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Obesity has reached epidemic proportions in the United States, currently ranking second among causes of preventable deaths. Our research group has shown in cultures of differentiated human adipocytes that 30 uM trans-10, cis-12 Conjugated Linoleic Acid (CLA) increases IL-6 and IL-8 secretion into the media and lowers mRNA levels of peroxisome proliferator-activated receptor gamma (PPARγ). Human adipose tissue explants were incubated with either vehicle or 30 uM trans-10, cis-12 CLA for 8, 24, or 72 h. Trans-10, cis-12 CLA treatment increased IL-6 and IL-8 secretion into the media ($p < 0.05$). Trans-10, cis-12 CLA treatment also increased the mRNA levels of IL-6 and IL-8 ($p < 0.05$) while having no significant effect on PPARγ or PPARγ targets. These data demonstrate for the first time that CLA induces IL-6 and IL-8 gene expression and protein secretion in cultures of human subcutaneous adipose tissue explants.

IMPACT OF TRANS-10, CIS-12 CONJUGATED LINOLEIC ACID ON INTERLEUKIN (IL)-6 AND IL-8 AND ADIPOGENIC GENES IN CULTURES OF HUMAN ADIPOSE TISSUE EXPLANTS

by

James Nathan Provo

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of the Requirements for the Degree
Master of Science

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2005

Approved by

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Committee Chair

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

Committee Chair ___________________________

Committee Members ___________________________

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

Significance

Obesity is currently the second leading cause of preventable deaths in the U.S. (Mokdad et al. 2004). The estimated annual medical spending attributed to obesity is $92.6 billion, or approximately 9.1% of U.S. Health expenditures (Finkelstein et al. 2003). Traditionally, low-fat diets are recommended to prevent obesity. However, specific isomers of the polyunsaturated fatty acid conjugated linoleic acid (CLA) reduces body fat in animals (Azain et al. 2000, Ryder et al. 2001, Park et al. 1999, deDeckere et al. 1999, Ostrowska et al. 2003) and in some humans (Gaullier et al. 2004). However, the antiobesity actions of CLA in humans is controversial because recent studies have shown mixed results. Some CLA studies report no significant decrease in adiposity (Malpuech-Brugere et al. 2004) while others report a significant decrease (Gaullier et al. 2004). What is needed is research demonstrating the extent to which trans-10, cis-12 CLA is operative in human adipose tissue. Our research group demonstrated that treatment of cultures of newly differentiated human adipocytes for 7-21 d with 30 uM trans-10, cis-12 CLA decreased the triglyceride (TG) content compared to cultures treated with 30 uM cis-9, trans-11 CLA or vehicle (Brown et al. 2004). Our group has also shown that trans-10, cis-12 CLA induces the release of proinflammatory cytokines, (e.g., IL-6 and IL-8),

that precede a decrease in adipogenic gene expression and delipidation (Brown et al. 2004). However, it is not known whether CLA affects cytokines and adipogenic gene expression in intact adipose tissue as it does in cultures of newly differentiated adipocytes. Therefore, the hypothesis was that CLA increases cytokine secretion and suppresses adipogenic gene expression as it does in cultures of adipocytes. To test this hypothesis, the following specific aim was pursued: ***Determine the extent that trans-10, cis-12 CLA increases IL-6 and IL-8 gene expression and protein secretion and decreases peroxisome proliferator activated receptor (PPAR)γ2 and its downstream targets in human adipose tissue explants.***

This research is important because it will provide a better understanding of how CLA decreases adiposity. A better understanding of these effects will facilitate the development of dietary strategies to control the development of obesity, a major cause of preventable morbidity and mortality in the U.S.

Biological Effects of Conjugated Linoleic Acid (CLA)

Dr. Michael Pariza's group at the University of Wisconsin Madison was the first to discover CLA, a geometric and positional isomer of linoleic acid, while studying carcinogens in grilled beef (Ha et al. 1987). CLA exists primarily as two naturally occurring isomers, cis-9, trans-11 and trans-10, cis-12 CLA. Animal studies with crude mixtures of CLA isomers have demonstrated beneficial biological effects on cancer (Palombo et al. 2002, Yang et al. 2002, Cho et al. 2003), diabetes (Houseknecht et al. 1998, Belury et al. 2003), atherosclerosis (Kritchevsky et al. 2002, Toomey et al. 2003),

immune function (Yu et al. 2002, Akahoshi et al. 2002, Yang et al. 2003), and body composition (Park et al. 1999a, Park et al. 1999b, Park et al. 2001, Ryder et al. 2001, Terpstra et al. 2002, Ostrowska et al. 2003, Wargent et al. 2005). Animals fed an equal mixture (~ 1%, w/w) of cis-9, trans-11 CLA and trans-10, cis-12 CLA had decreased body fat, increased lean body mass, and reduced excess weight gain. However, because the mixtures used in these studies contained two or more CLA isomers, it is difficult to pinpoint which isomer is responsible for the decreasing adiposity. Current studies demonstrate that trans-10, cis-12 CLA is the isomer primarily responsible for reduced adiposity *in vivo* (reviewed by House et al. 2005). For example, trans-10, cis-12 CLA decreased adiposity in pigs (Tischendorf et al. 2002, Wiegand et al. 2002, Ostrowska et al. 2003), hamsters (de Deckere et al. 1999, Gavino et al. 2000, Navarro et al. 2003), and rodents (Tsuboyama-Kasaoka et al. 2000, Ryder et al. 2001, Terpstra et al. 2002, Yamasaki et al. 2003). *In vitro* evidence also supports the notion that trans-10, cis-12 CLA is the isomer responsible for suppressing preadipocyte differentiation and promoting mature adipocyte delipidation. For example, trans-10, cis-12 CLA treatment decreased TG content in 3T3-L1 preadipocytes, inhibiting their differentiation into mature adipocytes (Brodie et al. 1999, Evans et al. 2001, Kang et al. 2003, Granlund et al. 2005). 3T3-L1 adipocytes treated with trans-10, cis-12 CLA altered fatty acid metabolism and reduced TG content (Park et al. 1999a, Choi et al. 2000, Granlund et al. 2005). In addition, our group has shown that 30 uM trans-10, cis-12 CLA decreased TG content in primary cultures of human differentiating preadipocytes (Brown et al. 2003) and in cultures containing newly differentiated adipocytes (Brown et al. 2004).

Adipocyte Biology

The process of adipocyte differentiation commonly referred to as adipogenesis, is regulated by numerous transcription factors including PPAR and CCAAT/enhancer-binding proteins (C/EBP). The activity of these transcription factors, and to a lesser extent the expression of these genes, can be altered by a variety of hormones, growth factors, and nutrients. These alterations can either promote or inhibit adipocyte differentiation, thereby inducing a number of metabolic changes in adipose tissue.

One of the main transcription factors involved in the regulation of adipogenesis is PPARγ. PPARγ is one member of the PPAR family of nuclear hormone receptors whose activity is modulated by ligand binding and heterodimerization with retinoid X receptor (RXR). Of the three members of the PPAR family, PPARγ is the most important regulator of adipogenesis in adipose tissue. This is supported by the following research: 1) activation of PPARγ2, an isoform resulting from alternative splicing of the PPARγ gene, by high affinity, synthetic ligands such as thiazolidinediones (TZD) upregulates genes involved in adipocyte differentiation (Sewter et al. 2002); 2) peroxisome proliferator response elements (PPRE) have been found in the promoter sequences of genes abundantly expressed during adipogenesis, including adipocyte fatty acid binding protein (aP2) and lipoprotein lipase (LPL) (Lemberger et al. 1996); 3) PPARγ2 knockout animals had fewer mature adipocytes in adipose tissue (He et al. 2003, Imai et al. 2004); and 4) nonprogenitor cells ectopically expressing PPARγ2 and grown in adipogenic conditions expressed markers of adipocyte differentiation (Rosen et al. 1999).

The critical role of PPARγ2 in adipogenesis and maintenance of the adipocyte phenotype led researchers to study effectors such as growth factors and nutrients that decrease or prevent these molecular events driven by PPARγ. In addition, they were interested in the impact these changes would have on gene expression and adipocyte metabolism (e.g., glucose and fatty acid uptake, lipogenesis, β-oxidation, lipolysis). Several groups have reported that cytokines such as tumor necrosis factor alpha (TNFα) and IL-6 and specific fatty acids such as octanoate suppress PPARγ2 gene expression and activity *in vivo* and/or *in vitro*. For example, 24 h TNFα (0.2 nmol/L) treatment suppressed PPARγ gene expression in 3T3-L1 adipocytes. PPARγ target genes regulating insulin-stimulated glucose uptake (e.g., GLUT4) and fatty acid uptake (e.g., LPL) were also downregulated by TNFα (Ruan et al. 2002). Xing et al. (1997) reported that treating 3T3-L1 preadipocytes with 25 ng/mL TNFα inhibited differentiation by reducing PPARγ mRNA levels. They also demonstrated that TNFα decreased PPARγ mRNA, protein, and DNA-binding activity in 3T3-L1 adipocytes compared to controls. Recently, Rotter et al. (2003) reported that 20 ng/mL TNFα or IL-6 treatment reduced mRNA levels for PPARγ and its downstream target GLUT4 in 3T3-L1 adipocytes, although TNFα decreased these parameters more rapidly than IL-6. 3T3-L1 adipocytes treated with 100-200 ng/mL IL-6 failed to maintain their adipocyte phenotype due to decreased PPARγ protein levels and reductions in the mRNA levels aP2, fatty acid synthase (FAS), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In addition,

de novo lipogenesis and insulin-stimulated glucose transport were suppressed by IL-6 consistent with decreased expression of GLUT4 (Lagathu et al. 2003).

Concentrations of 1-3 mmol/L of octanoate, a medium-chain fatty acid (MCFA), attenuated PPARγ gene and protein expression in 3T3-L1 adipocytes (Farmer et al. 2002). Additionally, octanoate-treated adipocytes had higher ratios of the inactive phosphorylated PPARγ protein than the nonphosphorylated active protein, which suggests octanoate works to inhibit adipogenesis by suppressing PPARγ gene expression and activation (Farmer et al. 2002). Another study reported decreased mRNA levels of PPARγ in rats fed MCFAs (Han et al. 2003). Taken together, these studies with cytokines and/or medium-chain fatty acids suggest suppression of PPARγ2 gene expression and activity are associated with the following effects in differentiating adipocytes: 1) decreased lipid accretion during differentiation (Xing et al. 1997, Farmer et al. 2002, Han et al. 2003); 2) decreased expression of PPARγ downstream adipogenic target genes regulating adipocyte differentiation (Lagathu et al. 2003, Farmer et al. 2002, Han et al. 2003; and 3) decreased insulin-stimulated glucose uptake and *de novo* lipogenesis (Lagathu et al. 2003). In addition, treatment with cytokines or MCFA decreased PPARγ gene expression in mature adipocytes and adipose tissue resulting in: 1) reduced expression of adipocyte marker genes (Ruan et al. 2002, Rotter et al. 2003, Lagathu et al. 2003, Han et al. 2003); 2) delipidation of adipocytes via decreased insulin-stimulated glucose uptake (Rotter et al. 2003) and *de novo* lipogenesis (Han et al. 2003); and 3) reduced LPL activity resulting in decreased fatty acid uptake in adipose tissue (Han et al. 2003).

Consistent with these data, our group has shown that trans-10, cis-12 CLA, but not cis-9, trans-11 CLA, suppressed the gene expression of PPARγ2 and its downstream targets in cultures of human preadipocytes (Brown et al. 2003) and adipocytes (Brown et al. 2004). The trans-10, cis-12 CLA-dependent downregulation of PPARγ2 and its target genes resulted in reduced glucose and fatty acid uptake, incorporation into lipid, and oxidation in differentiating preadipocytes (Brown et al. 2003) and mature adipocytes (Brown et al. 2004).

Collectively, these studies have increased our understanding of the molecular and metabolic alterations induced by PPARγ modulators in differentiating and mature adipocytes. While they provide evidence of PPARγ modulators, such as CLA as potential anti-obesity agents, these benefits may be outweighed by their potential to promote insulin resistance (DeLany et al. 1999, Tsuboyama-Kasaoka et al. 2000, Roche et al. 2002) and/or lipodystrophy (Tsuboyama-Kasaoka et al. 2000, Clement et al. 2002, Takahashi et al. 2003).

Cytokines

Following the discovery of leptin [reviewed in (Flier et al. 1998)], a hormone secreted by adipose tissue, scientists began to appreciate that adipose tissue functions as an endocrine organ. A number of other adipose-derived secretory molecules, such as hormones and cytokines, have demonstrated pleiotrophic roles in adipocyte differentiation and metabolism. For example, the cytokine TNFα inhibits differentiation (Xing et al. 1997, Ruan et al. 2002) and increases lipolysis (Gasic et al. 1999, Zhang et al. 2002). The

cytokine IL-6 decreases adipogenesis (Lagathu et al. 2003), insulin-stimulated glucose uptake, *de novo* lipogenesis, and increases lipolysis (Path et al. 2001, Rotter et al. 2003, Lagathu et al. 2003) in cultures of mature adipocytes. Further research identified and characterized cytokine cell membrane receptors in murine and human cell types, including adipocytes. Cytokine receptors are signal transduction receptors, which become activated when a ligand (i.e., cytokine) binds to the receptor. Upon activation, a signal is transmitted within the cell that causes a number of responses such as changes in gene expression and metabolism. This suggests a role for cytokines in molecular signaling mechanisms, or cross talk that enables adipocytes to communicate with other adipocytes or with non-adipocytes, impacting their gene expression and metabolism as shown in Figure 1. Recent evidence obtained from murine 3T3-L1 cells and human adipose tissue demonstrate that a significant portion of cytokines are synthesized and secreted from the non-adipocytes, or supporting stromal vascular (SV) cells compared to the adipocytes (Weisberg et al. 2003, Fain et al. 2003, Harkins et al. 2004). In addition, research with recombinant cytokines (Souza et al. 2003) and cytokine neutralization antibodies (Hotamisligil et al. 1999, Brown et al. 2004) have verified that cytokines activate intracellular signaling in multiple cell types. These signaling pathways induce changes in downstream gene expression and metabolism in adipocytes (Brown et al. 2004). Figure 1 summarizes some of the metabolic processes in adipocytes, such as insulin-stimulated glucose and fatty acid uptake, lipolysis, and lipogenesis, that can be mediated by cytokines. Taken together, these data provide strong evidence of cross talk in adipose tissue where cytokines, secreted primarily from non-adipocytes, bind to

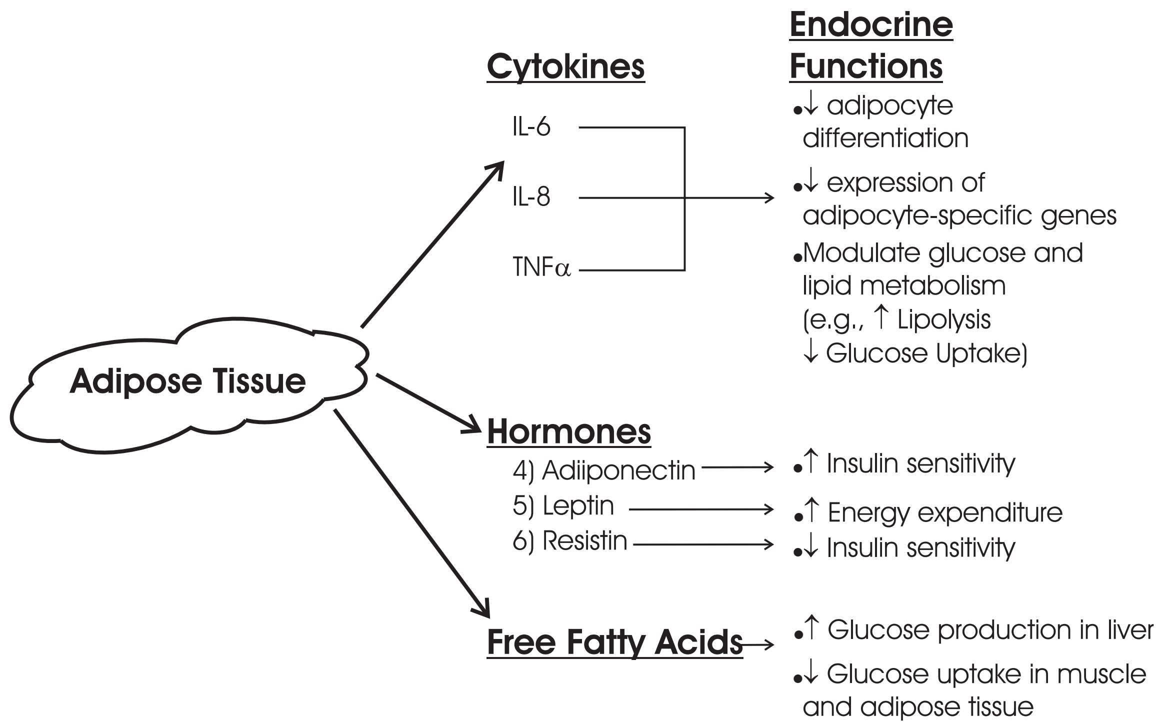


Figure 1. The pleiotropic functions of molecules secreted from adipose tissue. Potential functions of cytokines, hormones, and free fatty acids secreted from adipose tissue are illustrated (adapted from Morrison et al. 2000).

membrane-bound receptors on adipocytes, transducing signals that alter adipocyte gene expression and metabolism.

Cytokines and Inflammation

Recent studies have shown that secretion of proinflammatory cytokines increases with adiposity (Xu et al. 2003, Weisberg et al. 2003). Proinflammatory cytokines hypersecreted during obesity are associated with insulin resistance and type 2 diabetes (Pickup et al. 1997, Bastard et al. 2000, Kern et al. 2001, Pradhan et al. 2001, Muller et al. 2002). Human studies have demonstrated higher plasma levels of IL-6 (Bastard et al. 2000, Kern et al. 2001) and TNFα (Hotamisligil et al. 1995, Moller et al. 2000) are associated with insulin resistance and type 2 diabetes. As a result of these and other related findings, many research groups are focusing on cytokines and their involvement in the molecular mechanisms linking obesity, inflammation, and the development of insulin resistance, type 2 diabetes, and hyperlipidemia. Through their respective receptor systems, the cytokines TNFα and/or IL-6 can activate inflammatory signaling pathways such as nuclear factor-κB (NFκB), Jun NH_2-terminal kinase (JNK), and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Ruan et al. 2003, Minden et al. 1994, Zhang et al. 2002).

It is now generally accepted that the inflammatory response observed in obesity is initiated in adipose tissue (Xu et al. 2003, Weisberg et al. 2003). However, the mechanisms triggering the initial inflammatory response in adipose tissue remain unclear as do the predominant cellular source of cytokines. It is well-established that adipose tissue is compromised of a number of different cell types, among them macrophages,

preadipocytes, and reticuloendothelial cells (Weisberg et al. 2003, Xu et al. 2003, Abderrahim-Ferkoune et al. 2004). More importantly, each of these cell types has been shown to secrete proinflammatory cytokines (Fain et al. 2003, Harkins et al. 2004, Weisberg et al. 2003). Wellen et al. 2003 proposed one of the best theoretical models to date explaining the mechanism by which the inflammatory response is triggered and maintained in adipose tissue of the obese. According to the model, cross-talk (e.g, molecular signaling) between adipocytes and non-adipocytes (i.e., macrophages, preadipocytes, and reticuloendothelial cells) is mediated by cytokines that can activate inflammatory pathways in adipocytes. The activation of inflammatory pathways modulates adipocyte glucose and lipid metabolism, ultimately causing delipidation and insulin resistance in adipose tissue.

Recent evidence supports the concept that cytokines (e.g., TNFα, IL-6, and IL-8) can activate inflammatory pathways (e.g,, NFκB, JNK, and ERK1/2) modulating adipocyte glucose and lipid metabolism (Hirosumi et al. 2002, Ruan et al. 2002, Ryden et al. 2002, Zhang et al. 2002, Ruan et al. 2003, Souza et al. 2003, Engelman et al. 2005). In addition, studies suggest that cytokine-induced activation of these inflammatory pathways can suppress adipocyte differentiation (Chae et al. 2003, Souza et al. 2003, Suzawa et al. 2003) and promote the delipidation of differentiating and mature adipocytes (Zhang et al. 2002, Souza et al. 2003), leading to the development of insulin resistance and type 2 diabetes.

Recent studies with differentiating adipocytes provide evidence that known activators of ERK1/2 and JNK, transcription factors belonging to the mitogen-activated

protein kinase (MAPK) signaling family, can phosphorylate PPARγ, thereby reducing its transcriptional activity (Hu et al. 1996, Adams et al. 1997, Camp et al. 1997). Following growth factor treatment, adipocytes expressing PPARγ mutated at consensus MAP kinase phosphorylation sites have greater PPARγ transcriptional activity, due to less ERK-mediated inhibitory phosphorylation of PPARγ, while adipocytes expressing wild-type PPARγ have greater ERK-mediated phosphorylation of PPARγ, resulting in a decrease in PPARγ transcriptional activity (Hu et al. 1996). Importantly, 3T3-L1 cells treated with growth factors and ectopically expressing wild-type PPARγ have a lower degree of adipocyte differentiation, indicated by decreased gene expression of PPARγ downstream targets regulating glucose and lipid metabolism (e.g., aP2 and adipsin) and reduced TG stores. This evidence suggests cytokines can activate inflammatory pathways in differentiating adipocytes that mediate the phosphorylation of PPARγ. Phosphorylation of PPARγ decreases its activity resulting in a concomitant downregulation of adipogenic genes regulating adipocyte glucose and lipid metabolism during the differentiation process.

In mature adipocytes, cytokines can also activate the ERK pathway and induce delipidation by stimulating lipolysis. Souza et al. (2003) demonstrated that 10 ng/mL TNFα increased ERK activity in 3T3-L1 adipocytes. To determine whether the ERK activation was necessary for TNFα-stimulated lipolysis, 3T3-L1 cells were pretreated with PD98059, an upstream inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) blocking ERK activation.

Pretreatment with PD98059 attenuated TNFα-induced lipolysis. Souza et al. (2003) proposed that TNFα (10 ng/mL) activates ERK which then phosphorylates PPARγ reducing its transcriptional activity. They also demonstrated a decrease in the protein expression of perilipin, a PPARγ target gene. Therefore, they attribute TNFα-induced lipolysis in 3T3-L1 adipocytes to decreased PPARγ activity and a concomitant decrease in the protein expression of perilipin. In summary, the data of Souza et al. 2003 suggest that TNFα activates ERK in mature adipocytes, decreasing PPARγ activity and perilipin protein expression. The decrease in perilipin induces lipolysis in 3T3-L1 adipocytes. This finding is supported by data demonstrating that overexpression of perilipin blocks TNFα-induced lipolysis (Souza et al. 1998).

In addition to suppressing adipocyte differentiation and stimulating lipolysis in mature adipocytes, recent evidence has shown that cytokines can activate inflammatory pathways in adipocytes resulting in impaired insulin signaling. Mature 3T3-L1 adipocytes treated for 24 h with TNFα activate NFκB leading to decreased expression of a number of genes including GLUT4 and proteins [(e.g., GLUT4, insulin receptor substrate 1 (IRS-1), and protein kinase B (AKT)] critical to insulin signaling and glucose uptake (Ruan et al. 2002). 3T3-L1 adipocytes treated with TNFα and stably expressing a non-degradable (i.e., non-phosphorylatable) form of the inhibitor of NFκB (i.e, IκB-α) had higher levels of IκB-α than wild-type adipocytes, further implicating TNFα's role in inducing NFκB. Ruan et al. (2003) also reported a potential mechanism by which TNFα-induced NFκB activation inhibits the transcriptional activity of PPARγ.

Using a reporter gene assay in HeLa cells, they demonstrated that a member of the NFκB family, p65, decreases basal and PPARγ ligand-induced transcriptional activity. They also showed that the decrease in PPARγ activity is not due to inhibitory binding of p65 to NFκB response elements (NFκB RE) in the promoter region of PPARγ responsive reporter genes. This would suggest p65 binds directly to PPARγ to reduce its activity or that it associates with known co-activators of PPARγ. Decreased PPARγ activity could potentially explain the suppression of proteins regulating insulin signaling and glucose uptake in adipocytes. Therefore, these data from 3T3-L1 adipocytes support the following concepts: 1) TNFα activates NFκB inflammatory signaling in mature adipocytes; 2) NFκB activation decreases PPARγ activity, resulting in decreased expression of PPARγ-regulated proteins critical to insulin signaling and glucose uptake; 3) decreased expression of insulin signaling proteins can reduce glucose uptake in adipocytes; and 4) reduced glucose uptake decreases the TG content of mature adipocytes by reducing the amount of substrates available for *de novo* TG sysnthesis.

These data examining the effects of proinflammatory cytokines on adipocyte glucose and lipid metabolism taken together with data reported by Fain et al. 2003, Weisberg et al. 2003, and Harkins et al. 2004 demonstrating the predominant cellular source (i.e., SV cells vs. adipocytes) of cytokines in adipose tissue support the theoretical model proposed by Wellen et al. 2003. Figure 2 summarizes the following sequence of molecular and metabolic events involved in the cross-talk between adipocytes and non-adipocytes: 1) Macrophages recruited to adipose tissue by unknown signal(s) infiltrate adipose tissue and reside there; 2) non-adipocytes residing in adipose tissue

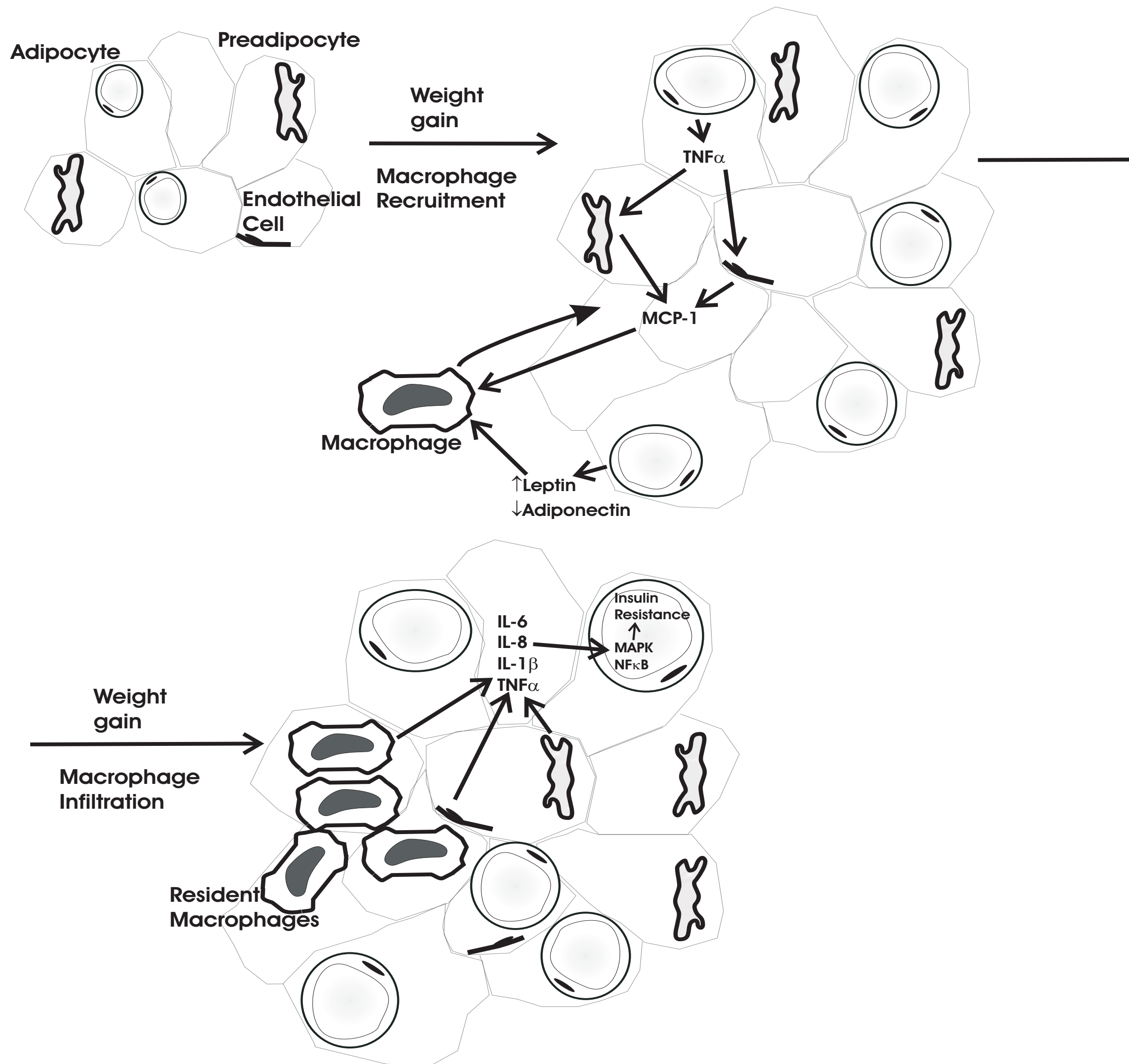


Figure 2. Cross-talk mechanisms between insulin receptor signaling and inflammatory pathways. As the number and size of adipocytes in adipose tissue increases with obesity, adipocytes secrete low levels of the cytokine, TNFα. TNFα acts in a paracrine fashion, stimulating the non-adipocyte cells in adipose tissue [(i.e, stromal vascular cells (SVCs))], such as preadipocytes and endothelial cells, to produce and secrete monocyte chemoattractant protein-1 (MCP-1) (Xu et al. 2003). Recent evidence suggests that MCP-1 can act as a signal, attracting macrophages to adipose tissue where they take up residence (Xu et al. 2003). In addition, increased secretion of leptin and/or decreased secretion of adiponectin by adipocytes has been shown to stimulate transport of macrophages to adipose tissue (Sierra-Honigmann et al. 1998). Evidence suggests residing macrophages and SVCs are the predominant source of secreted cytokines (e.g., IL-6, IL-1β, IL-8, and TNFα) in adipose tissue (Fain et al. 2003, Xu et al. 2003, Weisberg et al. 2003, Harkins et al. 2004). These cytokines can act in an autocrine/paracrine fashion to activate inflammatory signaling pathways in adipocytes, leading to insulin resistance (adapted from Wellen et al. 2003)

(macrophages, preadipocytes, and reticuloendothelial cells) and adipocytes secrete cytokines (e.g., TNFα, IL-6, and IL-8) which can act in an autocrine/paracrine fashion to activate inflammatory signaling pathways (e.g. JAK/STAT, MAPK, NF-κB) in adipocytes; 3) cytokine-mediated activation of inflammatory pathways modulates adipocyte glucose and lipid metabolism; and 4) these alterations promote insulin resistance and delipidation in adipocytes. Figure 2 provides a possible explanation for *in vivo* studies linking elevated circulating cytokines with insulin resistance (Uysal et al. 1997, Kern et al. 1995, Moller et al. 2000, Bastard et al. 2000, Kern et al. 2001) and hyperlipidemia (Boden et al. 1997).

Finally, one could hypothesize from this model that inflammatory pathway inhibitors have the potential to antagonize or attenuate insulin resistance and delipidation in adipose tissue by inhibiting production of cytokines that activate inflammatory pathways and/or attenuating the activation of inflammatory pathways modulating adipocyte glucose and lipid metabolism. Indeed, recent studies have demonstrated that synthetic (Ruan et al. 2003) and endogenous PPARγ ligands (Straus et al. 2000) can attenuate the activation of inflammatory pathways (e.g,, NFκB) in various cell lines. As anti-inflammatory compounds, they have the potential to act as therapeutic agents preventing cytokine-induced alterations in adipocyte glucose and lipid metabolism that lead to delipidation. Ruan et al. 2003 reported that troglitazone (TGZ), a member of the thiazolidinedione (TZD) class of anti-diabetic compounds and synthetic ligand for the PPARγ receptor, inhibits the TNFα-induced and NFκB-mediated downregulation of several 3T3-L1 adipocyte genes. Among those genes inhibited by TGZ are those that

prevent the release of fatty acids [(e.g., glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT)] and those involved in insulin signaling [(e.g., c-Cbl associate protein (CAP)]. Using a reporter gene assay in HeLa cells they showed that relative luciferase activity for the NFκB promoter was abolished by PPARγ and further decreased by TGZ. This demonstrates that TGZ inhibits p65-dependent transcriptional activity of NFκB gene expression. Furthermore, while the data shows TGZ inhibits NFκB gene expression, NFκB activation and DNA binding were unaffected in 3T3-L1 adipocytes according to an ELISA anaylsis of TNFα-induced p65 activity. Based on these data, their conclusion was that TGZ does not inhibit NFκB activity or gene expression, but instead functions to block NFκB's transcriptional downregulation of key adipocyte genes regulating glucose and fatty acid uptake. These molecular events could potentially explain the insulin sensitizing effects of TZD's in adipose tissue.

Endogenous PPARγ ligands such as 15d-PGJ_2 (a metabolite of Prostaglandin J_2) can also act in a PPARγ-dependent manner to block the activation of inflammatory pathways (e.g., NFκB) thereby preventing the transcription of NFκB target genes (Straus et al. 2000). To determine the effects of PPARγ on NFκB activity, experiments were designed using PPARγ-negative HeLa cells or HeLa cells transiently cotransfected with PPARγ-expression plasmids. NFκB activity was measured using a reporter gene assay coupled to firefly luciferase. The inhibition of NFκB-activity was significantly reduced in HeLa cells transiently expressing PPARγ and the synthetic PPARγ agonist BRL49653

inhibited NFκB activity only in the cells transiently expressing PPARγ. These data suggest PPARγ expression was requisite for the inhibition of NFκB.

Collectively, these data provide evidence that synthetic and endogenous PPARγ agonists can antagonize cytokine-induced activation of inflammatory signaling pathways. By blocking multiple steps in these pathways, PPARγ agonists prevent the downregulation of adipocyte genes regulating glucose and fatty acid uptake. This is one of the mechanisms through which PPARγ agonists are believed to protect adipose tissue from delipidation and the development of insulin resistance and type 2 diabetes.

Current Knowledge About the Mechanisms by Which CLA Decreases Adiposity

Once research determined the trans-10, cis-12 CLA isomer was responsible for reductions in body fat, studies focused on clarifying the molecular mechanisms responsible for CLA's adipocyte delipidation. These studies focused primarily on trans-10, cis-12 CLA's regulation of adipocyte metabolism by measuring: 1) activity or gene expression of enzymes involved in lipid and energy metabolism; and/or 2) activity or gene expression of transcription factors regulating adipocyte differentiation and maintenance. Evidence from these studies support the concept that trans-10, cis-12 CLA modulates the enzymatic activity of steroyl-CoA desaturase-1 (SCD-1) and LPL, thereby altering lipid metabolism in differentiating and mature adipocytes. Trans-10, cis-12 CLA decreased the activity of SCD-1 in 3T3-L1 adipocytes, which is thought to reduce the availability of monounsaturated substrates for TG synthesis, resulting in decreased TG content in adipocytes (Choi et al. 2000). LPL activity also decreased during trans-10, cis-

12 CLA treatment, reducing lipid uptake into adipocytes (Park et al. 1997). Animal studies using the trans-10, cis-12 CLA isomer also showed significant reductions in activity or gene expression of enzymes involved in lipid and energy metabolism. Mice fed a diet containing 1% CLA (e.g., 50% cis-9, trans-11, 50% trans-10, cis-12 CLA) weighed less than controls, due to a decrease in gene expression of FAS and acetyl-CoA carboxylase (ACC), two key enzymes regulating lipid synthesis (Tsuboyama-Kasaoka et al. 2000). Mice fed CLA-enriched diets (e.g., 50% cis-9, trans-11, 50% trans-10, cis-12 CLA) had greater liver mRNA levels for enzymes regulating β-oxidation, which reduced TG content in the liver (Takahashi et al. 2003). Collectively, these data suggest that CLA alters the activity or gene expression of key enzymes controlling adipocyte lipid and energy metabolism.

Initially, PPARα expression was thought to be responsible for many of metabolic changes observed in adipocytes following trans-10, cis-12 CLA treatment (Moya-Canarena et al. 1999). Subsequent experiments with PPARα null mice showed trans-10, cis-12 CLA could still decrease adiposity, suggesting CLA's effects were not dependent on PPARα activation (Peters et al. 2001). Work by Granlund et al. (2003) and our group (Brown et al. 2003) suggested trans-10, cis-12 CLA's antiadipogenic effects are due instead to decreased PPARγ gene expression and/or activity. PPARγ promotes differentiation and lipid accumulation in adipocytes by forming a heterodimer with RXR and binding to PPRE inducing genes responsible for lipid and carbohydrate metabolism.

Theoretically any molecule that antagonizes ligand binding to PPARγ or its binding to the PPRE of a target gene could downregulate the expression of PPARγ target

genes involved in adipocyte metabolism. In support of this hypothesis, our group found that trans-10, cis-12 CLA decreased the expression of PPARγ and its target genes (i.e., aP2, GLUT4, perilipin, LPL) in cultures of differentiating human adipocytes (Brown et al. 2003) and mature adipocytes (Brown et al. 2004). Reductions in gene expression induced by CLA were preceded by the secretion of the proinflammatory cytokines IL-6 and IL-8, suggesting they may have some role in mediating the reductions in adipocyte TG content (Brown et al. 2004). A working model presented in Figure 3 by Brown et al. (2004) summarizes our proposed molecular mechanisms by which trans-10, cis-12 CLA mediates adipocyte delipidation.

Gap in the Knowledge Base

Recent studies have demonstrated that adipocytes express receptors for the proinflammatory cytokines TNFα, IL-6, and IL-8 (Gerhardt et al. 2001, Zhang et al. 2002, Lagathu et al. 2003). Treating human and murine adipocytes with IL-6 or TNFα alters adipocyte metabolism by stimulating lipolysis and decreasing insulin-stimulated glucose uptake, cellular TG content, and/or *de novo* lipogenesis (Path et al. 2001, Rotter et al. 2003, Lagathu et al. 2003). These cellular effects can be attributed primarily to decreased PPARγ adipogenic target genes and PPARγ itself (Rotter et al. 2003, Lagathu et al. 2003, Path et al. 2001).

Our group demonstrated in cultures of SV cells containing newly

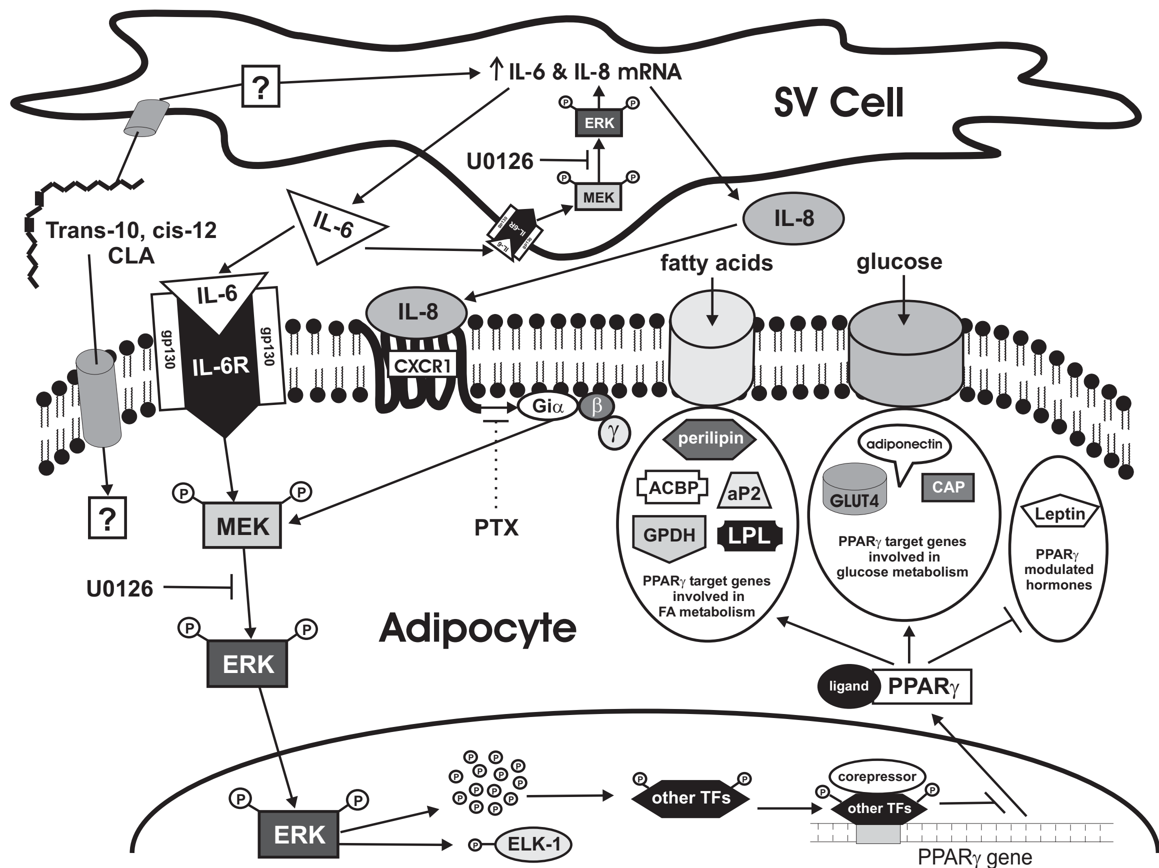


Figure 3. CLA-induced hypersecretion of cytokines controls the repression of PPARγ target gene expression. CLA either enters into the adipocyte or the supporting stromal vascular (SV) cells, initiating an autocrine/paracrine signaling network. In the SV cell, CLA through an unidentified mechanism, increases the mRNA expression and secretion of IL-6 and IL-8. The secreted IL-6 can work in an autocrine manner, binding to the SV cell transmembrane IL-6 receptor (IL-6R) activating MEK/ERK or in a paracrine manner, binding to the IL-6R in neighboring adipocytes also activating MEK/ERK. Secreted IL-8 only works in a paracrine manner, by binding to its obligate receptor (CXCR1) and further amplifying MEK/ERK signaling in the adipocyte. The collective paracrine actions of both IL-6 and IL-8 in the adipocyte result in sustained phosphorylation of MEK and ERK and the concomitant translocation of ERK into the nucleus. The ERK-dependent phosphorylation of other transcription factors (other TFs) may repress the expression of PPARγ. Collectively, ERK-dependent repression of PPARγ gene expression blocks the ability of PPARγ to modulate its traditional target genes, resulting in decreased expression of genes involved in fatty acid (FA) uptake and metabolism (e.g., aP2, LPL, and GPDH) and glucose uptake and metabolism (e.g., GLUT4, CAP, and adiponectin) (taken from Brown et al. 2004).

differentiated adipocytes that 30 uM trans-10, cis-12 CLA treatment for 24 h increased IL-6 and IL-8 gene expression and protein secretion to a greater extent than controls. This increase in cytokine expression and secretion precede a time-dependent downregulation of PPARγ2 and its target genes regulating adipocyte differentiation. The changes in human adipocyte metabolism are similar to what is observed following IL-6 and TNFα treatment, namely reduced glucose uptake and *de novo* lipogenesis (Path et al. 2001, Rotter et al. 2003, Lagathu et al. 2003).

However, it has yet to be demonstrated the extent to which primary culture of human adipose tissue explants, a model more resembling of *in situ* conditions in human adipose tissue, respond similarly to trans-10, cis-12 CLA treatment, *vis a vis* induce proinflammatory synthesis and secretion and downregulate PPARγ2 and its downstream target genes.

Hypothesis and Specific Aims

Based on the preliminary data from our group presented above, the following central hypothesis was formulated: ***Human adipose tissue explants treated with trans-10, cis-12 CLA have increased gene expression and protein secretion of IL-6 and IL-8 and decreased gene expression of PPAR-γ2 and its downstream targets compared to controls.***

To test this central hypothesis, the following specific aim will be pursued: Determine the extent that trans-10, cis-12 CLA increases IL-6 and IL-8 gene expression and protein secretion and decreases PPARγ2 and its downstream target genes in human adipose tissue explants.

CHAPTER II

TRANS-10, CIS-12 CONJUGATED LINOLEIC ACID INDUCES INTERLEUKIN (IL)-6 AND IL-8 SECRETION AND GENE EXPRESSION IN CULTURES OF HUMAN ADIPOSE TISSUE EXPLANTS

Abstract

Previously, we reported that primary cultures of human stromal vascular (SV) cells containing newly differentiated adipocytes treated with 30 uM trans-10, cis-12 CLA for 24 h had greater gene expression and protein secretion of IL-6 and IL-8 than vehicle controls (Brown et al. 2004). In addition, cultures treated with 30 uM trans-10, cis-12 CLA had decreased mRNA levels of PPARγ2 and several of its downstream target genes. However, the effects of trans-10, cis-12 CLA treatment on human adipose tissue explants are unknown. Therefore, we examined the extent to which trans-10, cis-12 CLA increased IL-6 and IL-8 gene expression and protein secretion, and decreased mRNA levels of PPARγ2 and several of its downstream targets in human subcutaneous adipose tissue explants. Explants (~ 500 mg each) from human subcutaneous adipose tissue were treated with either vehicle (BSA) or 30 uM trans-10, cis-12 CLA (CLA) for 8, 24, or 72 h. Trans-10, cis-12 CLA treatment increased IL-6 and IL-8 secretion into the media (Main effect of treatment: $p < 0.05$). Evaluation of the treatment by time interactions revealed that cultures treated with trans-10, cis-12 CLA for 72 h had higher ($p < 0.05$) levels of IL-6 and IL-8 in the media compared to the BSA controls. Similarly, trans-10, cis-12 CLA treatment increased the mRNA levels of IL-6 and IL-8 (Main effect of

treatment: $p < 0.05$). Evaluation of the treatment by time interactions revealed that cultures treated with trans-10, cis-12 CLA had higher ($p < 0.05$) levels of IL-6 after 24 h and higher ($p < 0.05$) levels of IL-8 after 8 and 24 h of treatment compared to the vehicle controls. However, CLA had no significant effect on the expression of PPARγ or its target genes. These data demonstrate for the first time that trans-10, cis-12 CLA induces IL-6 and IL-8 gene expression and protein secretion in cultures of human subcutaneous adipose tissue explants.

Introduction

CLA refers to a group of naturally occurring isomers of linoleic acid. Approximately 4-6 mg/g of CLA is found in ruminant meats and dairy products. The two major CLA isomers occurring in nature are cis-9, trans-11 CLA and trans-10, cis-12 CLA. CLA, specifically the trans-10, cis-12 CLA isomer, reduced adiposity *in vivo* and fatty acid incorporation into TG in adipocytes *in vitro* (Park et al. 1999, Kang et al. 2003, Brown et al. 2003, Brown et al. 2004, Granlund et al. 2005). Trans-10, cis-12 CLA has also been shown to decrease mRNA levels for PPARγ2 and several of its adipogenic downstream target genes (i.e., GLUT4, aP2, perilipin, LPL, GPDH, and adiponectin), thereby decreasing TG accumulation in newly differentiating and promote delipidation of mature human adipocytes (Brown et al. 2003, Brown et al. 2004) or 3T3-L1 adipocytes (Granlund et al. 2005). Interestingly, CLA increased mitogen-activated protein kinase kinase/extracellular signal-related kinase kinase (MEK/ERK) signaling which was essential for CLA's suppression of the expression of adipogenic target genes and the hypersecretion of IL-6 and IL-8 (Brown et al. 2004). Based on these observations, the effect of trans-10, cis-12 CLA treatment on IL-6 and IL-8 gene expression and protein secretion in human subcutaneous adipose tissue explants was examined. Furthermore, the extent to which trans-10, cis-12 CLA treatment reduced the mRNA levels of PPARγ2 and its adipogenic target genes was examined. In this study, we show that trans-10, cis-12 CLA-treated explants had greater gene expression and protein secretion of IL-6 and IL-8 than vehicle controls. In addition, trans-10, cis-12 CLA treatment had no significant effect on the mRNA levels of PPARγ2 and several of its downstream targets (e.g.,

GLUT4, aP2, perilipin, LPL), with the exception of adiponectin, which was decreased after 72 h of treatment with CLA compared to controls.

Materials and Methods

Chemicals and Reagents

Trans-10, cis-12 CLA (+ 98% pure) was purchased from Matreya, Inc. (Pleasant Gap, PA) and fatty acid free bovine serum albumin (>98%) was purchased from Sigma Chemical (St. Louis, MO). TURBO DNA-free was purchased from Ambion (Austin, TX). Reverse transcription kit and Syber Green real time PCR kit were purchased from Qiagen, Inc. (Valencia, CA). Gene specific primers for quantitative real time PCR (Table 1) were purchased from Invitrogen, Inc. (Carlsbad, CA) or Integrated DNA Technologies, Inc. (Coralville, IA). ELISA kits for IL-6 (#D6050) and IL-8 (# D8000C) were purchased from R & D Systems, Inc. (Minneapolis, MN). All other chemicals and reagents were purchased from Sigma Chemical Co. unless otherwise indicated.

Fatty Acid Preparation

Trans-10, cis-12 CLA was complexed to fatty acid-free (>98%) bovine serum albumin (BSA) at a 4:1 molar ratio using 1 mM BSA stocks as previously described (Brown et al. 2004).

Culture of Human Subcutaneous Adipose Tissue Explants

Abdominal adipose tissue was obtained from females with a body mass index < 30.0 during elective surgery with consent from the Institutional Review Board (IRB) at University of North Carolina-Greensboro and the Moses Cone and Wesley Long IRB. Adipose tissue was weighed out into 500 mg pieces and cut into ~ 5 to 10 mg fragments

for explant culture. Explants were incubated in 5 mL of Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12 HAM) containing 50 ug/mL gentamicin, 100 U/mL penicillin, 100 U/mL streptomycin, 25 mg/mL fungizone, and 1% BSA for approximately 1 h. At the conclusion of the incubation period, tissue explants were centrifuged for 30 sec at 400 x g to remove erythrocytes, then resuspended in the same media without BSA for 24 h before treatments were added. To initiate the experiments, explants were transferred to fresh tubes and incubated continuously in serum-free media containing either vehicle (BSA) or 30 uM trans-10, cis-12 CLA for 0, 8, 24, or 72 h. At the conclusion of each time point, tissue explants were frozen in liquid nitrogen and stored at -80 °C for RNA isolation and conditioned media was harvested and frozen at –80 °C for ELISA.

RNA Isolation and Quantitative Real Time PCR Analysis

Tissue explants were pulverized in liquid nitrogen prior to homogenization in guanidinium thiocyantate solution. Following homogenization, surface lipids were removed to ensure better RNA quality. Total RNA was isolated using the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski et al. 1987). DNA was removed from RNA preparations using TURBO DNA-free, and the first strand synthesis was performed using oligo dT15 primers and Omniscript RT kit (Qiagen).

Real time quantitative PCR was performed in a Smartcycler (Cepheid, Sunnyvale, CA) using QuantiTect SYBR Green PCR Kit (Qiagen) for 40 cycles. Gene-specific primer pairs used for real time quantitative PCR are shown in Table 1. To account for

variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene hOS-9 (Weisberg et al. 2003) was simultaneously quantified in a separate tube for each sample. A melting curve was also generated for every PCR product to check the specificity of the PCR. Following melting curve analyses, standard curves for each target gene were constructed using serial dilutions of a reference cDNA sample. Relative mRNA quantification of target genes with comparable amplification efficiencies to the endogenous reference gene was determined using the ΔΔCt method. Relative mRNA quantification of target genes with amplification efficiencies different from the endogenous reference gene was determined using "Guidelines for relative quantification with different amplification efficiencies" (from "Critical Factors for Successful Real-Time PCR" –www.qiagen.com).

Immunoassay

Aliquots of conditioned media were measured for immunodetectable IL-6 and IL-8 using a sandwich immunoassay from R & D Systems, Inc. Both immunoassays used an antihuman monoclonal antibody for capture and a rabbit antihuman antibody for detection. Samples of conditioned media were diluted 1:20-1:100 for IL-6 and 1:30-1:80 for IL-8. IL-6 and IL-8 concentrations (pg/g of tissue) were determined based on the standard curve of the assay.

Statistical Analyses

Data are expressed as the mean $\pm$ S.E. Data were analyzed using two-way analysis of variance (Main Effects: Time (n=3) and Treatment (n=2), followed by Student's t-tests for multiple comparisons of the time by treatment interactions. Differences were

Table 1. Primer sequences for quantitative real-time PCR

Target Gene		Primer Sequence	Accession Number
Adiponectin	Sense Antisense	5'-GCAGAGATGGCACCCCTG–3' 5'-GGTTTCACCGATGTCTCCCT–3'	NM_ 004797
aP2	Sense Antisense	5'-ATATGAAAGAAGTAGGAGTGGGCTTT–3' 5'-CCATGCCAGCCACTTTCC–3'	NM- 001442
GLUT4	Sense Antisense	5'-GCTACCTCTACATCATCCAGAATCTC-3' 5'-CCAGAAACATCGGCCCA–3'	NM__0010 42 00584
hOS-9	Sense Antisense	5'-TAAACGCTACCACAGCCAGACC–3' 5'-AGCCGAGGAGTGCGAATG–3'	NM_ 006812
IL-6	Sense Antisense	5'-AAATGCCAGCCTGCTGACGAA–3' 5'-AACAACAATCTGAGGTGCCCATGCTAC-3'	NM_ 000600
IL-8	Sense Antisense	5'-GAATGGGTTTGCTAGAATGTGATA–3' 5'-CAGACTAGGGTTGCCAGATTTAAC–3'	NM_ 00584
PPARγ	Sense Antisense	5'-AGCAAACCCCTATTCCATGCTA–3' 5'-ATCAGTGAAGGAATCGCTTTCTG–3'	NM_ 005037
TNFα	Sense Antisense	5'-TCTTCTCGAACCCCGAGTGA–3' 5'-CCTCTGATGGCACCACCAG–3'	NM_ 000594

considered significant if $p<0.05$. All analyses were performed using JMP IN v4.04 (SAS Institute, Cary, NC) software.

Results

Trans-10, cis-12 CLA increases IL-6 and IL-8 protein secretion from human adipose tissue explants

To determine the extent to which trans-10, cis-12 CLA increased the amount of IL-6 and IL-8 secreted from human adipose tissue explants, explants were incubated for 8, 24, or 72 h with 30 uM trans-10, cis-12 CLA or BSA vehicle. Conditioned media was collected at each time point and IL-6 and IL-8 concentrations were measured using ELISA. CLA treatment increased IL-6 and IL-8 secretion into the media (Main effect of treatment: $p < 0.05$). As shown in Figures 4 and 5, trans-10, cis-12 CLA treatment for 72 h increased IL-6 and IL-8 levels in the media compared to BSA vehicle.

Trans-10, cis-12 CLA increases gene expression of IL-6 and IL-8

To determine the extent to which CLA-mediated secretion of IL-6 and IL-8 in human adipose tissue explants was the result of increased gene expression, explants were treated with 30 uM trans-10, cis-12 CLA or BSA vehicle for 8, 24, or 72 h. CLA treatment increased IL-6 and IL-8 mRNA levels (Main effect of treatment: $p < 0.05$). Messenger RNA levels for IL-6 (Fig. 6) were higher at 24 h in trans-10, cis-12 CLA-treated explants compared to the vehicle controls. Explants treated with CLA had higher IL-8 mRNA levels at 8 and 24 h when compared to BSA controls (Fig. 7).

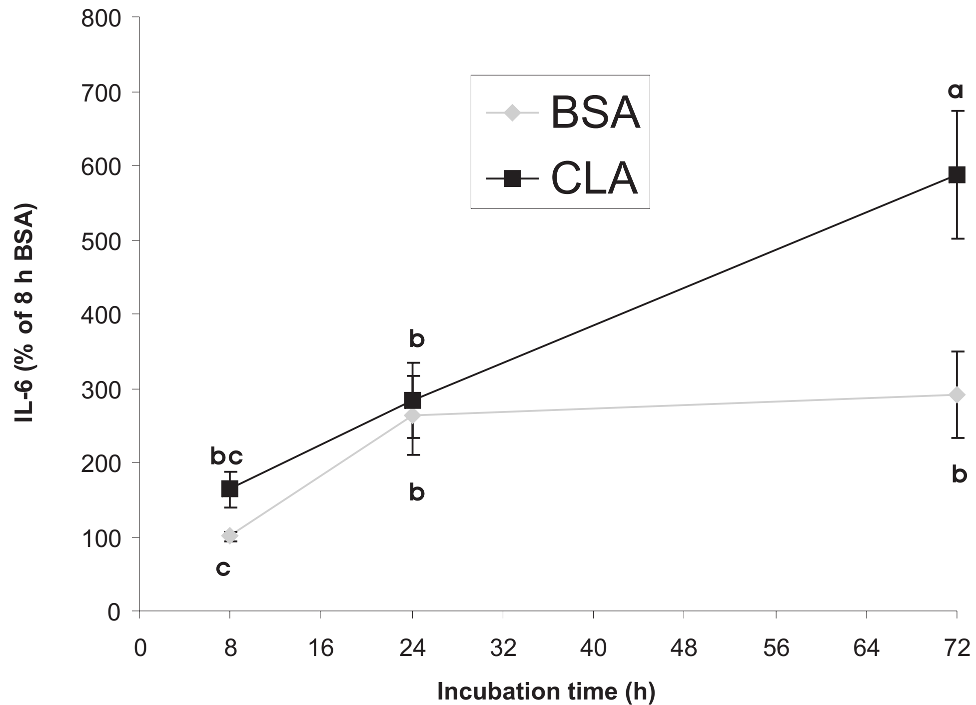
The effects of trans-10, cis-12 CLA on PPARγ2 and several of its downstream target genes

To determine the effect of trans-10, cis-12 CLA on PPARγ2 and PPARγ2 target genes in human adipose tissue, explants were treated with 30 uM trans-10, cis-12 CLA or BSA vehicle continuously for 8, 24, or 72 h. As shown in Figure 8, trans-10, cis-12 CLA had no significant effect on PPARγ2 mRNA levels at each time interval compared to vehicle. In addition, cultures treated with 30 uM trans-10, cis-12 CLA had similar levels of mRNA for GLUT4 (Fig. 9) and aP2 (Fig. 10) when compared to BSA controls. However, explants treated with 30 uM trans-10, cis-12 CLA for 72 h had higher adiponectin mRNA levels compared to BSA controls (Fig. 11).

Discussion

These data are the first to show that trans-10, cis-12 CLA increases the gene expression and protein secretion of IL-6 and IL-8 in cultures of human adipose tissue explants. Similar to cultures of SV cells containing newly differentiated adipocytes, we propose in our working model that trans-10, cis-12 CLA increases cytokine gene expression and secretion in human subcutaneous adipose tissue through the following mechanism(s): 1) CLA activates a membrane protein or enters the SV cells in adipose tissue by simple diffusion and is converted to a metabolite; and 2) the membrane protein or CLA metabolite activates a signal activating NFκB and ERK ½, inducing the synthesis of the cytokines such as IL-6 and IL-8, predominantly from the SV cells.

This working model is supported by the following preliminary data from our research group (Chung et al. 2005 submitted to J. Biol. Chem.): 1) the non-differentiated SV cells produced 10-fold and 7-fold more IL-6 and IL-8, respectively, in response to 24 h treatment with CLA than did differentiated cultures of adipocytes treated with CLA 2)



<u>Figure 4.</u> Trans-10, cis-12 CLA increases IL-6 secretion. Human adipose tissue explants were incubated continuously for 8, 24, or 72 h with 30 uM trans-10, cis-12 CLA (CLA) or BSA vehicle (BSA). At each time point, conditioned media was collected for IL-6 determination using an ELISA. IL-6 concentrations are expressed as a percentage of 8 h BSA vehicle. Means ($\pm$ S.E.; n = 5-8) for IL-6 measurements were obtained from three independent experiments from different human subjects. Means not sharing a common superscript differ, $p < 0.05$.

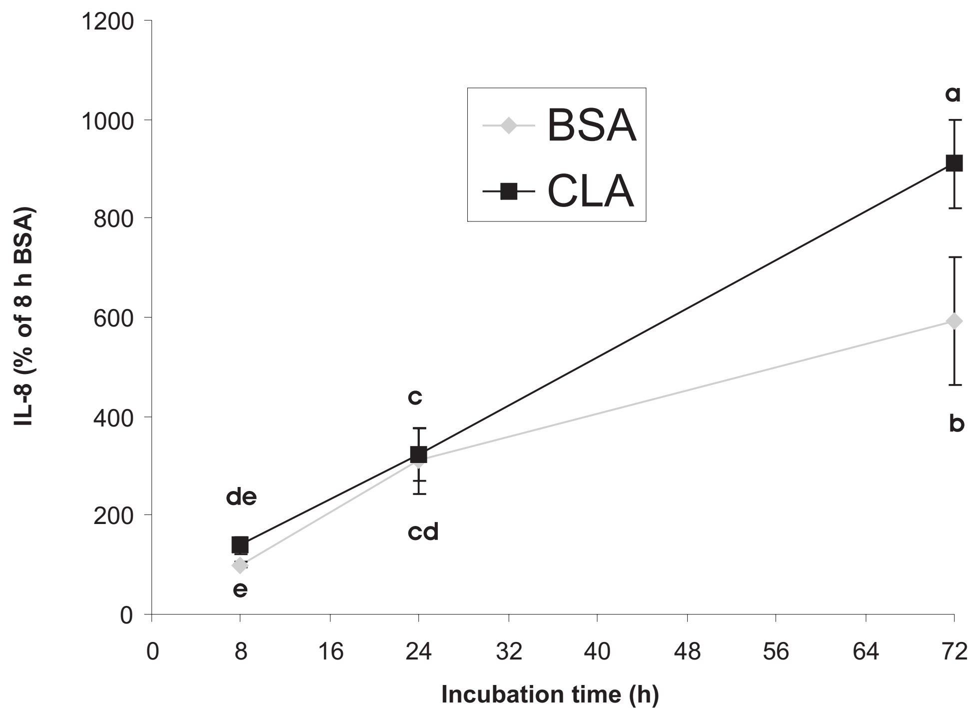


Figure 5. Trans-10, cis-12 CLA increases IL-8 secretion. Human adipose tissue explants were incubated continuously for 8, 24, or 72 h with 30 uM trans-10, cis-12 CLA (CLA) or BSA vehicle (BSA). At each time point, conditioned media was collected for IL-8 determination using an ELISA. IL-8 concentrations are expressed as a percentage of 8 h BSA vehicle. Means ($\pm$ S.E.; n = 5-8) for IL-8 measurements were obtained from three independent experiments from different human subjects. Means not sharing a common superscript differ, $p < 0.05$.

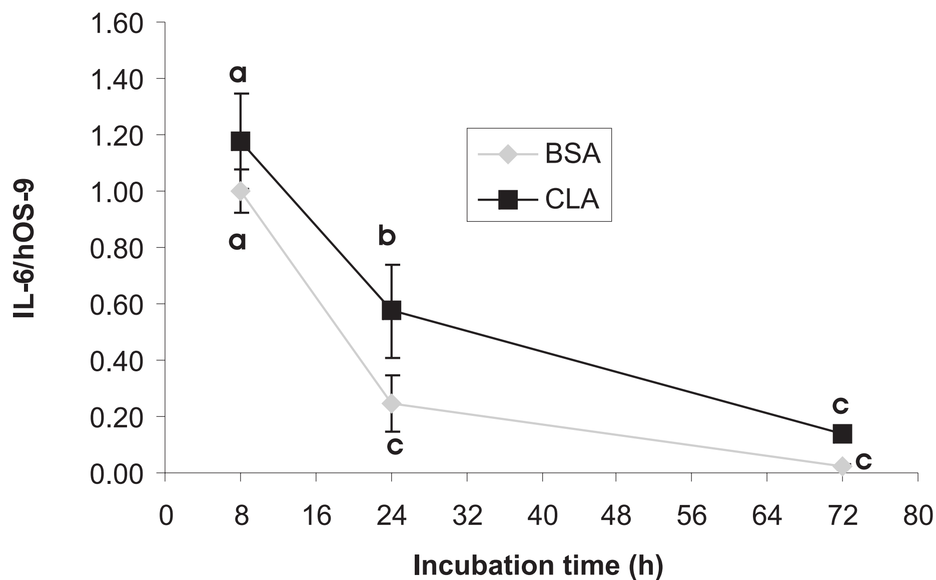


Figure 6. **Trans-10, cis-12 CLA-induced alterations in IL-6 gene expression.** Human adipose tissue explants were incubated with 30 uM trans-10, cis-12 CLA (CLA) or BSA vehicle (BSA) for 8, 24, or 72 h. Explants were frozen in liquid nitrogen at each time interval and RNA was harvested for first strand cDNA synthesis. Real time quantitative PCR analyses were performed to determine IL-6 expression relative to 8 h BSA vehicle control. The internal control gene amplified in osteosarcoma (hOS-9) was used to normalize the expression data. Means (± S.E.; n = 2-4) for IL-6 expression were obtained from one human subject. Means not sharing a common *superscript* differ, $p < 0.05$.

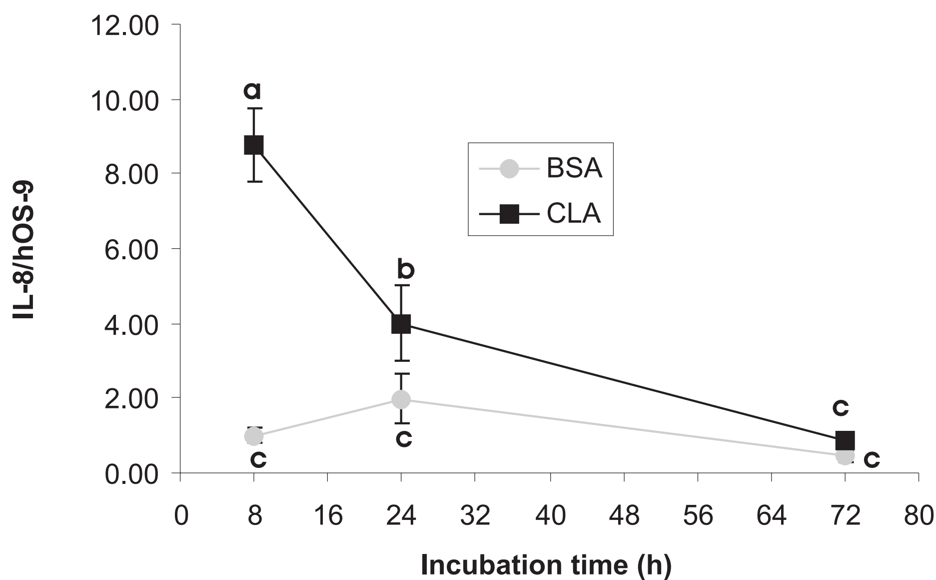
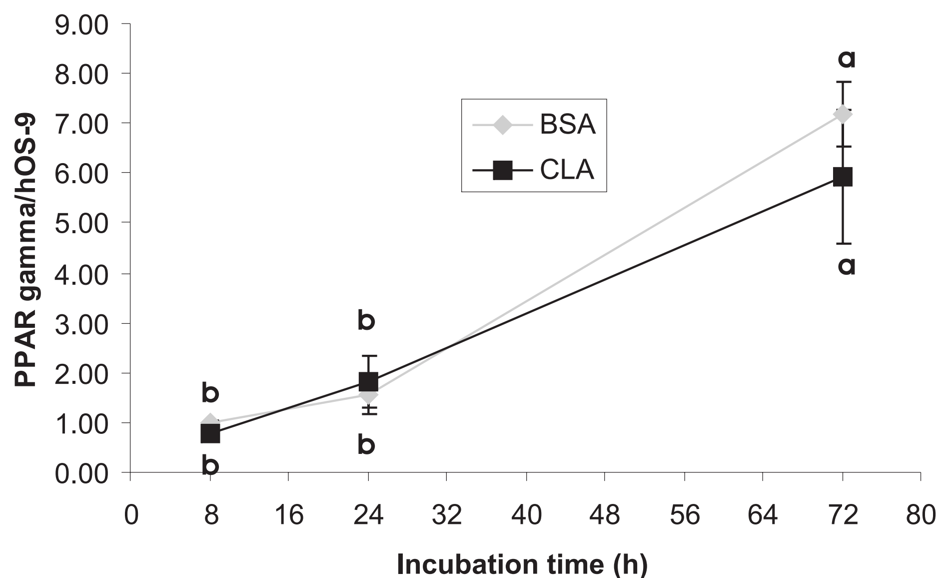
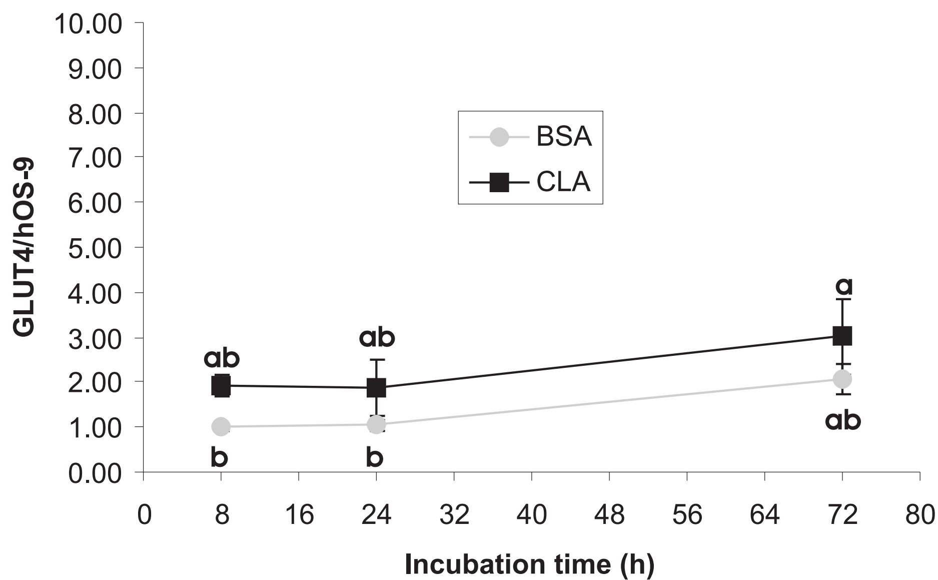


Figure 7. Trans-10, cis-12 CLA-induced alterations in IL-8 gene expression. Human adipose tissue explants were incubated with 30 uM trans-10, cis-12 CLA (CLA) or BSA vehicle (BSA) for 8, 24, or 72 h. Explants were frozen in liquid nitrogen at each time interval and RNA was harvested for first strand cDNA synthesis. Real time quantitative PCR analyses were performed to determine IL-8 expression relative to 8 h BSA vehicle control. The internal control gene amplified in osteosarcoma (hOS-9) was used to normalize the expression data. Means ($\pm$ S.E.; n = 2-4) for IL-8 expression were obtained from one human subject. Means not sharing a common *superscript* differ, $p <$ 0.05.



<u>Figure 8.</u> Trans-10, cis-12 CLA-induced alterations in PPAR gamma gene expression. Human adipose tissue explants were incubated with 30 uM trans-10, cis-12 CLA (CLA) or BSA vehicle (BSA) for 8, 24, or 72 h. Explants were frozen in liquid nitrogen at each time interval and RNA was harvested for first strand cDNA synthesis. Real time quantitative PCR analyses were performed to determine PPAR gamma expression relative to 8 h BSA vehicle control. The internal control gene amplified in osteosarcoma (hOS-9) was used to normalize the expression data. Means ($\pm$ S.E.; n = 2-4) for PPAR gamma expression were obtained from one human subject. Means not sharing a common *superscript* differ, $p < 0.05$.



<u>Figure 9.</u> Trans-10, cis-12 CLA-induced alterations in GLUT4 gene expression. Human adipose tissue explants were incubated with 30 uM trans-10, cis-12 CLA (CLA)or BSA vehicle (BSA) for 8, 24, or 72 h. Explants were frozen in liquid nitrogen at each time interval and RNA was harvested for first strand cDNA synthesis. Real time quantitative PCR analyses were performed to determine GLUT4 expression relative to 8 h BSA vehicle control. The internal control gene amplified in osteosarcoma (hOS-9) was used to normalize the expression data. Means ($\pm$ S.E.; n = 2-4) for GLUT4 expression were obtained from one human subject. Means not sharing a common *superscript* differ, $p < 0.05$.

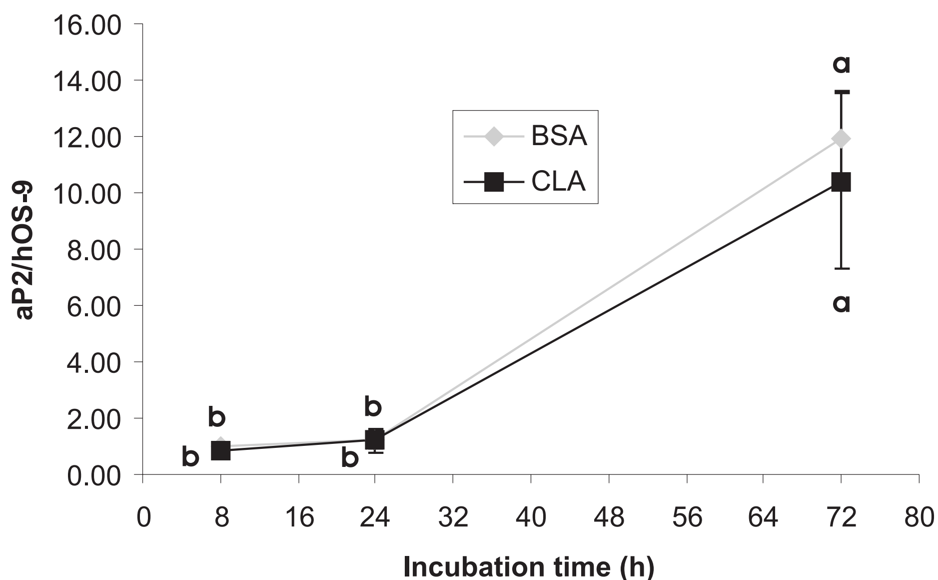
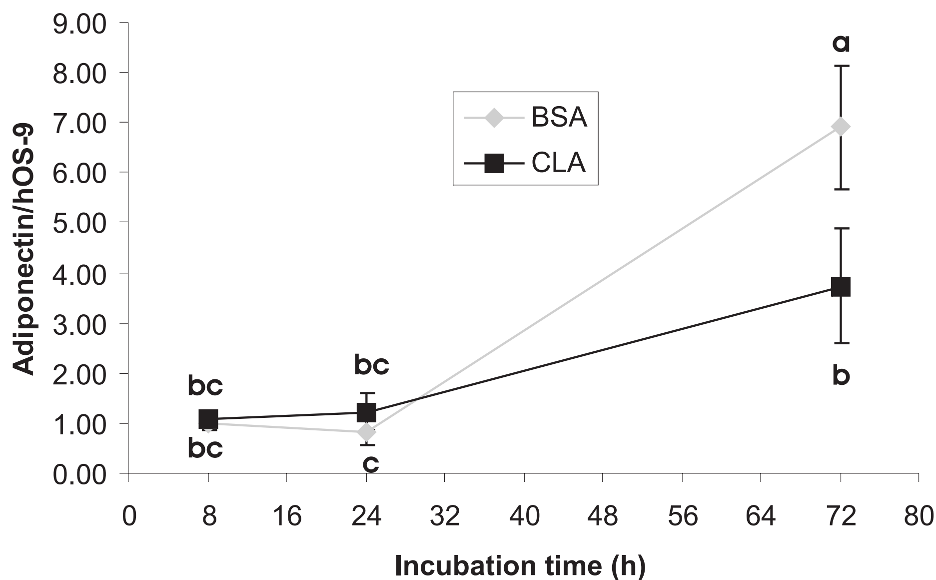


Figure 10. Trans-10, cis-12 CLA-induced alterations in aP2 gene expression. Human adipose tissue explants were incubated with 30 uM trans-10, cis-12 CLA (CLA) or BSA vehicle (BSA) for 8, 24, or 72 h. Explants were frozen in liquid nitrogen at each time interval and RNA was harvested for first strand cDNA synthesis. Real time quantitative PCR analyses were performed to determine aP2 expression relative to 8 h BSA vehicle control. The internal control gene amplified in osteosarcoma (hOS-9) was used to normalize the expression data. Means (± S.E.; n = 2-4) for aP2 expression were obtained from one human subject. Means not sharing a common *superscript* differ, $p < 0.05$.



<u>Figure 11.</u> Trans-10, cis-12 CLA-induced alterations in adiponectin gene expression. Human adipose tissue explants were incubated with 30 uM trans-10, cis-12 CLA (CLA) or BSA vehicle (BSA) for 8, 24, or 72 h. Explants were frozen in liquid nitrogen at each time interval and RNA was harvested for first strand cDNA synthesis. Real time quantitative PCR analyses were performed to determine adiponectin expression relative to 8 h BSA vehicle control. The internal control gene amplified in osteosarcoma (hOS-9) was used to normalize the expression data. Means ($\pm$ S.E.; n = 2-4) for adiponectin expression were obtained from one human subject. Means not sharing a common *superscript* differ, $p < 0.05$.

cytokine secretion in differentiated cultures of adipocytes treated with CLA was preceded by IκBα degradation, IKK phosphorylation, and increased p50 and p65 translocation to the nucleus indicating NFκB activation; 3) NFκB p50 and p65 DNA binding in CLA-treated cultures of differentiated adipocytes was increased by approximately 120% and 80%, respectively, when compared to BSA control; and 4) NFκB selective inhibitors block the acute induction of IL-6 gene expression in CLA-treated differentiated adipocytes. In further support of our model, Harkins et al. 2003 demonstrated that LPS-stimulated SV cells from adipose tissue of obese mice had higher levels of secreted IL-6 than adipocytes. In addition, LPS-stimulated SV cells from adipose tissue of obese mice had greater levels of IL-6 mRNA than adipocytes. Similarly, the non-adipocyte fraction isolated from human subcutaneous and visceral adipose tissue accounted for 96% and 92% of secreted IL-6 and IL-8, respectively (Fain et al. 2003). Finally, Chen et al. (1999) demonstrated that IL-6 and IL-8 possess a nuclear factor κB response element (κBRE). Collectively, these data support our working model explaining the mechanisms and source of CLA's proinflammatory response (i.e., increased secretion and mRNA levels of IL-6) in human subcutaneous adipose tissue. Additionally, it should be mentioned that LPS treatment (10 ug/mL) increased the secretion and gene expression of IL-6 and IL-8 (data not shown) in human subcutaneous adipose tissue explants, demonstrating the responsiveness of these explants to immunomodulatory effectors.

Our hypothesis was that trans-10, cis-12 CLA would decrease the mRNA expression of PPARγ2 and PPARγ2 adipogenic targets (i.e., GLUT4, adiponectin, and aP2). However, treatment with 30 uM trans-10, cis-12 CLA treatment only decreased

adiponectin mRNA levels after 72 h compared to controls. While a 30 uM concentration is sufficient to exert anti-adipogenic effects (i.e., decreased gene expression of PPARγ2 and its downstream adipogenic genes) in newly differentiated human adipocytes treated for up to 9 d (Brown et al. 2004), a higher concentration might be needed to deliver a comparable amount of CLA to the cells in the interior of human adipose tissue explants. Brown et al. 2004 reported the greatest decrease in mRNA levels of PPARγ2 and PPARγ2 downstream targets in cultures treated with CLA for 9 d. This also suggests a longer CLA treatment might be required to decrease mRNA levels of adipogenic genes. Fried et al. 1998 have demonstrated adipose tissue explants cultured for up to 7 d are still responsive to hormones such as insulin and are still capable of secreting IL-6. Evidence from studies with murine 3T3-L1 adipocytes and mature human adipocytes support the concept that trans-10, cis-12 CLA is a modulator of PPARγ2, the master regulator of adipocyte differentiation. In these studies, trans-10, cis-12 CLA treatment decreased the gene expression of PPARγ2 and its adipogenic downstream targets in murine and human preadipocytes (Kang et al. 2003, Brown et al. 2003) and mature adipocytes (Granlund et al. 2003, Brown et al. 2004). Synthetic PPARγ antagonists exert similar effects such as: 1) downregulation of PPARγ2 and its target genes regulating adipogenesis; 2) reduced conversion of preadipocytes to adipocytes; and 3) increased adipocyte delipidation to those observed in trans-10, cis-12 CLA-treated cultures. These outcomes strengthen the argument that CLA's antiadipogenic effects depend largely on PPARγ2 modulation (Rieusset et al. 2002, Mukherjee et al. 2000). However, future research is needed to determine whether trans-10, cis-12 CLA decreases mRNA levels of PPARγ2 and its

downstream adipogenic targets in human adipose tissue explants and whether these decreases are associated with the secretion of the proinflammatory cytokines IL-6 and IL-8.

In summary, our data with human adipose tissue explants demonstrates that trans-10, cis-12 CLA increases the gene expression and secretion of IL-6 and IL-8 in cultures of human adipose explants. These data support previous data from our research group showing that trans-10, cis-12 CLA increases IL-6 and IL-8 secretion and gene expression in cultures of differentiated adipocytes.

CHAPTER III

EPILOGUE

Here we demonstrated for the first time that trans-10, cis-12 CLA increased the gene expression and secretion of the proinflammatory cytokines IL-6 and IL-8 in human adipose tissue explants. We also hypothesized that trans-10, cis-12 CLA would decrease the expression of PPARγ2 and its downstream adipogenic genes (e.g., GLUT4, adiponectin, and aP2), although these data were inconclusive. If time and resources were not limiting, the following experiments could be conducted to further support these findings and determine the degree to which CLA exerts anti-adipogenic effects in human adipose tissue explants.

In our experiment to determine CLA's anti-adipogenic effects on human subcutaneous adipose tissue explants, the concentration of trans-10, cis-12 CLA used was 30 uM. At this concentration, only one significant difference was obtained between explants treated with CLA or BSA for mRNA levels of adipogenic genes (i.e., PPARγ2, GLUT4, adiponectin, and aP2). Adiponectin mRNA for explants treated with CLA was decreased at 72 h compared to BSA vehicle. Interestingly, of the adipogenic genes measured in our experiment, Brown et al. 2004 reported that CLA treatment decreased adiponectin mRNA by 100% at 72 h in cultures of newly differentiated adipocytes. While a 30 uM concentration is sufficient to exert anti-adipogenic effects (i.e., decreased gene expression of PPARγ2 and its downstream adipogenic genes) in newly

differentiated human adipocytes treated for up to 9 d (Brown et al. 2004), a higher concentration might be needed to deliver a comparable amount of CLA to the cells in human adipose tissue. In addition, Brown et al. 2004 reported the greatest decrease in mRNA levels of PPARγ2 and PPARγ2 downstream targets in cultures treated with CLA for 9 d. This suggests a longer treatment could be required to observe a significant decrease in the mRNA levels of adipogenic genes. Therefore, my first experiment would be a dose response/time course study that would allow me to investigate the anti-adipogenic effects of CLA when manipulating these variables. The concentrations of CLA used would still need to be physiologically relevant, so 30 uM and 60 uM could be used. In addition, adipose tissue explants could be cultured up to 7 d because Fried et al. 1998 have demonstrated adipose tissue explants cultured for this length are still viable and secrete IL-6. Real time PCR would then be used to determine if CLA decreases the mRNA levels of PPARγ2, GLUT4, adiponectin, and aP2, which are genes involved in regulating adipogenesis. If a decrease in mRNA is demonstrated, follow up experiments could be conducted to determine if the protein levels of PPAR PPARγ2, GLUT4, adiponectin, and aP2 have decreased similar to mRNA levels. Homogenization and protein determination could be carried out using the method outlined by Lappas et al. 2003. Following protein determination, immunoblotting would be used to determine CLA's effect on the level of each protein.

Here we demonstrated that treatment with trans-10, cis-12 CLA induces the expression of IL-6 and IL-8 mRNA in adipose tissue explants. Preliminary data from our group has demonstrated that NFκB activation is requisite for CLA's induction of IL-6

gene expression in differentiated cultures of adipocytes at 3 h (Chung et al. 2005). Based on these data, a second experiment could be conducted to determine whether CLA induces the protein expression of NFκB and other transcription factors known to induce IL-6 gene expression (e.g., NF-IL6 and AP-1). If the expression of one or more of these transcription factors is induced by CLA, then protein expression of putative upstream kinases [e.g., ERK 1/2 and protein kinase C (PKC)] could also be assessed. Following protein determination, immunoblotting would be used to determine CLA's effect on the level of each protein.

If a CLA-mediated increase in protein expression could be demonstrated for NFκB, it would be interesting to determine the impact of NFκ inhibitors on CLA-induced IL-6 and IL-8 gene expression and protein secretion in adipose tissue explants. A third experiment could be designed to pre-treat adipose tissue explants with either PSI or Bay11-7082 (Lappas et al. 2004) that block different steps in the process of NFκB activation. PSI blocks the degradation of polyubiquitinated IκBα in the proteosome and Bay11-7082 inhibits the phosphorylation of IκBα by IκBα kinase. After pretreating the explants could be treated with CLA or vehicle to study the effect of each of these inhibitors on IL-6 gene expression and protein secretion. Bacterial lipopolysaccharide (LPS), which has been shown to induce NFκB activity (Lappas et al. 2004), could be used as a positive control for the experiment. IL-6 secreted into the media would be harvested and measured using ELISA and IL-6 gene expression in the explants would be measured using Real time quantitative PCR.

Finally, based on recent evidence from our research group and others, we propose that CLA increases cytokine (i.e., IL-6 and IL-8) secretion primarily in the non-adipocyte (i.e., SV cell) population in human subcutaneous adipose tissue explants and newly differentiated adipocytes (Brown et al. 2004). This hypothesis is supported by the following evidence: 1) Weisberg et al. 2003 and Xu et al. 2003 reported that the SV cells in the adipose tissue of mice had greater mRNA levels of the cytokines TNFα and IL-6; 2) Fain et al. 2004 reported that greater than the non-adipocyte population in human adipose tissue explants accounted for 93% of IL-6 secreted; and 3) Harkins et al. 2004 demonstrated that 3T3-L1 preadipocytes had significantly higher IL-6 mRNA and IL-6 secretion than differentiated 3T3-L1 adipocytes. To test this hypothesis, two different experimental designs could be used. The first design would use a buoyant density gradient to isolate differentiated adipocytes from SV cells grown in cell culture. The second design previously used by Fain et al. 2003 and Weisberg et al. 2003 would isolate adipocytes from SV cells by low-speed centrifugation (400 – 1000 x g) following collagenase digestion of adipose tissue.

Following CLA treatment, the first design requires that cultures of SV cells containing differentiated adipocytes be detached from the cell culture monolayer. To minimize cell lysis, a chelating agent or a microbial-derived trypsin could be used to lift the cells from the monolayer. To prevent handling of the adipocytes and remove excess gradient media from the tube, the bottom of the tube could be punctured and drained. SV cells would then be harvested and pelleted for RNA isolation using a guanidinium isothiocyanate (GTC) solution. After harvesting the SV cells and removing the gradient

media, the floating adipocytes would be lysed in GTC solution. The samples would then be analyzed for mRNA expression of cytokines (i.e., IL-6 and IL-8), adipocyte markers (aP2 and leptin) (Fain et al. 2003, Abderrahim-Ferkoune et al. 2004) and preadipocyte markers [(preadipocyte factor-1 (Pref-1) and Aortic carboxypeptidase-like protein(ACLP)] (Abderrahim-Ferkoune et al. 2004) using Real time PCR.

The second design would require adding human adipose tissue minced into small (~ 10 mg) fragments to a suspension containing bacterial collagenase (approximately 0.03 mg/mL) and Dulbecco's modified Eagle's medium/Ham's F12. The tissue is then digested in a rotary water bath shaker for approximately 1 to 2 h. The digested suspension is filtered through 200 to 250-um mesh fabric into a centrifuge tube and then spun at approximately 400-1000 x g for 1 min. Following centrifugation, the SV cells will form a pellet while the adipocytes remain in suspension. The medium and SV cells are removed from the tube and then fresh medium containing either 30 uM trans-10, cis-12 CLA or BSA vehicle is added to each cell fraction. IL-6 secreted into the media by each cell fraction would be harvested and measured using ELISA. Then the SV cells and adipocytes would be analyzed for mRNA expression of cytokines , adipocyte markers, and preadipocyte markers using Real time quantitative PCR. To normalize the cytokine ELISA data, the cytokine concentration (pg/mL) would be divided by the wet weight of adipose tissue before digestion. This is the method used by Fain et al. 2003 to normalize cytokine ELISA data from each cell fraction following tissue digestion.

In conclusion, the experiments discussed in this section would further validate our data demonstrating for the first time that trans-10, cis-12 CLA stimulates cytokine

secretion and induces cytokine gene expression in adipose tissue explants. In addition, they would also examine the effect of CLA on the following parameters: 1) adipogenic protein and gene expression; 2) protein expression of transcription factors and kinases (i.e., NFκB, NF-IL-6, AP-1, ERK, PKC) leading to cytokine secretion and gene expression; and 3) the induction of cytokine gene expression in the SV cell and adipocyte fraction isolated from human adipose tissue as well as the cytokine secretion from each cell population. Collectively, these experiments would help address the following research questions related to CLA's effects on human adipose tissue explants: 1) how CLA works mechanistically to induce a pro-inflammatory response (i.e., increased cytokine secretion and gene expression); 2) the predominant cellular source (i.e. SV cells vs. adipocytes) of the CLA-mediated response; and 3) whether or not CLA treatment exerts anti-adipogenic effects.

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