was N-linked or O-linked glycosylated since previous studies reported putative glycosylation sites on the CT-1 sequence (186). Interestingly, CT-1 mobility was not altered after samples were incubated with the appropriate glycosidases. Therefore, it is necessary to conduct further studies to fully understand the highly stable interaction between CT-1 and another approximately 10 kD molecule. Furthermore, it will prove valuable to characterize the substantially strong dimer interaction that is established between CT-1 monomers that is able to resist rigorous denaturing and dissociation protocols required to perform Western blot analysis.

The novel findings presented in this study demonstrate that adipose tissue is a potential physiological source of circulating CT-1, and that CT-1 expression is downregulated in murine T2DM/obesity. It is therefore pertinent to suggest that the regulation of CT-1 in T2DM/obesity could be a possible link between T2DM/obesity and cardiovascular disease. Further, elucidating the underlying mechanism that regulates the protein expression may present therapeutic targets for T2DM related cardiovascular disease. While we did not observe CT-1 regulation in human white adipose tissue in T2DM/obesity, it is important to note that we did not examine CT-1 expression in the heart, liver and skeletal muscle of patients with T2DM. Investigating these tissues may gain insight on how CT-1 expression is regulated in human T2DM/obesity.
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