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**TNF-alpha INHIBITION OF ADIPONECTIN EXPRESSION BY TARGETING
PPAR-gamma AND C/EBP IN ADIPOCYTES**

A Thesis

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science**

In

The School of Human Ecology

By

**Yanning Wang
Bachelor of Medicine, Jiangsu University School of Medicine, China, 2005
August 2009**

DEDICATION

I would like to dedicate this to my major professor Dr. Ye and my family.

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I will be forever grateful to my major professor Dr. Ye for guiding me and sharing his wealth of experience and knowledge to every stage of my project and to my further education. I will always be indebted to him.

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ABBREVIATIONS

C/EBP	CCAAT-enhancer-binding proteins
Chop	CCAAT/Enhancer-Binding Protein Homologous Protein
HDAC3	Histone deacetylases 3
MAPK	Mitogen-activated protein (MAP) kinases
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PPAR- γ	Peroxisome proliferator-activated receptors- γ
PPRE	PPAR- γ response element
SREBP	Sterol Regulatory Element Binding Proteins
SRE	SREBP regulatory element
TNF- α	Tumor necrosis factor- α
TZD	Thiazolidinedione
Trog	Troglitazone

ABSTRACT

Chronic inflammation is involved in the adipose tissue dysfunction through regulation of endocrine and storage function of adipocytes. As a representative proinflammatory cytokine, TNF- α was reported to inhibit expression of adiponectin. However, the mechanism of inhibition remains to be identified. Here, we provide experimental evidence that TNF- α inhibits adiponectin at both transcriptional and posttranscriptional levels. In three animal models (aP2-P65, ob/ob and high fat diets-fed mice), an increase in TNF- α expression was associated with a decrease in adiponectin expression. In 3T3-L1 adipocytes, TNF- α inhibition of adiponectin was observed at mRNA and protein levels. Luciferase reporter assay and mRNA stability tests suggest that the mRNA reduction is a consequence of inhibition of gene transcription and mRNA stability. TNF- α inhibited expression and function of PPAR- γ , an activator of adiponectin gene promoter. The inhibitory activity of TNF- α was blocked by chemical inhibitors of NF- κ B or recombinant I κ B α (ssI κ B α), suggesting that the I κ B α /NF- κ B pathway mediates the TNF- α signal. The inhibition was attenuated by troglitazone, C/EBPs were required for PPAR- γ expression and their activities were reduced by HDAC3, a nuclear receptor corepressor. The study suggests a signaling pathway of TNF/NF- κ B/HDAC3/C/EBPs/PPAR- γ -Adiponectin for inhibition of adiponectin transcription by TNF- α .

CHAPTER 1

INTRODUCTION

In the last decade, adipose tissue has been identified as an important endocrine organ for its production of multiple adipokines, such as adiponectin, leptin, interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) (Costa et al., 2006). Adipocytes control metabolism in the whole body through endocrine function (Kershaw et al., 2004). Adipokines play a vital role in maintaining homeostasis of glucose and lipid metabolism (Iwaki et al., 2003).

Adiponectin, a 30-kDa adipokine that is exclusively produced by adipocytes, presents in the circulation of healthy humans and mice at high concentrations (Whitehead et al., 2006). Adiponectin has been recognized as a key regulator of insulin sensitivity and energy homeostasis. It promotes glucose uptake and fuel oxidation in skeletal muscle, enhances hepatic insulin action and decreases vascular inflammation (Berg et al., 2001; Hu et al., 1996). In human and animals, adiponectin is shown to increase insulin sensitivity (Nawrocki et al., 2006) and protect against cardiovascular diseases (CVD) (Ouchi et al., 2006; Yamauchi et al., 2001); Hypoadiponectinemia is observed in obese individuals with insulin resistance, type 2 diabetes (Hotta et al., 2000), and CVD (Ouchi et al., 1999). The mechanism for hypoadiponectinemia remains to be investigated.

TNF- α is one of the candidate molecules for the pathogenesis of insulin resistance and type 2 diabetes. TNF- α was initially discovered as an antitumor factor, however it is now known as a proinflammatory cytokine involved in pathogenesis of a wide variety of metabolic diseases such as insulin resistance, diabetes and atherosclerosis (Aggarwal et al., 2006; Saghizadeh et al., 1996; Uysal et al., 1997). In animal models of type 2 diabetes and humans with type 2 diabetes, TNF- α mRNA and protein levels are elevated in the adipose tissue (Mishima et al., 2001;

Saghizadeh et al., 1996; Uysal et al., 1997). In vitro, TNF- α treatment reduced the expression and secretion of adiponectin in 3T3-L1 adipocytes (Fasshauer et al., 2002; Maeda et al., 2001). There is a significant inverse relationship between adiponectin and TNF- α expression in human adipose tissue (Aggarwal et al., 2006; Uysal et al., 1997), indicating that TNF- α may contribute to the decreased adiponectin in obesity (Fasshauer et al., 2002). However, the mechanisms by which TNF- α inhibits adiponectin expression are poorly understood. Our data suggest that TNF- α may inhibit adiponectin expression at two levels: transcription and posttranscription.

CHAPTER 2

REVIEW OF LITERATURE

Biological and Physiological Effects of Adiponectin

Adiponectin, a product of the apM1 gene, is the most abundant adipokine produced by adipose cells and serves as a key regulator of insulin sensitivity and tissue inflammation (Whitehead et al., 2006). Human adiponectin is a 244 amino acid protein of approximately 30-kDa, which consists of an N-terminal collagenous domain and a C-terminal globular domain. Adiponectin exists in the circulation in multiple oligomeric forms in serum, of which the most abundant forms are trimers and hexamers. The oligomers are generally divided into low-molecular weight (LMW) oligomers and high-molecular weight (HMW) multimers. The LMW form is dominant in circulation, whereas the HMW form is dominant in the cells (Pajvani et al., 2003). Recent data show that a reduction in circulating HMW, but not other isoforms, correlates to the onset and development of insulin resistance and type 2 diabetes, suggesting that the HMW isoform is the biologically active one (Pajvani et al., 2004). Adiponectin exists abundantly in human plasma at a concentration of 2 to 20 µg/ml. In women, circulating adiponectin is about 40% higher than that in men (13 vs. 7 µg/ml on average) (Arita et al., 1999). In Pima Indians, serum adiponectin is lower than that in Caucasians (median 3.3 vs. 4.9 µg/ml) (Valsamakis et al., 2003).

Adiponectin exhibits anti-diabetic properties (Maeda et al., 2002; Yamauchi et al., 2002). The direct target tissues of adiponectin include skeletal muscle, liver, adipose tissue and the vasculature. In skeletal muscle cells, adiponectin stimulates insulin receptor substrate-1 (IRS-1)-mediated phosphatidylinositol-3 kinase (PI-3K) function, increases glucose uptake (Maeda et al., 2002; Yamauchi et al., 2001) and fuel oxidation via up-regulation of adenosine monophosphate-

activated protein kinase (AMPK) activity (Yamauchi et al., 2002). In the liver, adiponectin suppresses hepatic glucose production and improves hepatic insulin sensitivity (Combs et al., 2002). Adiponectin has anti-atherogenic effects in the vascular system, where it inhibits the nuclear factor- κ B (NF- κ B) signaling pathway and decreases mRNA expression of adhesion molecules in endothelial cells (Maeda et al., 2001). In vascular smooth muscle cells, adiponectin suppresses the proliferation and migration of smooth muscle cells induced by platelet-derived growth factors, including platelet-derived growth factor (PDGF)-AA, PDGF-BB, and heparin-binding epidermal growth factor (HB-EGF) (Ouchi et al., 2001). Adiponectin suppresses macrophage transformation into foam cells by inducing anti-inflammatory mediators IL-10 and IL-1 receptor. It also reduces lipid accumulation and the expression of class A macrophage scavenger receptor (Wolf et al., 2004).

In contrast to other adipokines whose levels increase with fat mass, adiponectin secretion and plasma levels are inversely proportional to body fat content (Arita et al., 1999). In both animal models and humans, adiponectin levels are reduced in individuals with type 2 diabetes and coronary artery disease (Arita et al., 1999; Hotta et al., 2000; Weyer et al., 2001). Its levels are up-regulated when body weight is decreased. Studies suggest that adiponectin secretion is enhanced by insulin-sensitizing thiazolidinediones (TZDs), which serves as agonists for the peroxisome proliferator-activated receptor gamma (PPAR- γ) transcription factor (Maeda et al., 2001; Yamauchi et al., 2001). Furthermore, adiponectin is able to antagonize many effects of TNF- α in 3T3-L1 adipocytes (Maeda et al., 2001). In human adipose tissue, a significant inverse relationship between adiponectin and TNF- α has been reported (Kern et al., 2003). This relationship suggests that TNF- α may be responsible for the reduction of adiponectin in obesity.

Regulation of Adiponectin Gene Expression

Although the biological effects of adiponectin have been investigated intensively, the regulatory mechanisms of adiponectin gene expression are poorly understood. Transcriptional regulation of the adiponectin gene is controlled by several transcription factors such as PPAR- γ (Gustafson et al., 2003; Iwaki et al., 2003; Maeda et al., 2001), sterol regulatory element-binding protein-1c (SREBP1c) (Liu et al., 2005), CCAAT/enhancer-binding protein (C/EBP) (Park et al., 2004; Qiao et al., 2005; Saito et al., 1999), and nuclear factor-kappa B (NF- κ B) (Ruan et al., 2002).

PPAR- γ

PPAR- γ is a member of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors. Three subtypes of PPARs have been identified: PPAR- α , PPAR- β/δ and PPAR- γ . PPAR- α , which regulates uptake and β oxidation of fatty acids, is mainly found in the liver, muscle, kidney and heart (Haluzik and Haluzik, 2006; Tsuchida et al., 2005). PPAR- β/δ is expressed ubiquitously, with the highest levels found in brain, adipose tissue, skin and skeletal muscle. PPAR- β/δ has been reported to be involved in embryo implantation (Xie et al., 2008), skin proliferation and differentiation (Schmuth et al., 2004), preadipocyte proliferation, and the up-regulation of lipid metabolism in the muscle (Rodriguez-Calvo et al., 2008). PPAR- γ gene produces two protein isoforms: PPAR- γ 1 and PPAR- γ 2. PPAR- γ 1 is expressed at low levels in many tissues including adipose tissue and muscle, whereas PPAR- γ 2 is most abundantly expressed in the adipose tissue. As a transcription factor, PPAR- γ binds to specific DNA response elements after forming heterodimers with retinoid X receptors (RXRs) to regulate transcription of target genes (Savage, 2005). In addition, PPAR- γ may affect gene expression through indirect interaction with other transcription factors (Cunard et al., 2004). Growing

evidence suggests that PPAR- γ is the master transcriptional regulator involved in adipogenesis, energy storage and lipid redistribution (Sempé et al., 2006). The natural ligands for PPAR- γ include fatty acids and fatty acid derivatives, such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂, eicosapentaenoic acid, 9- and 13-hydroxyoctadecadienoic acids. Meanwhile, several classes of synthetic PPAR- γ ligands have been widely used in research and clinical practice. TZDs represent the best characterized synthetic PPAR- γ ligands with wide application in the clinical management of type 2 diabetes. Activation of PPAR- γ by TZDs improves glycemic control and insulin sensitivity. Hyperinsulinemic clamp studies reported that TZDs can improve whole-body insulin sensitivity by increasing glucose and lipid uptake, and glucose oxidation in the target tissues (Maeda et al., 2001).

Adiponectin expression is related to PPAR- γ activity since administration of TZDs increases both mRNA and protein expression of adiponectin by adipose tissue (Combs et al., 2002; Iwaki et al., 2003). Several papers have reported that negative regulation of PPAR- γ mRNA contributes to the inhibition of adiponectin expression by TNF- α (Iwaki et al., 2003; Maeda et al., 2001; Zhang et al., 1996). Treatment of 3T3-L1 adipocytes with TNF- α resulted in a time- and concentration-dependent reduction in adiponectin mRNA and protein (Zhang et al., 1996). The expression inhibition was a consequence of gene promoter suppression (Maeda et al., 2001). In vitro, the promoter activity of adiponectin was markedly enhanced by the TZDs, suggesting that the adiponectin promoter may have a functional PPAR- γ response element (PPRE). TNF- α activity was antagonized by TZDs in the adipose tissue, suggesting involvement of PPAR- γ in prevention of TNF-inhibition of adiponectin (Maeda et al., 2001). Two papers have reported a functional PPRE (-273/-285) in both human and mouse adiponectin promoter (Iwaki et al., 2003; Seo et al., 2004). Their results indicated that a PPAR- γ /retinoid X receptor

(RXR) heterodimer was directly bound to PPRE in the adiponectin gene promoter (Iwaki et al., 2003). However, it remains to be investigated how PPAR- γ is involved in prevention of TNF- α inhibition of adiponectin expression.

ADD1/SREBP1c

The sterol regulatory element-binding protein (SREBP) is a transcription factor that regulates sterol and lipid metabolism. It plays an important role during adipocyte differentiation and insulin-dependent gene expression (Shimano, 2007). The SREBPs, which are inserted into the endoplasmic reticulum (ER) membrane, are synthesized as large precursor proteins through two membrane-spanning domains. The C terminus of the SREBP interacts with Scap (SREBP-cleavage-activating protein) in the ER, serving as a sterol sensor (Edwards et al., 2000). In sterol-depleted cells, SREBPs undergo specific proteolytic events and become transcriptionally active after translocation into the nucleus, where they bind to the promoter of SREBP target genes (Espenshade, 2006). When cholesterol builds up in the ER membrane, the Scap/SREBPs complex is retained in the ER membrane and the proteolytic activation of SREBPs stops preventing SREBP activation. SREBPs belong to the basic-helix-loop-helix leucine-zipper class of transcription factors and their unique DNA binding domains allow them to recognize the distinct promoter motifs known as SREs and classic E-boxes (Osborne, 2000; Yokoyama et al., 1993).

The family of SREBPs consists of three different SREBP proteins: SREBP1a, ADD1/SREBP1c and SREBP2. SREBP1a and SREBP1c originate from a single gene but have different N-terminal transactivation domains (Bengoechea-Alonso and Ericsson, 2007). SREBP1a has a higher transcriptional potency than that of ADD/SREBP1c, but ADD/SREBP1c is the dominant isoform that is involved in insulin signaling and fatty acid and triglyceride metabolism

(Horton et al., 2002). ADD/SREBP1c is highly expressed in liver, adipose tissue and other tissues. In adipocytes, ADD/SREBP1c expression is activated at an early phase of adipocyte differentiation, suggesting a role in the induction of the adipogenic genes (Kim and Spiegelman, 1996). It up-regulates the expression of many genes involved in lipogenesis and adipogenesis including fatty acid synthase (FAS), acetyl CoA carbocylase, PPAR- γ , lipoprotein lipase, and resistin (Seo et al., 2004; Shimano, 2007).

It has been reported that ADD/SREBP1c mRNA expression and protein concentration are significantly lower in obese and diabetic animal models (Seo et al., 2004). Also in obese and diabetic humans, ADD/SREBP1c expression is correlated to the reduction in adiponectin levels in the adipose tissue, suggesting that there may be a common signaling pathway to regulate both adiponectin and ADD/SREBP1c gene expression in adipocytes (Kolehmainen et al., 2001; Mingrone et al., 2003). A recent clinical trial in a large middle-aged Austrian population indicated that the SREBP1c locus is associated with type 2 diabetes and plasma adiponectin levels. Sequence variations at the SREBP-1 gene locus might contribute to altered plasma adiponectin levels and thus increase type 2 diabetes risk (Felder et al., 2007). Seo et al. have reported there were two putative SREs in the adiponectin gene promoters in both human and mouse. Their location is bp -398 to -389 (SRE1) and bp -343 to -334 (SRE2) (Seo et al., 2004). DNase I footprinting and chromatin immunoprecipitation (ChIP) analysis with recombinant ADD/SREBP1c indicated that ADD/SREBP1c binds to these two SREs motifs. Luciferase reporter assay showed that coexpression of ADD/SREBP1c led to activation of the adiponectin gene promoter, and SRE mutations abolished the transactivation from ADD/SREBP1c. Adenovirus overexpression of ADD/SREBP1c in differentiated 3T3-L1 adipocytes resulted in

high levels of adiponectin mRNA and protein, suggesting that ADD/SREBP1c controls adiponectin gene expression in differentiated adipocytes (Seo et al., 2004).

C/EBP

The CCAAT-enhancer-binding proteins (C/EBP) belong to the large family of basic leucine zipper (bZip) transcription factors. All members of the C/EBP family have a C-terminal leucine zipper domain for dimerization and a basic domain for DNA binding, respectively (Johnson, 2005). To date, six members in the C/EBP family have been cloned and characterized: C/EBP α , C/EBP β (also known as NF-IL6, LAP, AGP/EBP, IL-6DBP or NF-M), C/EBP γ (also known as Ig/EBP or GPE1BP), C/EBP δ (also known as NF-IL6 β , CRP3 or CELF), C/EBP ϵ (also known as CRP1) and CHOP (also known as gadd153) (Cao et al., 1991; Ron and Habener, 1992). The different members of the C/EBP family can form either homodimers or heterodimers. C/EBPs interact with the CCAAT (cytidine-cytidine-adenosine-adenosine-thymidine) DNA element in many gene promoters, such as adiponectin (Saito et al., 1999), PEPCK (Park et al., 1990), aP2 (Hutton et al., 2004), and GLUT4 (Biswas et al., 2005).

Four members of the C/EBP family, C/EBP α , C/EBP β , C/EBP δ and CHOP are expressed in both white and brown adipose tissues and have been analyzed extensively with regard to their roles in adipogenesis (Cao et al., 1991; Ron and Habener, 1992). The four C/EBP isoforms are expressed sequentially at specific stages during adipogenesis. C/EBP β and δ are induced at a very early time during the differentiation of preadipocytes, and have been reported to activate the expression of PPAR- γ (Tanaka et al., 1997). C/EBP α is induced relatively late during the adipogenesis, usually after the induction of PPAR- γ , but occurring before the synthesis of many enzymes (Wu et al., 1998). Ectopic expression of C/EBP α in 3T3-L1 preadipocytes blocks mitosis and induces adipocyte differentiation in the absence of hormonal stimulants (Freytag et

al., 1994). Blocking of C/EBP α expression in 3T3-L1 preadipocytes by antisense RNA prevents adipocyte differentiation and inhibits the expression of several adipocyte genes (Lin and Lane, 1992). Although NIH-3T3 fibroblasts cannot differentiate into adipocytes in the presence of hormonal stimulants, a high level of recombinant C/EBP α results in their conversion into adipocytes in the absence of hormonal stimulants (Wu et al., 1998). Moreover, C/EBP α knockout mice display a severely reduced mass of adipose tissue, demonstrating that C/EBP α is an important regulator of adipogenesis (Wang et al., 1995). Similarly, ectopic expression of C/EBP β also can convert fibroblasts into adipocytes even in the absence of hormonal stimulants. Over expression of a dominant-negative form of C/EBP β inhibits adipocyte differentiation, suggesting that C/EBP β is also involved in adipocyte differentiation. C/EBP δ exhibits similar activities, but to a lesser extent. CHOP is C/EBP homologous protein that is known as the dominant-negative form of C/EBP family members. The CHOP protein heterodimerizes with other C/EBP family members (i.e., C/EBP β) to block DNA-binding of the C/EBP proteins. Previous reports indicated that during adipogenesis, CHOP is down regulated, leading to C/EBP β activation. Up-regulation of CHOP prevents adipogenesis by inhibition of C/EBP β and inhibition of expression of C/EBP α (Tang and Lane, 2000).

It has been reported that three C/EBP sites were identified in the human adiponectin promoter (Park et al., 2004; Saito et al., 1999). Deletion or mutation of the C/EBP element in the adiponectin promoter results in a great loss of the gene transactivation in 3T3-L1 adipocytes. Chromatin immunoprecipitation analysis (ChIP) showed that both C/EBP α and β occupied adiponectin promoter sites in adipocytes, indicating C/EBP binds directly to the proximal adiponectin promoter in vivo (Park et al., 2004). The adiponectin promoter activity was markedly inhibited when co-transfected with the dominant negative form of C/EBP. On the

contrary, the promoter activity was increased around 3-fold in 3T3-L1 adipocytes when a constitutively active form of C/EBP β was expressed. The increase was significantly suppressed by TNF- α in a treatment for 8 hr (Kita et al., 2005). Electrophoretic mobility shift assay demonstrated that the specific DNA-protein interaction was abolished by TNF- α treatment, suggesting that TNF- α may be involved in the suppression of C/EBP leading to inhibition of adiponectin expression (Kita et al., 2005).

Several papers reported that CHOP protein is involved in down-regulation of adiponectin expression in obesity and type 2 diabetes (Hosogai et al., 2007; Kim et al., 2006). In obese mice, adiponectin mRNA expression was decreased and mRNA of CHOP was significantly increased in the adipose tissue. And RNA interference of CHOP partly reversed the suppression of adiponectin mRNA expression in adipose tissue of obese mice, suggesting the role of CHOP in insulin resistance and type 2 diabetes by down-regulation of adiponectin expression in adipocytes (Chevillotte et al., 2007; Hosogai et al., 2007; Kim et al., 2006).

SP1/SP3

Specificity protein 1 (SP1) and other Sp proteins are members of a family of transcription factors which bind the GC/GT-rich DNA elements through three conserved Cys2/His2 zinc fingers that are present at their C-terminal domains (Li et al., 2004; Safe and Abdelrahim, 2005). SP1- SP4 proteins are involved in the expression of multiple genes in normal tissues and tumors (Safe and Abdelrahim, 2005) .

Among the Sp family of transcription factors, SP1 and SP3 are ubiquitously expressed in mammalian cells to regulate various genes. SP1 is a human transcription factor involved in gene regulation in the early development of an organism. SP3 belongs to a family of SP1 related genes, which is structurally similar to SP1 and with high homology in Sp1-binding domain (Li et al.,

2004). SP1 and SP3 genes encode 105- and 115-kDa proteins respectively. SP1 and SP3 have been shown to activate or repress gene promoter activity (Barth et al., 2002; Li et al., 2004).

One paper reported that Sp1/Sp3 was involved in the TNF- α induced inhibitory effect on adiponectin promoter activity. SP1 has stimulatory effects; SP3 has inhibitory effects on adiponectin promoter activity, which is mediated by a proximal SP1 binding site. The mechanism of TNF- α -induced inhibition of adiponectin gene expression is, at least in part, due to a decrease of transcriptional SP1 binding activity caused by TNF- α . First an electrophoretic mobility shift assay was used to investigate the transcriptional binding activity of SP1 protein in the adipocyte. The result indicated that SP1 is expressed in adipocytes and its DNA-binding activity is induced during differentiation from preadipocytes to adipocytes. Next an electrophoretic mobility shift assay was used to indicate an added recombinant SP1 factor as well as the natural SP1 factor in nuclear extracts from adipocytes, bind specifically to the proximal SP1 binding site within the adiponectin promoter. Also, mutation of the SP1 binding site within the proximal promoter fragment abolishes the SP1 binding shift of preadipocytes and adipocytes in the electrophoretic assay. The transient transfection of adipocytes with promoter constructs carrying the proximal SP1 binding site demonstrated that SP1 can induce the promoter activity. In the end, EMSA showed that treatment of preadipocytes and mature adipocytes with different doses of TNF- α (0.2nmol/l and 2nmol/l) leads to an inhibition of SP1 binding to the proximal promoter fragment (prom -28/-80). (Schaffler et al., 1999)

NF- κ B

Nuclear factor-kappa B is a ubiquitous transcription factor which is found in almost all animal cell types and involved in cellular responses to stimuli such as stress, cytokines, free radicals, and oxidized LDL (Gilmore, 2006). NF- κ B belongs to the Rel protein family which

includes RelA (p65), NF- κ B1 (p50), NF- κ B2 (p52), RelB, and c-Rel that exist as homo- and heterodimers (Liu and Malik, 2006). The most common form of NF- κ B is a heterodimer composed of p50/RelA (p50/p65). The p65, but not p50, subunit of NF- κ B contains trans-activating domain. All NF- κ B proteins share a highly conserved 300 amino acid Rel homology domain (RHD), which is responsible for DNA binding, dimerization and nuclear localization (Karin and Ben-Neriah, 2000). NF- κ B plays a vital role in the immune system (Baeuerle and Henkel, 1994) and its activities have been associated with many diseases including diabetes, atherosclerosis, cancer, and arthritis (Atreya et al., 2008; Sastre et al., 2008). Although activation of NF- κ B is a multi-step sequential process, NF- κ B activation is primarily controlled by inhibitor κ B (I κ B) that binds to NF- κ B and causes it to be retained in the cytoplasm. Most agents activate NF- κ B through the classic pathway that is dependent on phosphorylation-induced and proteasome-mediated degradation of I κ B, which results in the nuclear translocation of the transcription factor (Piotrowska et al., 2008). I κ B α is phosphorylated by the IKK (I κ B kinase) complex at two conserved serines (ser32 and ser36) on the N-terminal regulatory domain. The IKK complex contains three subunits: IKK α , IKK β and the IKK γ of which IKK γ (NEMO. NF- κ B essential modulator) serves as a regulatory subunit. IKK α and IKK β have kinase activities that are responsible for phosphorylation of I κ B (Basak et al., 2007). Studies have found IKK β , not IKK α , is essential for the degradation of I κ B α , because disruption of IKK β led to a complete loss of NF- κ B activity in mice (Li et al., 1999).

TNF- α is a key inflammatory cytokine involved in insulin resistance and type 2 diabetes. One study suggests that NF- κ B is an obligatory mediator responsible for inhibition of adipocyte-specific genes by TNF- α (Ruan et al., 2002). Nuclear translocation of p65 increased substantially at 15 to 60 min after TNF- α addition. Gel mobility shift assay in 3T3-L1 adipocytes confirmed

that after TNF stimulation, p65 activation and nuclear translocation occurred within 15 min and lasted at least 60 min, suggesting that NF- κ B is immediately involved in TNF- α action at the gene transcriptional level in 3T3-L1 adipocytes. A nondegradable NF- κ B inhibitor was utilized to assess the kinetics of TNF- α -induced change in gene expression. In wild type 3T3-L1 adipocytes, TNF- α , inhibited the mRNA levels of 61, 193, and 264 genes at 0.5 h, 1 h, and 2 h time points. However, in I κ B α -DN adipocytes, only 1.6%, 0.5%, and 1.9% of the number of genes were down regulated by TNF- α at the same time points. This suggests the requirement of NF- κ B activation in suppression of 98%-99% of the gene inactivated by TNF- α .

mRNA Stability

The level of mRNA expression is regulated at the transcriptional and posttranscriptional levels. mRNA stability is important in the posttranscriptional pathway and varies from one gene to another. It is affected by many variables such as structure, intracellular location and extracellular stimuli (Shim and Karin, 2002). The 3' poly (A) tail of eukaryotic mRNAs plays a key role in the nuclear processing of pre-mRNA, transport to the cytoplasm, translation and mRNA stability. Deadenylation is the first step in the degradation of mRNA. The presence of poly (A) tail can protect the mRNA from decaying by binding to poly (A) binding protein (PABP) at the mRNA 3' terminus, protecting it from degradation by deadenylases and exonucleases (Bernstein and Ross, 1989). Meanwhile, several regions that include the 5'-cap structure, the 5'-untranslated region (UTR), the protein coding region and the 3'-UTR have been identified as important structure elements which affect the half lives of mRNAs (Shim and Karin, 2002). In addition, c-Fos and c-Jun induced by extracellular stimuli have been reported to increase the half-life of mRNA (Chen and Shyu, 1995). Early studies of IL-2 have shown that activation of JNK is involved in extension of mRNA stability (Chen et al., 2000). Another MAPK p38 was

reported to participate in the mRNA stability of vascular endothelial growth factor (VEGF) (Levy et al., 1998) and cyclo-oxygenase 2 (Cox-2) (Lasa et al., 2000). Posttranscriptional regulation of mRNA stability is another control of adiponectin gene expression. One study has examined the effect of hypoxia on the stability of adiponectin mRNA. The mRNA degradation of adiponectin was accelerated under hypoxia compared with normoxia (Hosogai et al., 2007). Adiponectin mRNA does not contain a poly (A) tail and a classical AU rich motif, therefore, the mechanism for adiponectin mRNA-life is still unknown.

CHAPTER 3

TNF- α INHIBITION OF ADIPONECTIN BY TARGETING PPAR- γ AND C/EBP

Introduction

Modulation of adiponectin expression at the transcriptional level is an important step for understanding the hypoadiponectinemia in insulin resistance and type 2 diabetes. Regulatory elements and transcription factors involved in the regulation have been described in the proceeding chapter. For example, adiponectin transcription is regulated by PPAR- γ , C/EBPs, SREBP-1c (Seo et al., 2004), SP1, SP3 and NFATC4 (Barth et al., 2002; Kim et al., 2006). However, their activities in adiponectin regulation by TNF- α have not been demonstrated. A thorough understanding of those mechanisms will assist the development of treatments for metabolic syndromes. Therefore, our present study was performed to investigate the molecular mechanisms of adiponectin expression in response to TNF- α .

Materials and Methods

Animals

Male ob/ob mice (B6.V-Lepob/J, stock no. 000632), and male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The aP2-P65 transgenic mice were generated by microinjecting transgene aP2-P65 into the pronuclei of fertilized mouse eggs taken from superovulated C57BL/6 F1 females. The injected embryos were implanted into the oviducts of surrogate females of the same genetic background. These procedures were conducted in the transgenic core facility at the Pennington Biomedical Research Center. The aP2-P65 and ob/ob mice were fed a normal chow diet (12.8% calories from fat). In the diet-induced obesity model, male C57BL/6 mice (4 wk of age) were fed a high-fat diet (HFD. No. D12331. 58% calories from fat. Research Diets, New Brunswick, NJ) beginning at 5 wk of age. All of the mice were

housed in the animal facility at the Pennington Biomedical Research Center with a 12:12-h light-dark cycle and constant temperature (22–24 °C). The mice were kept 4 per cage and had free access to water and diet. All procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the Pennington Biomedical Research Center.

Materials and Reagents

Adiponectin antibody (MAB3832) was obtained from Chemicon International and PPAR- γ (sc-7373x) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to tubulin (ab7291) and β -Actin (ab6276) were obtained from Abcam (Cambridge, UK). For RNA isolation from cell culture: trizol (T9424), chloroform (C2432) and isopropanol (I9516) were from Sigma (St. Louis, MO). Of the chemical inhibitor SP600125 (EI-305) was purchased from Biomol (Plymouth Meeting, PA). Other chemical inhibitors, PD098059 (p-215) and SB203580 (s-8307) were from Sigma (St. Louis, MO), and 15d-PGJ2 (538927) was acquired from Calbiochem (San Diego, CA). TNF- α (T-6674) and insulin (I-9278) were purchased from Sigma (St. Louis, MO). Troglitazone (GR-210) was acquired from Biomol (Plymouth Meeting, PA). Lipofectamine 2000 reagent for transient transfection was purchased from Invitrogen (Carlsbad, CA). Real time RT-PCR reagents including MuLV reverse transcriptase, RNase inhibitor and PCR reagents were obtained from Applied Biosystems (Branchburg, NJ). The PPRE luciferase reporter was constructed utilizing the pGL3 basic luciferase vector. In this vector, the luciferase gene is driven by the thymidine kinase (TK) promoter (-105/-51) of herpes simplex virus. The PPAR- γ -specific reporter was generated by inserting three copies of the PPRE element of the rat acyl-CoA synthase gene

(⁵⁸³CCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT544) (Tugwood et al., 1992) upstream of the TK promoter. The supersuppressor I κ B α expression vector was originally obtained from Dr. Inder M. Verma (Salk Institute).

Cell Culture

3T3 preadipocytes were purchased from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and supplemented with 10% fetal bovine serum and 5% CO₂ at 37 °C. Differentiation of preadipocytes to adipocytes was achieved by allowing cells to reach confluence and adding DMEM supplemented with 10% fetal bovine serum, 5 μ g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 μ M dexamethasone. After 48h, the medium was replaced with fresh DMEM plus 10% fetal bovine serum supplemented with 5 μ g/ml insulin. Five days later, the fully differentiated adipocytes were used in the experiments.

HEK293 cell line was purchased from the ATCC and maintained in the DMEM containing high glucose and supplemented with 5% fetal bovine serum and 5% CO₂ at 37 °C.

Transfection and Luciferase Assay

The luciferase reporter driven by adiponectin gene promoter (–1,300/+18) was kindly provided by Dr. Jianhua Shao (Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, KY). The reporter plasmids were transfected into 3T3-L1 adipocytes by electroporation using the Nucleofector II electroporator (Amaxa Biosystems, Cologne, Germany). Program T030 was used in electroporation. For TNF- α and troglitazone treatment, the cells were kept in serum-free medium for 16 h and treated with TNF- α or troglitazone for 24 h before the reporter assay. Mouse PPAR- γ gene promoter (–2000/+45) was generated by Dr. Jin Zhang.

HEK 293 cells, 3T3-L1 fibroblasts transient transfection was conducted in triplicate in 12-well plates. Cells (1.5×10^5 /well) were plated for 16 h and transfected with plasmid DNA utilizing Lipofectamine 2000 reagent according to the manufacturer's instruction. In co-transfection system, the expression plasmids for ssI κ B α , C/EBP α , C/EBP δ , HDAC3 RNAi were used. For TNF- α and troglitazone treatment, the cells were kept in serum-free medium for 16 h and treated with TNF- α or troglitazone for 24 h before reporter assay.

In all of the transient transfection experiments, the internal control reporter is 0.1 μ g/well of SV40-Renilla luciferase reporter plasmid, and the total DNA concentration was corrected in each well with a control plasmid. The luciferase reporter assay was conducted using a 96-well luminometer with the dual luciferase substrate system (Promega). The luciferase activity was normalized with the internal control Renilla luciferase activity, and a mean value together with a standard error of the triplicate samples were used to determine the reporter activity. Each experiment was repeated at least three times.

The Supersuppressor I κ B α (ssI κ B α) Cell Line and 3T3-L1 Adipocytes

To make the ssI κ B α stable cell line, 3T3-L1 fibroblasts were infected with pBabe retrovirus that carries the FLAG- ssI κ B α expression cassette. The positive clone was selected by culturing the infected cells in puromycin containing medium for 2 days followed by a screening of the FLAG epitope in the whole cell lysate using a western-blotting. 3T3-L1 adipocytes were obtained by differentiation of the fibroblasts in the standard adipogenic mixture as described above.

Western Blotting

Whole cell lysate protein was made in lysis buffer [1% Triton X-100, 50 mM KCl, 25 mM HEPES (pH 7.8), 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, 125 μ M dithiothreitol, 1 mM

phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate] via sonication to break both cytoplasmic and nuclear membranes. The protein (100 µg) in 50 µl of reducing sample buffer was boiled for 3 min and resolved in 8% mini-SDS-PAGE for 90 min at 100 volts. Then, the protein was transferred onto polyvinylidene difluoride membrane (162-0184; Bio-Rad, Hercules, CA) at 50 volts for 120 min. The membrane was blotted with the first antibody for 1–24 h and the secondary antibody for 30 min. The horseradish peroxidase-conjugated secondary antibodies (NA934V or NA931; GE Healthcare, Little Chalfont, UK) were used with chemiluminescence reagent (NEL-105; PerkinElmer Life Sciences, Wellesley, MA) for generation of the light signal. To detect multiple signals from one membrane, the membrane was treated with a stripping buffer (59 mM Tris-HCl, 2% SDS, 0.75% 2-mercaptoethanol) for 20 min at 37 °C after each cycle of blotting to remove the bound antibody. All of the experiments were conducted three or more times. The intensity of the western blotting signal was quantified using a computer program, PDQuest 7.1 (Bio-Rad).

RNA extraction

Total RNA from 3T3-L1 adipocytes was isolated from each individual rat following the RNA extraction protocol. 1 ml TRIZOL was added and incubated at room temperature for 5 min. Then 200 µl chloroform was added and the samples vortexed thoroughly each sample for 3 min. Each sample was centrifuged at 13000 x g for 15 min at 4 °C. The upper aqueous phase RNA was collected and then 500 µl isopropanol was added, and then samples were vortexed for 10 min, then centrifuged at 13000 x g for 20 min at 4 °C. Supernatant was discharged and pellet washed with 1 ml 75% ethanol. Then the supernatant was spun at 7500 x g for 5 min at 4 °C. Again, the the supernatant was discharged and the RNA pellet was dried for 10 min. Then 30 µl of RNase-free water was used to dissolve the RNA pellet and the pellet was

stored at -70 °C. For RNA quantification, 2 ul of sample RNA was used to measure the optical density (OD) 260 and (OD) 280 nm using Nanodrop via UV spectrophotometry.

Quantitative Real Time RT-PCR

Quantitative Real time RT-PCR was used to determine TNF- α (Mm00443258_m1, Applied Biosystems), adiponectin (Mm00456425_m1, Applied Biosystems), PPAR- γ (Mm00440945-m1, Applied Biosystems), P65 (Mm00501346_m1), Applied Biosystems), C/EBP α (5'-GCGAGCACGAGACGTCTATAGA-3';5'-GCCAGGAACCTCGTCGTTGAA-3'), C/EBP β (5'-AGCGGCTGCAGAAGAAGGT-3',5'-GGCAGCTGCTTGAACAAGTTC-3') and C/EBP δ (5'-CATCGACTTCAGCGCCTACAT-3', 5'-TGAAGAGGTCGGCGAAGAGT-3') mRNA levels in the samples. Mouse 18s rRNA was used as an internal control to normalize the RNA levels in each sample. Real time RT-PCR reaction mixture was 10 ul total volume, including 15 ng of sample RNA, 1 X PCR buffer, 5.5mM MgCl₂, dATP, dCTP, dUTP and dGTP each 0.3 mM, 200 nM forward primers, 200 nM reverse primers, 100 nM Taqman probes, 1 U RNase inhibitor, 2.5 U MuLV reverse transcriptase, 0.25 U AmpliTaq Gold DNA polymerase and RNase-free H₂O. Each sample was tested in duplicate. Reverse transcription was carried out at 48 °C for 30 min. PCR was carried out at 95 °C for 10 min for one cycle, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Data was output as Ct number (cycle threshold number) and the data within the linear region of the amplification curve were analyzed according to ABI's User Bulletin #2. The relative amounts of adiponectin and PPAR- γ mRNA were calculated using $\Delta\Delta C_t$ method and normalized over 18S ribosomal RNA gene.

Statistical Analysis

Each experiment was conducted at least three times with consistent results. The digital data was presented as a mean \pm SEM. In the reporter assay, triplicates were used at each point.

The data were analyzed using Student's t test with significance $p < 0.05$.

Results

Expression of TNF- α and adiponectin in aP2-P65 and obese mouse models.

Previous studies have demonstrated that adiponectin was significantly down-regulated in the WAT of ob/ob mice and high fat diet (HFD)-fed mice (Barth et al., 2002). The reduction was associated with increased expression of TNF- α . In cultured cells, TNF- α was shown to inhibit adiponectin mRNA. To confirm the cause/result relationship in vivo, we used aP2-P65 mice which have enhanced TNF- α expression by over expression of RelA (p65) in the adipose tissue. RelA (p65) gene is a member of NF- κ B protein complex. Our previous study showed that aP2-P65 transgenic mice exhibit a high activity of chronic inflammation. TNF- α mRNA was increased in the adipose tissue in the absence of obesity (Fig. 1A), while adiponectin mRNA was dramatically reduced (Fig. 1B). These results exclude possibilities that are associated with obesity and support the idea that TNF- α inhibits adiponectin in adipose tissue.

TNF- α inhibits adiponectin gene expression in mature 3T3-L1 adipocytes

To explore the mechanism of adiponectin inhibition, we tested an in vitro model of TNF- α treatment in 3T3-L1 adipocytes. Adiponectin mRNA and protein were reduced by TNF- α (Fig. 2). At 4 h with TNF- α (20 ng/ml) treatment, the adiponectin mRNA was reduced by 50% in qRT-PCR assay (Fig. 2A). Adiponectin protein was measured in the cell supernatant by western blotting. Adiponectin expression was significantly reduced after 8h with TNF- α treatment (Fig. 2B). These data confirm that adiponectin expression is suppressed by TNF- α in adipocytes.

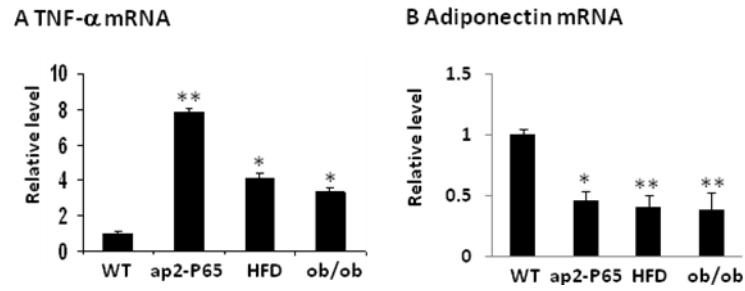


Figure 1. Adiponectin and TNF- α expression in white adipose tissue of wild type, ap2-P65, HFD-fed mice and ob/ob mice. Epididymal fat tissues were collected from the mice at the age of 20 weeks, and the total RNA was extracted and subjected to quantitative real time RT-PCR analysis for TNF- α mRNA (A) and adiponectin mRNA (B). In this figure, each bar represents mean \pm SEM (n=3). *, P<0.05, **, P<0.01.

A Adiponectin mRNA expression B Adiponectin protein in medium

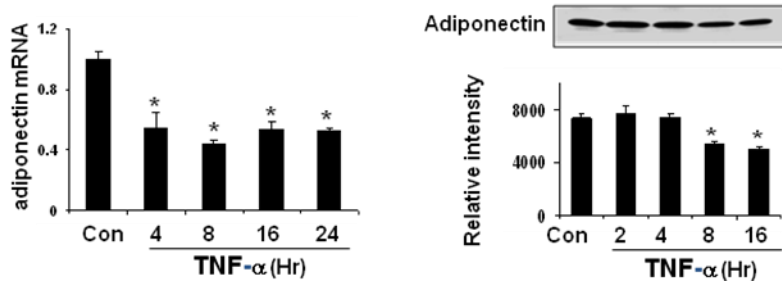


Figure 2. Inhibition of adiponectin expression by TNF- α in 3T3-L1 adipocytes. A: inhibition of adiponectin mRNA by TNF- α . The cells were serum starved in DMEM supplemented with 0.25% BSA overnight and treated with TNF- α (20 ng/ml) for different times as indicated. The total RNA was extracted and subjected to quantitative real time RT-PCR analysis for adiponectin mRNA. B: Inhibition of adiponectin secretion by TNF- α . The cells were serum starved overnight and treated with TNF- α for different times as indicated. Culture medium from 3T3-L1 adipocytes was analyzed by western blot with anti-adiponectin antibody. Quantification of adiponectin protein in the western blot was determined by densitometry. In this figure, each bar represents mean \pm SEM (n=3). *, P< 0.05.

TNF- α inhibits the adiponectin transcription and mRNA stability.

Since both transcriptional and post-transcriptional events influence mRNA levels, our goal was to find out which event is important for adiponectin inhibition. We tested transcription using the -2.0 kb human adiponectin promoter in a reporter assay. A luciferase reporter driven by the promoter was transfected into 3T3-L1 adipocytes in a transient transfection. The reporter was enhanced by troglitazone (a PPAR- γ activator) by 4.5 fold (Fig. 3A). The induction was completely inhibited in the presence of TNF- α (20 ng/ml) (Fig. 3A). The data indicates that TNF- α may target PPAR- γ in the transcriptional inhibition of adiponectin.

To determine whether the posttranscriptional events are involved in the reduction of adiponectin mRNA, we examined the effects of TNF- α on the stability of adiponectin mRNA. In the study, mRNA synthesis was blocked by Actinomycin D (5 μ g/ml) and the remaining adiponectin mRNA was measured at multiple time points thereafter. The mRNA stability is indicated by reduction rate of mRNA. The qRT-PCR result suggests that the mRNA degradation of adiponectin was significantly accelerated by TNF- α (Fig. 3B). The mRNA is very stable with only 10% reduction at 16 hrs. In the TNF-treated cells, the mRNA was reduced by 50% at 12 hrs. These results together suggest that TNF- α inhibits the adiponectin expression at both transcriptional and post-transcriptional levels.

TNF- α inhibits PPAR- γ gene expression through NF- κ B pathway

Since PPAR- γ is a key transcription activator in the adiponectin gene promoter, a loss of PPAR- γ function may account for the adiponectin inhibition. We conducted more investigation into the mechanism in 3T3-L1 adipocytes. As shown in Fig. 4A, the expression of PPAR- γ mRNA was significantly decreased in the presence of 20 ng/ml TNF- α . There was an inhibition of 40% observed as early as 4h with TNF treatment. The PPAR- γ protein was decreased

consistently by 70% at 8 hr (Fig. 4B). These data indicate that TNF- α suppresses the expression of PPAR- γ mRNA and protein in the differentiated adipocytes. We also examined the PPAR- γ mRNA stability. The stability was not changed in the absence or presence of TNF- α , indicating that TNF- α does not affect PPAR- γ mRNA stability. Several signaling pathways are activated by TNF- α , such as the IKK/NF- κ B pathway and MAPKs (ERK, JNK, and p38) pathways. To determine which pathway is required for the TNF- α inhibition of PPAR- γ expression, kinase-specific chemical inhibitors were used to evaluate each pathway. These inhibitors include IKK2 inhibitor 15dPGJ2 (15-deoxy- Δ 12,14-prostaglandin J2), JNK inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one), p38 MAPK kinase inhibitor SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole) and MEK/ERK inhibitor PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one). 3T3-L1 adipocytes were pretreated with the inhibitors before TNF- α exposure. The inhibitor activity was determined with the PPAR- γ mRNA. 15dPGJ2 that inhibits NF- κ B activity through inhibition of IKK β activity blocked the TNF- α activity (Fig. 4D). The inhibitors to MAPKs were unable to block the TNF activity (Fig. 4D). To further ascertain whether NF- κ B activation is involved in TNF- α mediated PPAR- γ gene expression, a PPAR- γ reporter system was used in the transient transfection of NIH3T3 fibroblasts, at which the reporter activity was induced by the synthetic troglitazone (Fig. 4E). TNF- α led to a 40% reduction in the PPAR- γ reporter and this reduction was completely blocked by expression of the super suppressor I κ B α (ssI κ B α), a non-degradable mutant of I κ B α (I κ B α ^{S32A/S36A}) that blocks NF- κ B activation (Gao et al., 2006). These data suggest that NF- κ B mediates the suppression of PPAR- γ by TNF- α .

IKK/NF- κ B pathway mediates TNF- α activity on adiponectin

To determine the role of NF- κ B pathway on adiponectin expression, we examined the adiponectin mRNA after NF- κ B was inhibited. 15dPGJ2 blocked the TNF- α inhibition of adiponectin (Fig. 5A). In the ssI κ B α 3T3-L1 cell line that has a reduced NF- κ B activity, adiponectin inhibition was attenuated significantly (Fig. 5B). The adiponectin protein expression was examined during the adipogenesis of ssI κ B α 3T3-L1 cells. In this study, cells were exposed to TNF- α during the course of adipogenesis for 7 days. As shown in Fig. 5C, in the presence of TNF- α , adiponectin was completely blocked in the 3T3-L1 control cells, but not in the ssI κ B α 3T3-L1 cells, suggesting that ssI κ B α cells are resistant to TNF- α activity. These results indicate that TNF- α activity is dependent on activation of the I κ B α /NF- κ B pathway, and IKK/NF- κ B pathway in the inhibition of adiponectin gene transcription.

Troglitazone antagonizes TNF- α activity by inhibition of NF- κ B

It was reported that NF- κ B activity was inhibited by PPAR- γ ligands thiazolidinediones (Xie et al., 2005). Here we tested troglitazone on suppression of NF- κ B activity. Incubation with troglitazone increased adiponectin mRNA expression (Fig. 6A) and adiponectin protein secretion (Fig. 6B) into the media in a dose-dependent manner. Troglitazone treatment for 24 h induced the adiponectin mRNA expression (Fig. 6A). The adiponectin protein was also increased (Fig. 6B). The inhibition of adiponectin by TNF- α was partly reversed by troglitazone in 3T3-L1 adipocytes (Fig. 6 A and B). To determine inhibition of NF- κ B by troglitazone, we examined mRNA levels of NF- κ B target genes, such as TNF- α and I κ B α . Upon TNF- α stimulation, TNF- α and I κ B α mRNA levels increased dramatically (Fig. 6 C and D). Troglitazone inhibited I κ B α mRNA by 40% (Fig. 6C), and TNF- α mRNA by 75% (Fig. 6D). The data support that troglitazone antagonizes the action of TNF- α through inhibition of the NF- κ B pathway.

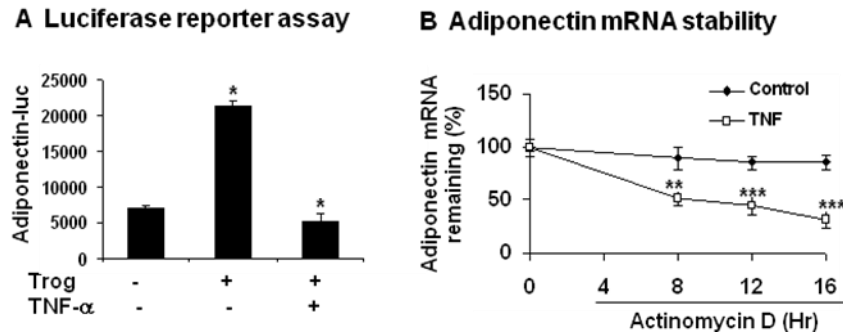


Figure 3. Adiponectin transcription and mRNA stability pathway. A. Inhibition of adiponectin gene promoter by TNF- α . The transcriptional activity of mouse adiponectin promoter ($-1,300/+18$) was analyzed in 3T3-L1 adipocytes in a transient transfection. After 24 h transfection, the cells were serum starved overnight and treated with Troglitazone (10 μ M) and TNF- α (20 ng/ml) for 16 h. B. Influence of TNF- α on adiponectin mRNA stability. De novo mRNA transcription was inhibited by the addition of actinomycin D (5 μ g/ml), and 3T3-L1 adipocytes were incubated with TNF- α treatment (20 ng/ml, open square) or not (filled square) for the indicated time intervals. The residual mRNAs of adiponectin were quantified by qRT-PCR. Values represent percentage of residual mRNA versus mRNA level at time 0. Results are means \pm SEM (n=3). *, P< 0.05. ***, P< 0.001.

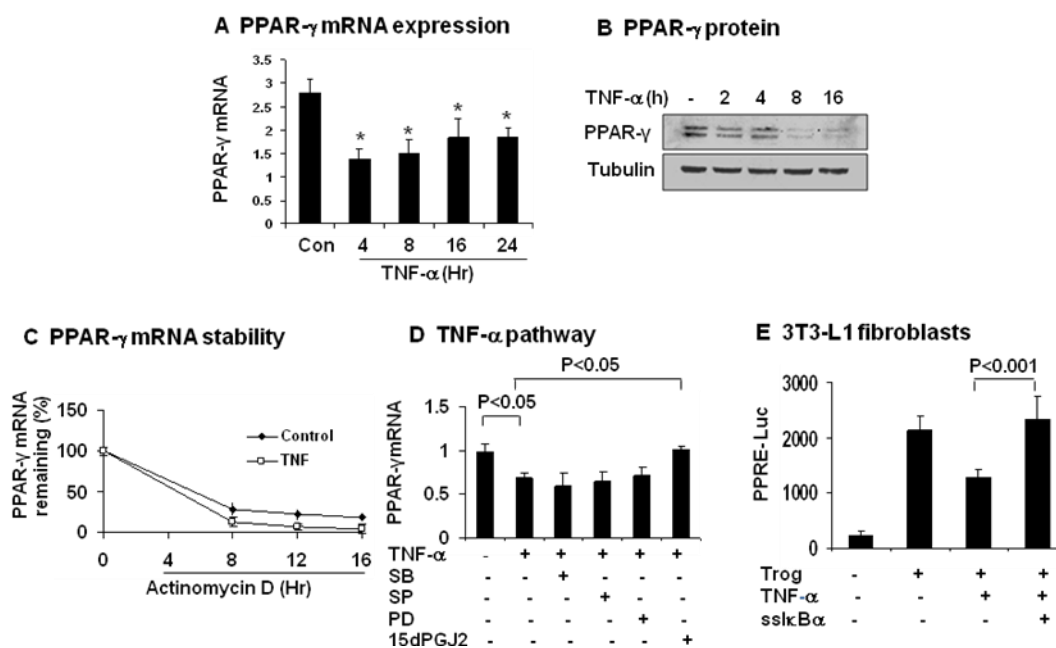


Figure 4. TNF- α inhibits PPAR- γ expression through the NF- κ B pathway. A: Inhibition of PPAR- γ mRNA expression by TNF- α in 3T3-L1 adipocytes. The cells were serum starved overnight and treated with TNF- α (20 ng/ml) for different times as indicated. The total RNA was extracted and subjected to qRT-PCR analysis for PPAR- γ mRNA. B: Inhibition of PPAR- γ protein expression by TNF- α . The cells were serum starved overnight and treated with TNF- α (20 ng/ml). The PPAR- γ protein was determined in the whole cell lysate in a western blot with anti-PPAR- γ antibody. C: Influence of TNF- α on the PPAR- γ mRNA stability. De novo mRNA transcription was inhibited by the addition of actinomycin D (5 μ g/ml), and 3T3-L1 adipocytes were incubated with TNF- α treatment (20 ng/ml, open square) or not (filled square) for the indicated time intervals. The residual mRNAs of PPAR- γ was quantified by qRT-PCR. Values represent percentage of residual mRNA versus mRNA level at time 0. D: Blocking of TNF- α activity by the NF- κ B inhibitor. The 3T3-L1 adipocytes were pretreated with the pharmacological inhibitors for 30 min before addition of TNF- α . The inhibitors were SB203580 (SB;10 μ M), SP600125 (SP;25 μ M), PD98059 (PD;40 μ M), 15dPGJ2 (15 μ M). mRNAs were determined in the cells 16 h later. E: The transcriptional activity of PPAR- γ was analyzed in 3T3-L1 fibroblasts using the PPRE (3X)-luciferase reporter system in the transient transfection. The expression vector of ssIkB α was co-transfected to block NF- κ B activation. At 24 h after transfection, the cells were serum starved overnight and treated with Troglitazone (10 μ M) and TNF- α (20 ng/ml). In this figure, each bar represents mean \pm SEM (n=3). *, P< 0.05.

TNF- α inhibited C/EBP α but induced C/EBP δ expression

C/EBPs were reported as key transcription activators in the adiponectin gene (Weyer et al., 2001). To elucidate whether C/EBPs are involved in the TNF- α inhibition of adiponectin, we next examined the expression and function of C/EBPs. As expected, TNF- α treatment significantly decreased the mRNA level of C/EBP α (Fig. 7A). However, C/EBP δ mRNA level was induced by TNF- α (Fig. 7C). C/EBP β mRNA was not dramatically changed by TNF- α (Fig. 7B). At the protein level, C/EBP α was reduced at 8 h, while C/EBP β and C/EBP δ proteins were modestly induced by TNF- α (Fig. 7D). To confirm the regulation of C/EBPs by TNF- α in vivo, the expression was determined in aP2-P65 transgenic mice. As shown in Fig. 7E, C/EBP α mRNA was decreased by 48%, while C/EBP δ was up-regulated by 30% in the aP2-P65 transgenic mice. No change was found in C/EBP β . Based on these results, C/EBPs may participate in TNF signaling. To test the possibility, we examined the PPAR- γ promoter activity in the transient transfection. Transfection of C/EBP α or C/EBP δ induced the PPAR- γ (Fig. 7F). The transactivation activity of C/EBP δ activity was inhibited by TNF- α , and the inhibition was blocked by ssI κ B α (Fig. 7G), suggesting that NF- κ B activity was required for inhibition of the promoter activity. NF- κ B may inhibit C/EBP function through HDAC3 as indicated by an early study in which we observed that HDAC3 activity was induced by NF- κ B (Gao et al., 2006). To test the possibility, HDAC3 activity was reduced by RNAi-mediated gene knockdown. The TNF- α activity was abolished by HDAC3 RNAi, but not by the control RNAi (Fig. 7H). These data suggest that HDAC3 is required for PPAR- γ inhibition by TNF- α and might be involved in suppression of C/EBP δ by NF- κ B.

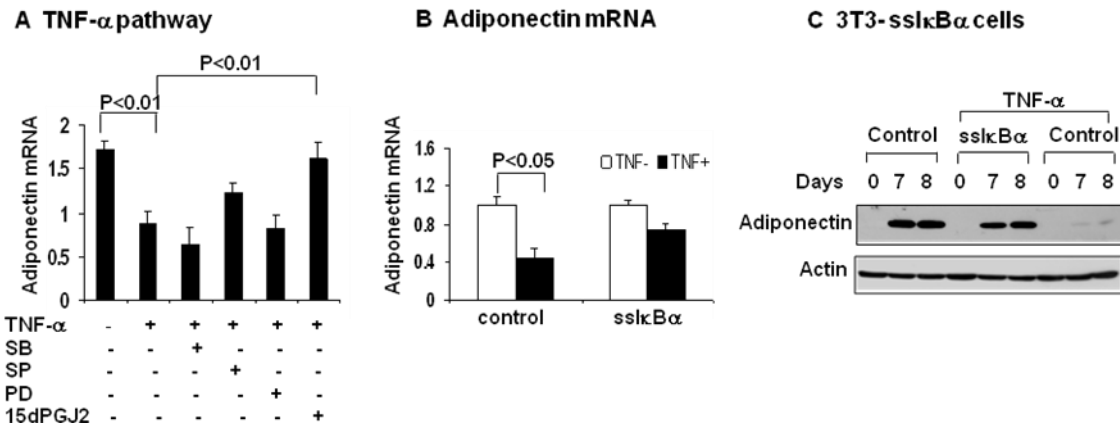


Figure 5. I κ B α /NF- κ B pathway mediates TNF- α activity. A: Blocking of TNF- α activity by the NF- κ B inhibitor. The 3T3-L1 adipocytes were pretreated with pharmacological inhibitors for 30 min before addition of TNF- α . The inhibitors were SB203580 (SB;10 μ M), SP600125 (SP;25 μ M), PD98059 (PD;40 μ M), and 15dPGJ2 (15 μ M). mRNA levels were determined in the cells 16 h later. B: Inhibition of TNF- α activity in 3T3-ssI κ B α stable cells. 3T3-L1 adipocytes and 3T3-ssI κ B α cells were serum starved overnight and treated with TNF- α (20 ng/ml) for 16 h. Adiponectin mRNA was determined by quantitative real time RT-PCR. C: Adiponectin expression during adipogenesis. The cells were treated with TNF- α (20 ng/ml) during adipogenesis. Adiponectin was determined in the whole cell lysate at 7 and 8 days of differentiation. In this figure, each bar represents mean \pm SEM (n=3).

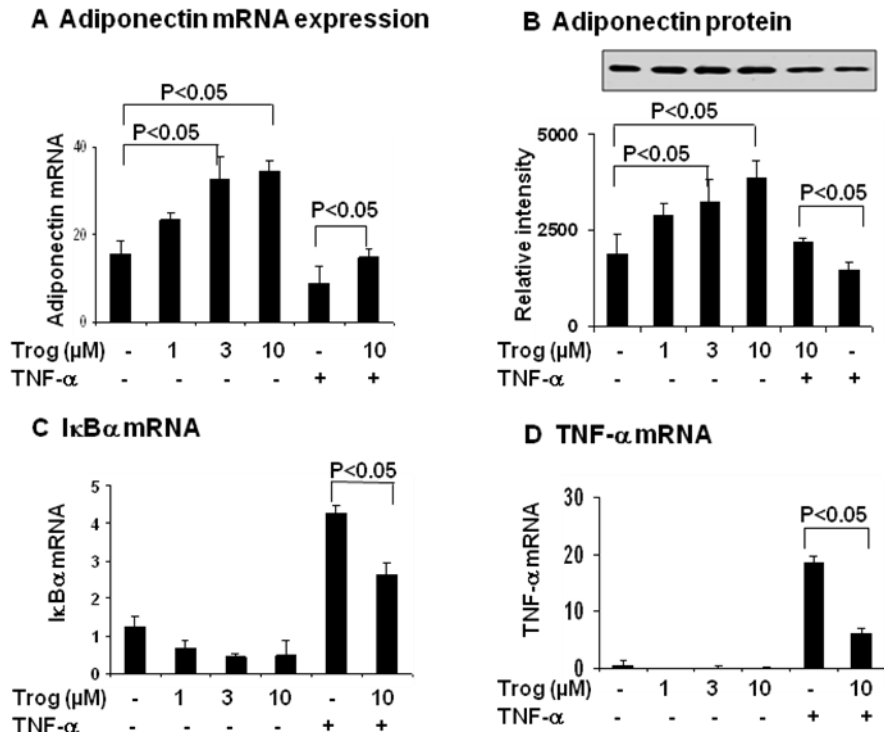


Figure 6. Troglitazone increases mRNA expression and plasma concentration of adiponectin through inhibiting NF-κB pathway. A, B: Dose effects of Troglitazone with or without TNF-α on the adiponectin mRNA level (A) and the secreted amount in medium (B) is shown. 3T3-L1 adipocytes were serum starved overnight and treated with the indicated concentration of troglitazone, 0, 1, 3, 10 μM plus TNF-α (20 ng/ml) for 24 h. The total mRNA was extracted and subjected to qRT-PCR analysis for adiponectin mRNA. The amount of adiponectin secreted into medium was measured by western blot. The adiponectin protein signal was determined by densitometry. C: NF-κB target genes (IκBα, TNF-α) were measured with troglitazone and TNF-α treatment. 3T3-L1 adipocytes were serum starved overnight and treated with the Trog, 0, 1, 3, 10 μM plus TNF-α (20 ng/ml) for 24 h. The total mRNA was extracted and subjected to qRT-PCR analysis for IκBα and TNF-α mRNA. In this figure, each bar represents mean ± SEM (n=3). *, P<0.05.

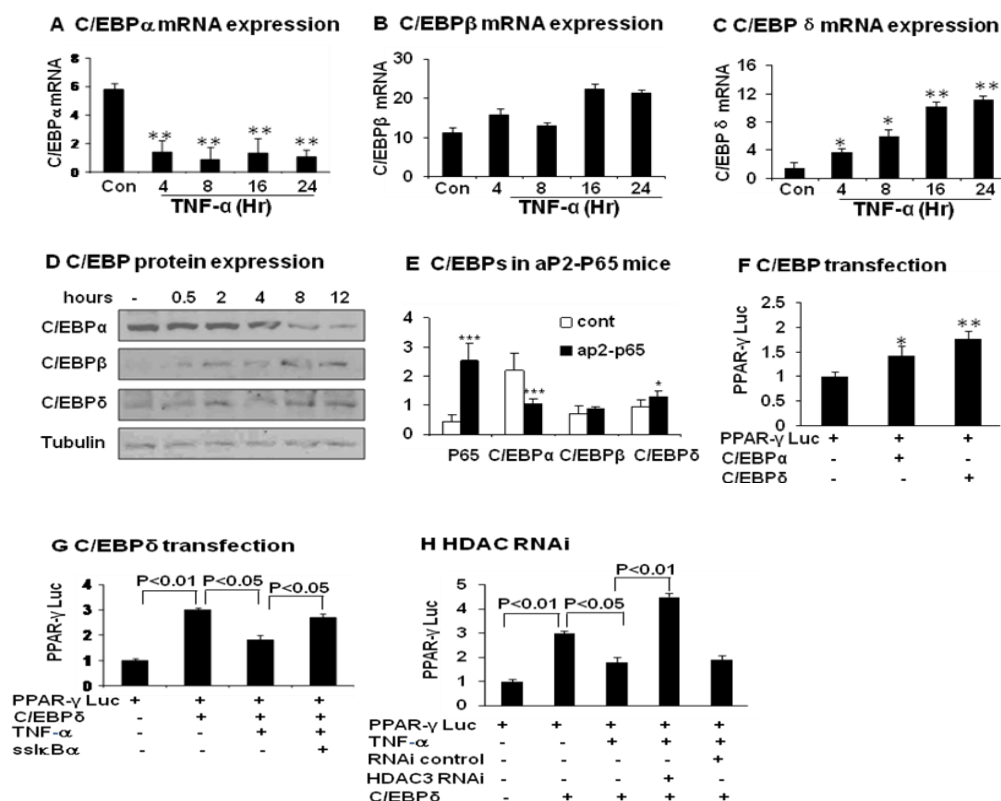


Figure 7. Effects of TNF- α on C/EBPs transcription. A,B,C: C/EBP α , C/EBP β and C/EBP δ mRNA expression levels with TNF- α treatment in 3T3-L1 adipocytes. The cells were serum starved overnight and treated with TNF- α (20 ng/ml) for different times as indicated. The total RNA was extracted and subjected to qRT-PCR analysis for C/EBP α , C/EBP β and C/EBP δ mRNA. D: C/EBP α , C/EBP β and C/EBP δ protein levels with TNF- α treatment in 3T3-L1 adipocytes. The cells were serum starved overnight and treated with TNF- α (20 ng/ml). The C/EBP protein was determined in the whole cell lysate in a western blot with anti-C/EBP α , β , δ antibody. E: C/EBP α , C/EBP β and C/EBP δ mRNA expression levels in aP2-P65 transgenic mice. Epididymal fat tissues were collected at the age of 20 weeks from wild type and aP2-P65 mice and the total RNA was extracted and subjected to quantitative real time RT-PCR analysis for C/EBP α , C/EBP β , C/EBP δ and P65. F: Stimulation of PPAR- γ gene promoter by C/EBP α and C/EBP δ . The transcriptional activity of mouse PPAR- γ promoter (-2000/+45) was analyzed in HEK 293 cells, which were transiently co-transfected with C/EBP α or C/EBP δ by Lipofectamine according to the manufacture's instructions. After 24 hours of transfection, cell lysates were measured by the luciferase assay system. G: The transcriptional activity of PPAR- γ was analyzed in HEK 293 cells using the luciferase reporter system in the transient transfection. C/EBP δ and ssIkB α were co-transfected. At 24 h after transfection, the cells were serum starved overnight and treated with TNF- α (20 ng/ml) for 24 h. H: Analysis of corepressor HDAC3 with RNAi-mediated gene knockdown in the PPAR- γ reporter assay. C/EBP δ and HDAC3 or RNAi control were co-transfected with the reporter system into HEK 293 cells. At 24 h after transfection, the cells were serum starved overnight and treated with TNF- α (20 ng/ml) for 24 h. In this figure, each bar represents mean \pm SEM (n=6). *, P<0.05; **, P<0.01.

Discussion

TNF- α , isolated 30 years ago, is a multifunctional cytokine implicated in apoptosis and cell survival as well as in inflammation and immunity. TNF- α was mainly secreted by macrophages, T lymphocytes, and natural killer (NK) cells which are stimulated with bacterial product. As a molecular with anti-tumor properties, TNF- α induces hemorrhagic necrosis in a certain type of tumors including sarcomas, metastatic melanomas (van Horssen et al., 2006). However, as a proinflammatory cytokine, TNF- α has been involved in a wide range of diseases such as type 2 diabetes, obesity and circulatory disease. In the present study, we focused on the TNF- α 's proinflammatory aspect. We investigated the mechanism of adiponectin inhibition by TNF- α treatment. We previously demonstrated that TNF- α is reversibly associated with adiponectin in adipose tissue in the absence of obesity in aP2-P65 mice. That study provided *in vivo* evidence that TNF- α may inhibit adiponectin expression. In the currently reported study, we investigated the mechanism. We demonstrated that transcription and posttranscription events are both involved in the adiponectin suppression by TNF- α . At the transcriptional level, we confirmed that PPAR- γ is a master transcription activator in the adiponectin gene. Meanwhile, our data suggest that C/EBPs may contribute to the adiponectin expression by increasing PPAR- γ expression. NF- κ B is a dominant mediator of TNF- α signal in the suppression of adiponectin expression. NF- κ B may act on C/EBPs for the suppression. We show here that C/EBPs enhance the promoter activity of PPAR- γ gene. Nuclear receptor co-repressor HDAC3 is required for NF- κ B inhibition of C/EBP function. The data suggest that TNF- α signal is mediated by NF- κ B/HDAC3/C/EBP/ PPAR- γ in the inhibition of adiponectin transcription.

PPAR- γ is a master nuclear factor in the control of adipogenesis (Gustafson et al., 2003; Iwaki et al., 2003; Ruan et al., 2003; Semple et al., 2006; Tsuchida et al., 2005). PPAR- γ

activates adiponectin transcription at a functional PPRE (peroxisome proliferator response element (Iwaki et al., 2003; Zhang et al., 1996). Our data suggest that TNF- α may inhibit adiponectin transcription by suppressing PPAR- γ activity. The suppression was observed at mRNA and gene promoter levels for PPAR- γ .

NF- κ B is required for TNF- α inhibition of adiponectin. TNF- α has been reported to inhibit a number of genes including GLUT-4, aP2, UCP-1 and transcription factor C/EBP α , and the signaling pathway of TNF- α is different for each distinct gene. For example, TNF- α inhibits the UCP-1 expression through ERKs and p38MAPK pathway (Valladares et al., 2001). Also ERK pathway is involved in the mechanism of TNF- α -induced lipolysis (Souza et al., 2003). NF- κ B signaling pathway was shown in our study to mediate TNF- α inhibition of PPAR- γ . By using a chemical inhibitor of NF- κ B (blocked I κ B to prevent degradation of I κ B, which inhibits NF- κ B) and ssI κ B α (suppresses NF- κ B) stable cell line, we showed that PPAR- γ mRNA was not inhibited by TNF- α if NF- κ B activation was completely blocked. In these conditions, TNF- α also loses the activity to inhibit adiponectin. Furthermore, troglitazone, a synthetic PPAR ligand attenuated TNF- α activity. The mechanism is also related to inhibition of NF- κ B. The role of NF- κ B is supported by reduced PPAR- γ expression in aP2-P65 adipocytes, in which NF- κ B is enhanced by over expression of the p65 subunit. Taken together, these results suggest that TNF- α may suppress adiponectin expression by activation of NF- κ B, which reduces PPAR- γ expression.

Our data suggest that C/EBPs may be involved in TNF- α regulation of PPAR- γ . C/EBPs regulate many adipocyte-specific genes, such as aP2 (Christy et al., 1989), GLUT4 (Yamamoto et al., 2002). Previous studies have reported that C/EBP α is required to fully activate the adiponectin gene (Qiao et al., 2005; Qiao et al., 2006). C/EBP δ was shown to interact with the

PPAR- γ gene promoter through a direct protein-DNA interaction. TNF- α has been shown to inhibit C/EBP α mRNA in 3T3-L1 adipocytes (Ron et al., 1992; Stephens and Pekala, 1991). C/EBP β and δ were shown to activate expression of PPAR- γ and C/EBP α during adipogenesis (Rosen and MacDougald, 2006). C/EBP α may control the transcriptional expression of PPAR- γ (Wu et al., 1999). We observed that TNF- α inhibits C/EBP α expression, but inducing C/EBP δ expression in fully-differentiated 3T3-L1 adipocytes. Therefore, C/EBP α and δ exhibit opposite expression patterns in response to an inflammation reaction. It is possible that C/EBP δ will replace C/EBP α in the PPAR- γ gene promoter in the response to TNF- α . However, the switch was not able to rescue PPAR- γ from the inhibition by TNF- α . Our reporter assay suggests that TNF- α may suppress the C/EBP δ activity by activation of HDAC3.

HDAC3 is a nuclear receptor co-repressor, which contains both nuclear export signal (180-313 aa in the central portion), and the nuclear localization signal (312-428 aa in the C-terminal) (Yang et al., 2002). Previous studies have reported that HDAC3 shuttles between the cytoplasm and nucleus, and that I κ B α is important in the control of HDAC3 shuttling. When I κ B α is degraded, HDAC3 enters the nucleus to inhibit PPAR- γ function (Gao et al., 2006). In the present study, we observed that HDAC3 is required for suppression of C/EBP δ activity in the PPAR- γ gene promoter. The data supports that HDAC3 may mediate NF- κ B signal in the suppression of C/EBP δ function.

Posttranscriptional regulation of mRNA stability is another pathway that controls adiponectin expression by TNF- α . In our study, TNF- α accelerated adiponectin mRNA degradation in adipocytes. This effect was independent of PPAR- γ effect since it was not rescued by a PPAR- γ activator. Adiponectin mRNA does not contain classical AU-rich motifs and poly (A) tail structures for mRNA stability. However, the mRNA has a long half life of more than 16

hrs. More studies are needed to explore the signaling pathway of the TNF- α regulation of adiponectin mRNA stability.

Although NF- κ B inhibited the promoter activity of PPAR- γ gene, we did not find the NF- κ B binding sequence in the promoter DNA. In the gene promoter, NF- κ B inhibited C/EBP response elements and the inhibition might be dependent on an indirect interaction. HDAC3, after activation by NF- κ B, may mediate the NF- κ B signal in the inhibition of C/EBPs. This possibility remains to be tested using ChIp assay in adipocytes.

In conclusion, our study demonstrates that TNF- α suppresses the adiponectin gene expression in differentiated 3T3-L1 adipocytes by inhibition of the adiponectin transcription level and mRNA stability. The inhibition is related to suppression of PPAR- γ activity. NF- κ B mediates the TNF- α signal to inhibit PPAR- γ expression. NF- κ B may target C/EBPs that are required for PPAR- γ expression. HDAC3 plays a role in the suppression of C/EBP δ by TNF- α .

CHAPTER 4

CONCLUSION

The work in this thesis focused on the mechanism of the TNF- α role in adiponectin gene expression in differentiated 3T3-L1 adipocytes. Previously, our in vivo animal data provided evidence that a decrease in adiponectin expression was associated with an increase in TNF- α . We measured mRNA expression and protein levels of adiponectin in 3T3-L1 adipocytes with TNF- α treatment. We found that adiponectin mRNA and protein levels are suppressed by TNF- α treatment in a time dependent manner. A Luciferase reporter assay indicated that adiponectin inhibition by TNF- α may happen at the gene transcription level. Meanwhile, TNF- α was found to increase adiponectin mRNA degradation in differentiated 3T3-L1 adipocytes. Thus adiponectin mRNA reduction is a consequence of inhibition of gene transcription and mRNA stability. PPAR- γ is a candidate transcription factor implicated in adiponectin expression. We examined the effects of TNF- α on the transcription factor PPAR- γ . Our data demonstrated that TNF- α down regulates PPAR- γ mRNA and protein levels and this inhibition is mediated by the I κ B α /NF- κ B pathway. Troglitazone, which can protect against adiponectin inhibition by TNF- α treatment, was shown to decrease NF- κ B target genes: TNF- α and I κ B α . C/EBPs, other important transcription factors implicated in adipogenesis, were tested in the research reported in this thesis. We observed C/EBP α and C/EBP δ functionally enhance PPAR- γ activity and may indirectly control adiponectin regulation. HDAC3, a nuclear receptor repressor, is involved in the TNF- α inhibition of PPAR- γ -C/EBPs interaction.

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VITA

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