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Obesity is an important health issue, having risen to epidemic proportions in the U.S. Use of conjugated linoleic acid (CLA), positional and geometric isomers of linoleic acid, has received recent attention due to its potential health benefits including the reduction of fat mass in animals. However, the effectiveness and safety of CLA consumption in humans remains unclear. Our group previously reported that *trans*-10, *cis*-12 CLA impaired the conversion of preadipocytes into lipid-filled adipocytes (e.g., differentiation) and caused adipocyte delipidation that involved inflammatory cytokines in a human cell model. However, the isomer-specific mechanism for these events was unknown. Thus, this research examined mechanisms by which *trans*-10, *cis*-12 CLA induced adipocyte delipidation, inflammation, and insulin resistance in primary cultures of human adipocytes. Delipidation of adipocytes by *trans*-10, *cis*-12 CLA was accompanied by increased lipolysis and changes in the morphology of lipid droplets and the expression and localization of proteins regulating lipid droplet metabolism. This process involved the translational control of adipose differentiated related protein (ADRP) through activation of mTOR/p70S6K/S6 signaling and transcriptional control of perilipin A. Prior to these morphological changes, it was shown that *trans*-10, *cis*-12 CLA promoted nuclear factor κB (NFκB) and mitogen activated protein kinase (MAPK) activation and subsequent induction of interleukin (IL)-6 which were, at least in part, responsible for *trans*-10, *cis*-12 CLA-mediated suppression of peroxisome proliferator activated receptor gamma (PPAR)γ target gene expression and insulin sensitivity in human adipocytes.

The essential role of NFκB on CLA-induced inflammation was confirmed by using RNA interference. Further studies were conducted examining the localization and characterization of the inflammatory response, including the type of cells involved, using lipopolysaccharide (LPS) as the inflammatory agent. It was demonstrated that LPS-induced, NFκB-dependent proinflammatory cytokine expression was predominantly from preadipocytes, which led to, at least in part, the suppression of PPARγ activity and adipogenic gene expression and insulin sensitivity. Collectively, these data support the emerging concept that adipose tissue is a dynamic endocrine organ with the capacity to generate inflammatory signals that impact glucose and lipid metabolism. Furthermore, human preadipocytes have the capacity to generate these inflammatory signals induced by *trans*-10, *cis*-12 CLA and LPS, subsequently causing insulin resistance in neighboring adipocytes. These studies also revealed that NFκB- and MAPK-signaling mediate inflammation and insulin resistance induced by CLA and LPS. Thus, although the *trans*-10, *cis*-12 isomer of CLA may decrease the size and lipid content of human adipocytes, it may also cause insulin resistance, which is a hallmark of type 2 diabetes.

MECHANISMS BY WHICH CONJUGATED LINOLEIC ACID CAUSES

HUMAN ADIPOCYTE DELIPIDATION

by

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Approved by

_____
Committee Chair

I would like to dedicate this work to my parents and my husband Sungyong who

inspire me with endless love, and to our wonderful daughters Jennifer and Jessica.

APPROVAL PAGE

This Dissertation has been approved by the following committee of the

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ACKNOWLEDGEMENTS

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

<u>Overview</u>

Adipose tissue plays a critical role in energy homeostasis and endocrine function. Obesity, characterized by excess accumulation of adipose tissue, is involved in the pathogenesis of multiple diseases (Saltiel, 2001; Spiegelman and Flier, 2001). These pathologies include insulin resistance, type 2 diabetes, dyslipidemia, cardiovascular disease, and fatty infiltration of the liver (Shulman 2000; Tilg and Diehl 2000). Currently more than 65% of the adult population in the United States is overweight (Skyler and Oddo 2002), and obesity and type 2 diabetes are the leading metabolic disease worldwide (Flegal et al. 1998; Zimmet et al. 2001). The incidence and the impact of this disease cluster, also referred to as Metabolic Syndrome, have risen to alarming levels, and there is great need for therapeutic and preventive measures against this major health epidemic.

Conjugated linoleic acid (CLA), a group of positional and geometric isomers of the essential fatty acid linoleic acid [18:2(n-6)], is found naturally in foods derived from ruminant animals. It has been clearly demonstrated that *trans*-10, *cis-12* isomer or mixed isomers of CLA (e.g. *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA) attenuate adiposity

in animals and some humans (reviewed in Pariza 2004; House et al. 2005). The potential use of CLA as a therapeutic strategy to prevent or treat human obesity has received recent attention in both popular and peer-reviewed publications. However, the isomer-specific, antiobesity mechanism of CLA remains largely unknown. Furthermore, there are only a few supplementation studies using specific CLA isomers that have examined CLA's effectiveness and safety in humans (Riserus et al. 2004 a,b). Recent data from our lab using a human adipocyte model demonstrated that *trans*-10, *cis*-12 CLA decreased the triglyceride (TG) content of the cultures through cytokine/chemokine signaling, including interleukin (IL)-6 and IL-8 (Brown et al. 2004). Production of these "adipokines" by CLA in cultures of human adipocytes raises a health concern, because proinflammatory adipokines are positively correlated with systemic dysregulation of metabolism including insulin resistance (reviewed in Wellen and Hotamisligil 2005). It is known that inflammatory stimuli elicit proinflammatory cytokine secretion through nuclear factor kappa B (NFκB)-dependent mechanisms in macrophages. Compared with the well-documented studies in macrophages, inflammation in fat tissue is a relatively new concept and less well understood. Evidence is slowly emerging that inflammation is a key player in the development of Metabolic Syndrome. However, identification of the mechanism by which CLA alters inflammation status in primary cultures of human adipocyte has been not reported. Furthermore, CLA appears to act through different cell signaling mechanisms depending on the species and tissue studied.

Therefore, examination of isomer-specific cellular and molecular mechanisms elicited by CLA in primary cultures of human adipocytes offers a unique opportunity to

gain new insights about direct CLA effects on gene expression, cell signaling, and metabolism in human (pre)adipocytes. Studies included in this body of work address potential mechanism by which CLA induces delipidation, inflammation, and insulin resistance in primary cultures of human adipocytes.

Background and Significance

Historical Review of CLA

Conjugated linoleic acid comprises a family of positional and geometric isomers of linoleic acid that are formed by biodegradation and oxidation processes in nature. The major dietary sources of these unusual fatty acids are derived from ruminant animals, in particular dairy products (Ha et al. 1987; Sehat et al. 1998). The main isomer of CLA, *cis*-9, *trans*-11CLA, can be produced directly by bacterial hydrogenation in the rumen or by delta-9 desaturation of the co-product vaccenic acid in most mammalian tissues including man. The second most abundant isomer of CLA is the *trans*-10, *cis*-12 form (Fig. 1.1).

Pariza et al. (1979) demonstrated that an extract from cooked ground beef inhibited mutagenesis in mice, and they subsequently identified the active agent as CLA, a new anti-carcinogen (Ha et al. 1987). This seminal discovery spurred workers in this field to investigate CLA's additional biological effects on cancer (Ip et al. 2002), atherosclerosis (Lee et al. 1994), growth efficiency (Chin et al. 1994), immune function (Bassaganya-Riera et al. 2002), lipid metabolism, and obesity (Park et al. 1999). On the basis of these studies, it has been suggested that supplementation of individual isomers or mixtures of

CLA is potentially effective in: 1) reducing the growth of tumors by inducing apoptosis (Park et al. 2004) or by inhibiting proliferation (Kemp et al. 2003); 2) decreasing the risk of cardiovascular disease by reducing atherosclerotic lesions (Valeille et al. 2004); and 3) enhancing immune competence by modulating inflammatory responses (Yamasaki et al. 2004).

In contrast to the aforementioned physiological benefits exerted by CLA isomers, it has been shown that the *trans*-10, *cis*-12 CLA isomer is solely responsible for reducing adiposity. It was first reported that 20-200 μM of a crude mixture of CLA isomers decreased triglyceride (TG) content in 3T3-L1 adipocytes (Park et al. 1997). Subsequently, it was demonstrated that CLA's ability to reduce body fat was primarily due to *trans*-10, *cis*-12 CLA *in vitro* and *in vivo* (Park et al. 1999). *Trans*-10, *cis*-12 CLA decreased adiposity in porcine (Ostrowska et al. 1999) and hamster (Navarro et al. 2003) models. In support of these *in vivo* data, our group has demonstrated that human adipose tissue is a target of *trans*-10, *cis*-12 CLA by showing that 3-30 μM *trans*-10, *cis*-12 CLA decreased the expression of markers of preadipocyte differentiation (Brown et al. 2003) and reduced TG content in primary human stomal vascular (SV) cultures containing newly differentiated adipocytes (Brown et al. 2004).

t10, c12 CLA

c9, t11 CLA

Linoleic Acid
(c9,c12)
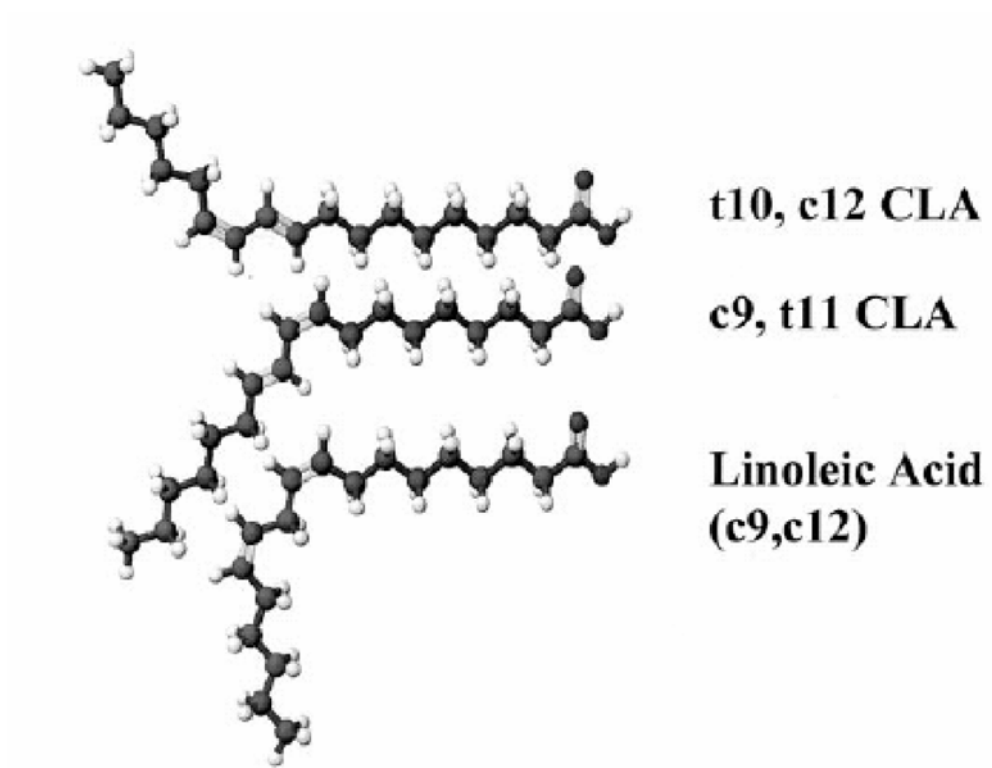
Figure 1. 1. Structures of the biologically active isomers of conjugated linoleic acid (CLA).

Anti-adipogenic Mechanisms of CLA

The evidence cited above has led researchers to appreciate that *trans*-10 *cis*-12 CLA decreases adipogenesis in adipocytes from both rodents and humans. However, the underlying mechanism by which *trans*-10, *cis*-12 CLA causes delipidation is conflicting, depending on cell type, metabolic status, and species. Several different mechanisms have been proposed for the anti-adipogenic mechanism of CLA supplementation.

The simplest explanation is that CLA induces a decrease in food intake. Several *in vivo* animal studies have reported that CLA feeding attenuates abdominal fat deposition coupled with reduced food or energy intake in mice and rats (Park et al. 1999; West et al. 1998; DeLany et al. 1999). However, a decrease in food intake seems to be marginal and cannot fully account for the marked reduction in fat deposition. It might be associated with inappropriate preparation of CLA supplementation (*e.g.* unpleasant smell of CLA) rather than increasing lipolysis, because reduced food intake is seldom reported in well controlled vehicle studies (Ostrowska et al. 1999; Yamasaki et al. 2003) or in human trials (Riserus et al. 2004, 2004).

One of the proposed mechanisms is that *trans*-10, *cis*-12 CLA inhibits the activities of lipid metabolism regulating enzymes and gene expression, such as lipoprotein lipase (LPL) and stearoyl-CoA desaturase (SCD-1). It has been proposed that CLA's inhibition of LPL activity is the key mechanism causing delipidation, *i.e*., reducing lipid uptake into adipocytes (Park et al. 1999). Alternatively, SCD-1, an enzyme responsible for introduction of *cis*-double bond at the C9 position of fatty acyl-CoA, has been proposed by Natambi's group as the target of *trans*-10, *cis*-12 CLA. CLA mediated inhibition of

SCD-1 results in limited availability of monosaturated fatty acids for TG esterification, thereby impairing lipid- and phospholipid-metabolism of adipocytes, and leading to a reduction of fat accretion (Choi et al. 2002). However, the proposed decrease in enzyme activities and gene expression of LPL and SCD-1 alone cannot account for the wide spectrum of changes in gene expression caused by *trans*-10, *cis*-12 CLA.

Recently, it is also proposed that *trans*-10, *cis*-12 CLA alters the structural characteristics of the plasma membrane. Plasma membranes, especially caveolin-rich areas which are composed of proteins that serve as ion channels, transporters, receptors, and signal transducers, play important roles in signal transduction in response to extracellular stimuli. Supported by gene array data, House et al. (2004) suggested that *trans*-10, *cis*-12 CLA reduces caveolin gene expression, resulting in disruption of adipocyte signaling pathway and leading to apoptosis of adipocytes. In agreement with the concept of programmed cell death, it has been suggested that the decrease in adiposity involves an apoptotic mechanism linked to an increase in tumor necrosis factor-α (TNF-α) production (Tsuboyama-Kasaoka 2003). Similarly, Evans et al. (2000) suggested that CLA induced-apoptosis contributes to CLA's TG lowering action in 3T3-L1 preadipocytes. These hypotheses seem to be valid, at least in rodent models, although CLA appears to trigger different mechanisms in humans. It has been reported that reductions in fat cell size rather than cell number were attributable to the decrease of fat deposition in the presence of CLA (Azain et al. 2000; Brown et al. 2004), which is contradictory to apoptosis. Consistent with these data, we showed that *trans*-10, *cis*-12 CLA had no significant impact on apoptotic cell death or on caveolin levels (Chung et al.

7

2005). Chapter II of this dissertation will address the cellular and molecular events relating to morphological changes of adipocytes by CLA supplementation.

Maintenance of energy homeostasis occurs through the induction of genes coding for enzymes that regulate rate determining steps in lipid and carbohydrate metabolism. Consequently, the metabolic effects of CLA are presumed to involve changes in gene expression. Control of lipid homeostasis in response to the body's energy requirements is primarily exerted through transcription factors of the nuclear hormone receptor family (Francis et al. 2003). Given the regulatory role of peroxisome proliferator-activated receptor(PPAR)s in lipid- and glucose-metabolism, a great deal of attention has been focused on the roles of CLA as a PPAR ligand. The PPARs are critical transcription factors in hepatic and adipose lipid metabolism, operating through a promoter sequence termed the PPRE (Desvergne et al. 1999).

It has been suggested that *trans*-10, *cis*-12 CLA activates PPARα, which stimulates β-oxidation, respiration, and energy expenditure resulting in body fat loss (Moya-Canarena et al. 1999). As one of the major targets of PPARα activation, uncoupling protein(UCP)s are predominantly expressed in the mitochondrial inner membrane. Overexpression of UCPs has been of particular interest in energy expenditure and oxidation (Adams. 2000). Several *in vivo* studies have reported an increase in UCP2 expression, suggesting that energy is expended with CLA treatment (Tsuboyama-Kasaoka et al. 2000; Ryder et al. 2001). Activation of PPARα and up-regulation of UCPs by CLA seems to contribute, at least in part, to CLA's anti-adipogenic action in small animals, where energy expenditure is important for controlling energy homeostasis such

8

as in the mouse and rat (Summarized in Fig 1.2). However, PPARα null mice still respond to CLA (Peters et al. 2001). CLA's impact on energy metabolism depends on the animal model or tissue studied along with the dose and isomer of CLA, (West et al. 2000; Ealey et al. 2002; Takahashi et al. 2002).

It has also been proposed that *trans*-10, *cis*-12 CLA is a more potent modulator of PPARγ rather than PPARα (Granlund et al. 2003). PPARγ promotes adipocyte maturation and lipid storage. Therefore, antagonistic actions of CLA on PPARγ can inhibit adipocyte differentiation by negatively modulating PPARγ target gene expression. Both *in vivo* studies in mice and in 3T3-L1 adipocytes have confirmed that, upon CLA supplementation, there is a decrease in PPARγ gene expression, thereby suppressing preadipocytes differentiation into mature adipocytes (Granlund et al. 2003; Brown et al. 2003, Brown et al. 2004). In support of this hypothesis, our group has reported that *trans*-10, *cis*-12 CLA down-regulates PPARγ-target genes, including glucose transporter 4 (Glut4), perilipin, LPL, adipocyte specific fatty acid binding protein (ap2), fatty acid synthase (FAS), acetylCoA carboxylase (ACC), and PPARγ itself in differentiating human preadipocytes (Brown et al. 2003) and mature adipocytes (Brown et al. 2004). Surprisingly, we also found that *trans*-10, *cis*-12 CLA increases proinflammatory "adipokines" (*i.e.*, IL-6, IL-8) secretion prior to modification of adipogenic gene expression. These data suggest that CLA's attenuation of TG content is mediated, at least in part, by inflammatory signaling pathways, raising the concern about the safety of CLA supplementation.
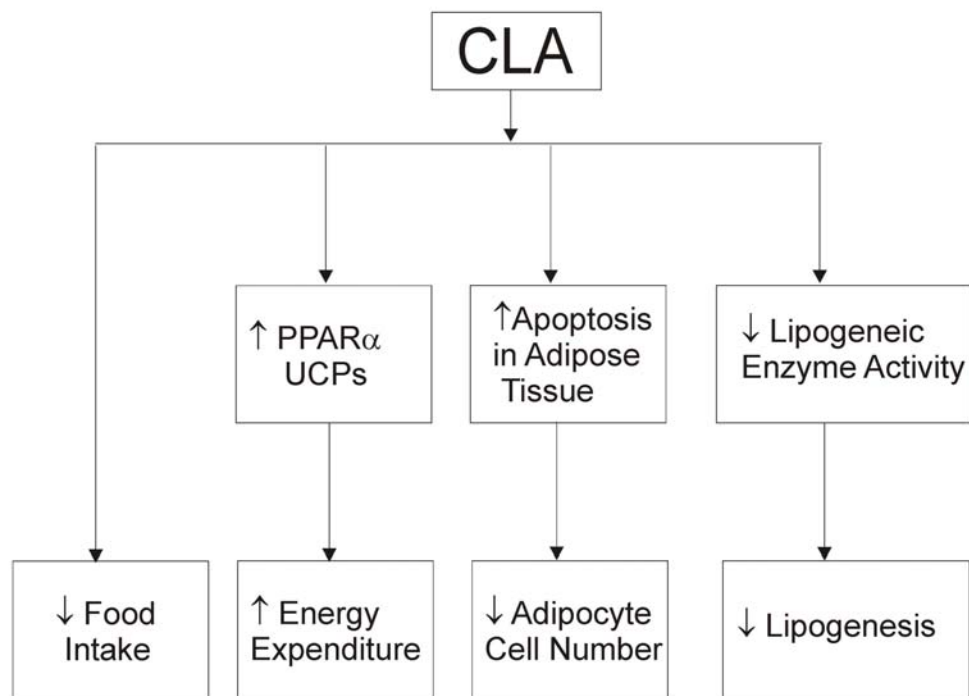
Figure 1. 2. Multiple mechanisms by which CLA reduces obesity in small animals.

Potential Metabolic Complications Associated with CLA Supplementation

Even though *trans*-10, *cis*-12 CLA is effective in decreasing adiposity, most dietary supplements for human consumption contain a 1:1 ratio of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. Apart from the obvious health benefits observed from the animal studies using the mixed isomers of CLA, human studies indicate that some of these beneficial effects are considerably less evident, and are conflicting compared to animal studies (Blankson et al. 2000; Gaullier et al. 2005; Desroches et al.2005). Recently, several researchers have raised concerns about the potential safety of CLA. The concerns include the induction of fatty liver in animals (Clement et al. 2002), insulin resistance (reviewed in Brown and McIntosh 2003) and lipodystrophy (Riserus et al. 2004a, b), elevated markers of oxidative stress (Riserus et al. 2002), lipid peroxidation (Basu et al. 2000 a, b), enhanced C-reactive protein (Smedman et al. 2005), and impairment of endothelial function (Taylor et al. 2006). The possible deleterious effects of CLA intake appear to be due mainly to the *trans*-10, *cis*-12 isomer. Riserus et al. conducted (2001, 2002a,b, 2004a,b) a series of human clinical trials with each individual CLA isomer. Their data suggested that *trans*-10, *cis*-12 CLA enhanced risk profiles for people with the Metabolic Syndrome. These included unfavorable changes in serum lipid composition (*i.e.*, elevated VLDL coupled with reduced HDL), hyperinsulinemia, impaired insulin sensitivity, resulting in increased risk of cardiovascular disease (Riserus et al. 2001, 2002a,b, 2004a,b). Furthermore, CLA may cause inflammation based on increase levels of serum C-reactive proteins (CRP) (Riserus et al. 2002; Smedman et al, 2005), a biomarker of inflammation.

In contrast, studies conducted in a number of animal and *in vitro* models suggested that CLA has anti-inflammatory and immune-ameliorating effects (Yu et al. 2002). However, only a limited number of CLA studies have been performed in humans. In addition to this paradoxal observation from Riserus's group, Brown et al. (2004) reported that *trans*-10, *cis*-12 CLA induced proinflammatory cytokine secretions and insulin resistance in primary cultures of human adipocytes. Therefore, it is of particular interest to determine if CLA's TG lowering actions are mediated by cytokine secretion and low-grade inflammation which are known to cause insulin resistance (reviewed in Wellen and Hotamisligil 2005).

Insulin Resistance and Inflammation: Role of NFκB

Insulin resistance is a characteristic feature of most cases of type 2 diabetes and is the defining pathophysiological defect in Metabolic Syndrome. An important recent development is the emergence of the concept that obesity is characterized by chronic low-grade inflammation. The potential relationships between inflammation and insulin resistance have been promoted by Hotamisligil and Spiegelman (Hotamisligil et al. 1993, 1994, 2003). Their earlier studies focused on production of tumor necrosis factor-α (TNF-α) and its ability to suppress insulin signaling mediated by the insulin receptor (IR) and insulin receptor substrates (IRSs). While the degree to which TNF-α itself mediates insulin resistance in human is controversial, findings by Hotamisligil's group clearly defined the potential for insulin resistance to be caused by cross-talk between inflammatory (*i.e.*, TNF-α) and metabolic (*i.e.*, inactivation of IR or IRS) signalings.

Similarly, anti-inflammatory medications such as aspirin can reverse insulin resistance, suggesting that inflammation may be directly involved in pathogenesis (Moller 2000; Hundal et al. 2002). A series of recently published papers demonstrated a positive relationship between obesity and inflammation in adipose tissue, linking adipokine secretion to metabolic dysfunction (i.e., insulin resistance) in adipocytes (Wellen et al. 2003; Xu et al. 2003; Stuart et al. 2003). These articles highlight the importance of adipose tissue as an endocrine organ, linking energy homeostasis to inflammatory cytokine secretion.

One potential mediator of insulin resistance and inflammation is activation of the IKKβ/NFκB axis. NFκB is a proinflammatory master switch that controls the production of a host of inflammatory markers and mediators including TNF-α, IL-1β, IL-6, IL-8, CRP and plasminogen activator inhibitor 1 (PAI-1). NFκB is inhibited by inhibitory κB proteins (IκBs) under basal conditions, and remains in the cytoplasm. Upon activation by proinflammatory stimuli, the kinase complex referred to as IKK (IκB kinase) is activated and catalyzes the phosphorylation of IκB. This leads to IκB degradation, which liberates NFκB to translocate to the cell nucleus and stimulate the transcription of inflammatory mediators as shown in Fig 1.3. Several investigations have demonstrated that activation of NFκB plays a significant role in local and systemic insulin resistance (Itani et al. 2002; Rotter et al. 2003; Shinha et al. 2004; Dongsheng et al. 2005). Concerning CLA modulation of NFκB, Locher et al. (2006) reported *cis*-9, *trans*-11 CLA may possess immuno-suppressive properties in dendritic cells by delaying LPS-induced NFκB

activation. Similarly, Chen et al. (2004) suggested CLA suppresses LPS induced-NFκB activation in RAW264.7 macrophages. However, no published studies have linked CLA-induced insulin resistance to NFκB activation in adipocytes. Our preliminary findings that *trans*-10, *cis*-12 CLA induces massive secretion of IL-6 and IL-8 prior to downregulation of PPARγ and suppression of glucose uptake in primary cultures of human of adipocyte (Brown et al. 2004), suggests that CLA decreases TG deposition via NFκB activation in adipocytes. Thus, the focus of this dissertation research is to examine the relationship between inflammation induced by CLA and LPS, and insulin resistance in primary cultures of newly differentiated human adipocytes.
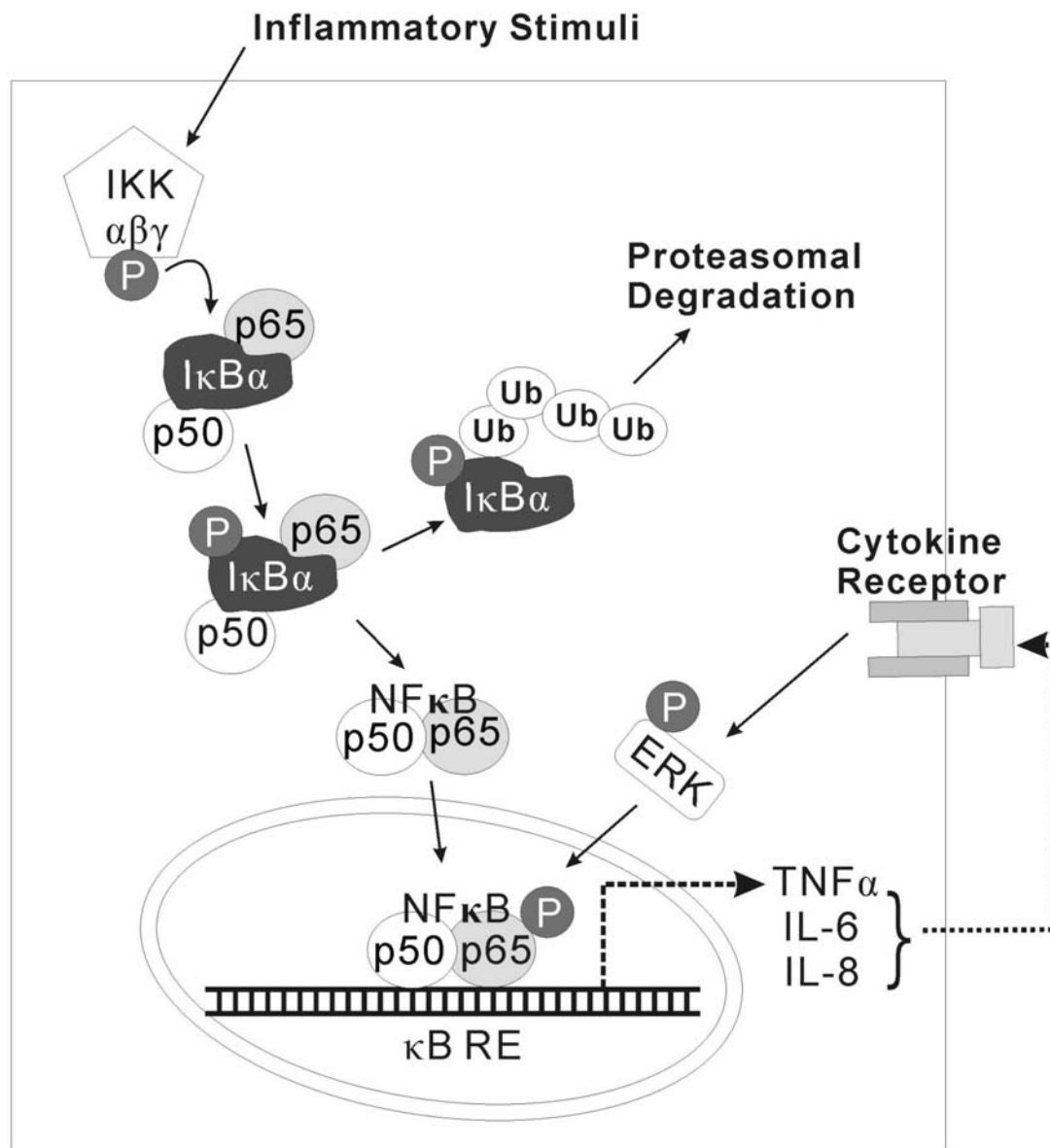
Figure 1. 3. Signaling pathways of NFκB activation by external inflammatory stimuli

Central Hypothesis and Specific Objectives

The central hypothesis for this dissertation research is that *trans*-10, *cis*-12 CLA promotes inflammation which antagonizes PPARγ and its downstream targets, leading to insulin resistance and delipidation in primary cultures of human adipocyte.

To test this hypothesis, the following three specific aims were investigated using primary cultures of newly differentiated human adipocytes as a cell model (Fig 1.4).

1. Determine the extent to which CLA alters lipolysis, and the expression and localization of lipid droplet coating proteins, key regulators of cellular TG content in adipocytes (Chapter II).

2. Determine the extent to which NFκB signaling plays an essential role in *trans*-10, *cis*-12 CLA induction of inflammatory cytokine expression and insulin resistance in primary cultures of newly differentiated human adipocytes (Chapter III).

3. Determine the role preadipocytes play in mediating LPS-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes (Chapter IV).
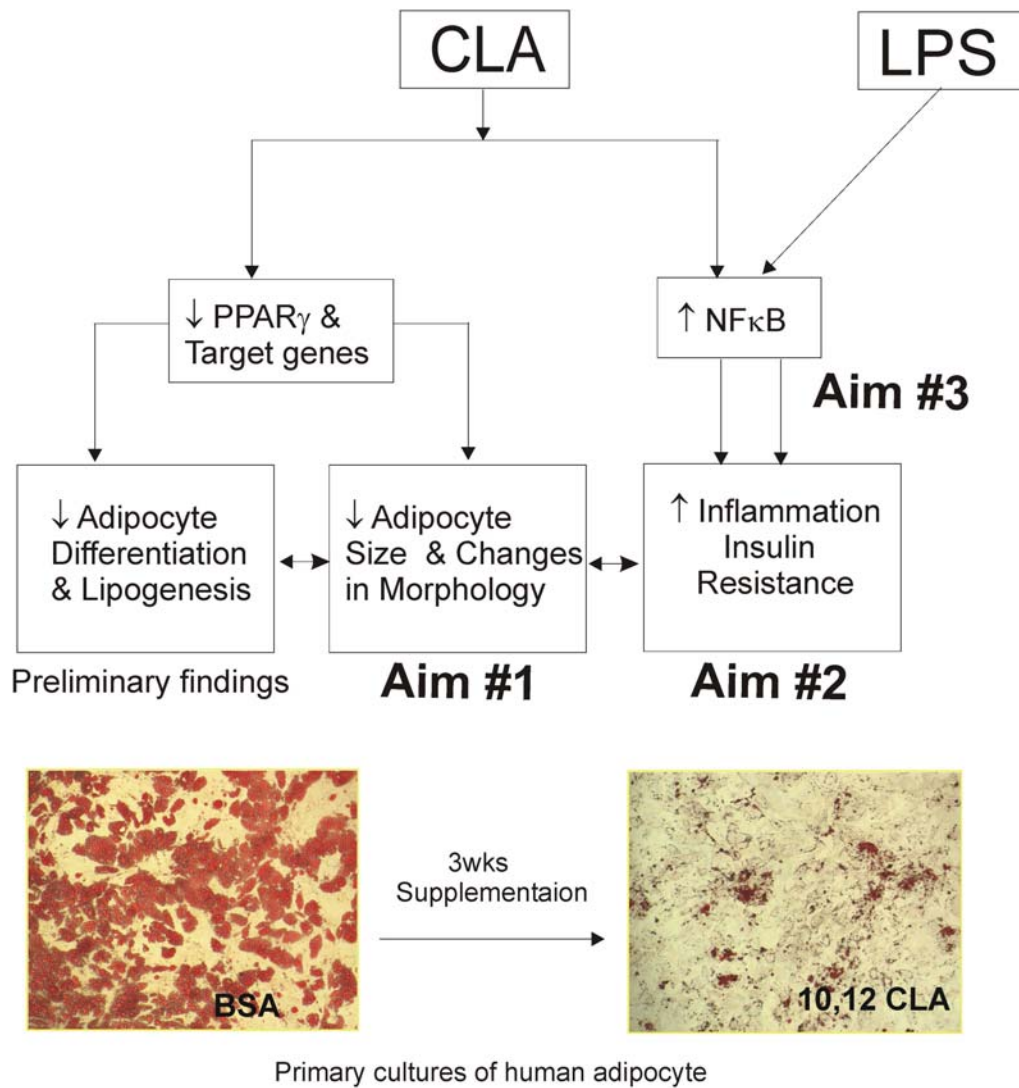
Figure 1.4. **The central hypothesis and aims of this dissertation research.** The central hypothesis is that *trans*-10, *cis*-12 CLA promotes inflammation through which CLA antagonizes PPARγ and the downstream targets, leading to insulin resistance and delipidation in primary cultures of human adipocyte. To test this hypothesis, in Aim #1 I focused on CLA induced morphological changes of adipocytes to provide cellular/molecular mechanisms of delipidation. In Aim #2, I focused on CLA-mediated NFκB activation and its metabolic consequences. Lastly, in Aim #3, I simulated inflammation using LPS to validate the role of preadipocytes in mediating inflammation that promotes insulin resistance in adipocytes.

CHAPTER II

TRANS-10, CIS-12 CLA INCREASES ADIPOCYTE LIPOLYSIS AND ALTERS LIPID DROPLET-ASSOCIATED PROTEINS: ROLE OF mTOR AND ERK SIGNALING

Abstract

Lipid droplet-associated proteins play an important role in adipocyte triglyceride (TG) metabolism. Here, we show that *trans*-10, *cis*-12 conjugated linoleic acid (CLA), but not *cis*-9, *trans*-11 CLA, increased lipolysis and altered human adipocyte lipid droplet morphology. Prior to this change in morphology, there was a rapid *trans*-10, *cis*-12 CLA-induced increase in the accumulation of perilipin A in the cytosol, followed by the disappearance of perilipin A protein. In contrast, protein levels of adipose differentiation-related protein (ADRP) were elevated in cultures treated with *trans*-10, *cis*-12 CLA. Immunostaining revealed that ADRP localized to the surface of small lipid droplets, displacing perilipin. Intriguingly, *trans*-10, *cis*-12 CLA increased ADRP protein expression to a much greater extent than ADRP mRNA without affecting stability, suggesting translational control of ADRP. To this end, we found that *trans*-10, *cis*-12 CLA increased activation of the mammalian target of rapamycin/p70S6 kinase/S6 ribosomal (mTOR/p70S6K/S6) pathway. Collectively, these data demonstrate that the

trans-10, *cis*-12 CLA-mediated reduction of human adipocyte TG content is associated with differential localization and expression of lipid droplet-associated proteins. This process involves both the translational control of ADRP through activation of mTOR/p70S6K/S6 signaling and transcriptional control of perilipin A.

Introduction

Conjugated linoleic acid (CLA) refers to a group of dienoic derivatives of linoleic acid. The two primary isomers of CLA found in ruminant meats and milk products and commercial preparations are *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. CLA isomers have potential anticancer (Ha et al. 1997; Belury 2002) and antiobesity properties (reviewed in Evans et al. 2002; Brown et al. 2003). Concerning the isomer specificity of CLA and obesity, numerous animal studies have demonstrated that *trans*-10, *cis*-12 CLA prevents the development of adiposity (Park et al. 1997, 1999a, b; Delany et al. 1999, Ostrowska 1999). Similarly, we have shown that *trans*-10, *cis*-12 CLA, but not *cis*-9, *trans*-11 CLA, inhibits differentiation of human preadipocytes into adipocytes and reduces the triglyceride (TG) content of mature or newly differentiated human adipocytes (Brown et al. 2001, 2003, 2004). However, the molecular mechanism(s) and physiological consequences of CLA supplementation are unclear, especially in humans.

Recently, we reported that *trans*-10, *cis*-12 CLA treatment of cultures of human stromal vascular (SV) cells containing newly differentiated adipocytes caused delipidation by activating mitogen-activated protein kinase kinase/extracellular signal related kinase (MEK/ERK) signaling (Brown et al. 2004). This relatively rapid activation

19

of MEK/ERK signaling by *trans*-10, *cis*-12 CLA was followed by a decrease in the mRNA levels of peroxisome proliferator-activated receptor gamma (PPARγ) and many of its downstream adipogenic target genes, including perilipin, glucose transporter 4 (GLUT4), adipocyte-specific fatty acid binding protein (aP2), lipoprotein lipase (LPL), and adiponectin. These CLA-mediated alterations were accompanied by decreased glucose and fatty acid uptake, leading to decreased cellular TG content (e.g., delipidation) of the cultures, and increased the number of cells containing small lipid droplets.

Given the important role of lipid droplet-associated proteins such as perilipin and adipose differentiation-related protein (ADRP) in facilitating lipid deposition or hydrolysis of fatty acids from lipid droplets, we hypothesized that these proteins were intimately involved in CLA's delipidation of adipocytes. The perilipins, exclusively found at the outer surface of lipid storage droplets in adipocytes and steroidogenic cells, provide a protective protein coat on the lipid droplet surface that shields stored TG against the basal (i.e., non-hormonally stimulated) lipolytic actions of cellular lipases, and serves as a cofactor for catecholamine-induce lipolysis (reviewed in Londos et al. 1999). Increased expression of perilipin increases TG storage (Brasaemle et al. 2000a; Souza et al. 1998), whereas tumor necrosis factor-alpha (TNF-α) stimulates lipolysis partly by terminating perilipin gene expression, leading to decreased TG storage (Souza et al. 1998). Perilipin ablation reduces fat mass, increases basal lipolysis, and alters lipid droplet morphology, including reducing adipocyte size (Tansey et al. 2001; Martinez-Botas et al. 2000). Protein kinase A (PKA)-mediated phosphorylation of hormone sensitive lipase (HSL) and perilipin promotes HSL movement to the lipid droplet and

perilipin movement away from the lipid droplet, thereby promoting lipolysis by providing HSL access to TG stores otherwise protected by unphosphorylated perilipin (Clifford et al. 2000; Sztalryd et al. 2003). In contrast to perilipin, ADRP is found on the surface of small lipid droplets during early adipocyte differentiation, but not on large lipid droplets in mature adipocytes (Brasaemle et al. 1997). ADRP is thought to play an important role in fatty acid flux in differentiating preadipocytes and many other cell types (Heid et al. 1998) by increasing fatty acid uptake kinetics (Gao et al. 1999) and concentrating unesterified fatty acids to the lipid droplet surface (Serrero et al. 2000).

Based on our data demonstrating that *trans*-10, *cis*-12 CLA causes delipidation and alters lipid droplet morphology (Brown et al. 2004), and the dynamic role perilipin and ADRP play in lipid metabolism, we examined the extent to which CLA altered perilipin and ADRP gene and protein expression and localization in primary cultures of SV cells containing newly differentiated adipocytes. Here, we demonstrate for the first time that *trans*-10, *cis*-12 CLA-mediated changes in lipid droplet morphology are associated with increased lipolysis and displacement of perilipin with ADRP on the surface of lipid droplets in human adipocytes. In contrast to the previously described MEK/ERK signaling-mediated reduction of perilipin A mRNA level (Brown et al. 2004), *trans*-10, *cis*-12 CLA-mediated induction of ADRP protein expression is mediated by a marked activation of the translational control mammalian target of rapamycin (mTOR) pathway.

Materials and Methods

Materials

     All cell culture ware and scintillation cocktail (ScintiSafe) were purchased from Fisher Scientific (Norcross, GA). [1-^{14}C] oleic acid and Western Lighting Plus Chemiluminescence Substrate were purchased from PerkinElmer Life Science (Boston, MA). Gene specific primers for real time quantitative (q) PCR and NUPAGE precast gels and buffers for SDS-PAGE were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Cambrex/BioWhittaker (Walkersville, MD). Isomers of CLA (+ 98% pure) were purchased from Matreya (Pleasant Gap, PA). ADRP monoclonal antibody was purchased from Research Dignostics Inc (Flander, NJ). Perilipin and HSL antibodies were generous gifts from Dr. C. Londos and Dr. F. Kraemer, respectively. Antibodies of caveolin-1 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rodamine red and fluorescein isothiocyanate-conjugated IgG were purchased from Jackson Immunoresearch (West Grove, GA). Rapamycin, pertussis toxin (PTX), Calphostin C, and protein phosphatase 2 (PP2), a c-SRC kinase inhibitor, were purchased from Calbiochem (La Jolla, CA). Total and phospho-specific antibodies used to measure translational control and U0126 and LY-294002 were obtained from Cell Signaling Technology (Beverly, MA). All the other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Cell Cultures of Human SV Cultures Containing Newly Differentiated Adipocytes

Abdominal adipose tissue was obtained from females between 20-50 years of age with a body mass index (BMI) $\leq$ 30 during liposuction or elective surgery with consent from the Institutional Review Board at the University of North Carolina-Greensboro. SV cells were isolated and cultured as defined previously (Brown et al. 2001, 2003) or purchased from Zen Bio, Inc (RTP, NC). Under these isolation and culturing conditions, ~50-70 % of the cells differentiated into adipocytes. Experimental treatment of cultures of SV cells containing newly differentiated adipocytes began on day 12-15 of differentiation.

Preparation of Fatty Acid

Both isomers of CLA were complexed to fatty acid (FA)-free (>98%) bovine serum albumin (BSA) at a 4:1 molar ratio using 1 mM BSA stocks as we described previously (Brown et al. 2003).

Lipolysis ($[^{14}C]$-Oleic Acid Release)

Cultures were seeded at 4×10^4 cells/cm^2 in 48 well cell culture plates and allowed to differentiation for 12 days as described in the cell culture protocol. The lipolysis experiments were conducted based on Guan et al. (Guan et al. 2002) with minor modifications. Before preloading the cultures with $[^{14}C]$-oleic acid, cultures were serum-starved in DMEM-F12 Ham for 12 h. Then 20 ul Hanks Balanced Salt Solution (HBSS) containing 6.25 nmole of $[^{14}C]$-oleic acid (specific activity 50 mCi/mmol) was added to

the cultures for additional 12 h. Approximately 90% of [^{14}C]-oleic acid was sequestered by the cultures during this incubation. The medium was then removed, and the cultures were washed four times, resulting in a background radioactivity <1000 dpm. Each well was treated with 250 ul fresh DMEM containing either 30 μM *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, BSA vehicle, or 10 μM isoproterenol in the presence of 40 μM phloretin (a fatty acid-reuptake inhibitor). After 3 h of treatment, 200 ul of medium was collected from each well and delivered to the liquid scintillation counting vial to measure [^{14}C]-oleic acid release to the medium as described previously (Brown et al. 2004). The amount of cellular protein per well did not differ among treatments; thus, data are expressed on a per well basis.

Preparation of Cytosolic Fractionations and Immunoblotting

Cytosolic fractions were prepared according to Clifford et al. (2000). Cells were lysed in ice-cold 50 mM Tris-HCl buffer pH 7.4 containing 225 mM sucrose, 1 mM EDTA, 1 mM bensamidine, 1 ug/ml pepstatin, 1 ug/ml leupeptin, 1 ug/ml antipain and 50 mM NaF. Following lysis, cells remained on ice for 15 min to allow the floating fat cake to solidify. The lysate was then vortexed vigorously for 1 min and centrifuged at 13,000g at 4°C for 15 min. The cytosolic fraction was aspirated from below the solidified fat cake. Total cell lysates preparation, protein determination, and immunoblotting procedures were the same as we described previously (Brown et al. 2004).

Immunofluorescence Microscopy and Phase Contrast Images

Cells were cultured on coverslips for immunofluorescence microscopy and stained as described previously (Brown et al. 2004). For perilipin immunostaining, cells were fixed with 3.7% paraformaldehyde in phosphate buffered saline (PBS) for 20 min, then coverslips were blocked and quenched in PBS containing 0.1% saponin, 10 mM glycine and 1.25 mg/ml rabbit IgG for 1 h. Immunodetection was carried out by incubating cells with 1:400 dilution of goat-anti perilipin antiserum for 6 h followed by three washes with PBS. Coverslips were then incubated with 1:200 dilution of Rhodamine red conjugated rabbit anti-goat IgG for 1 h (Fig 2.1). For perilipin and ADRP double staining, coverslips were blocked again with PBS containing 1.25 mg/ml goat IgG and incubated with 1:50 dilution of a mouse-anti ADRP for 12 h followed by addition of a fluorescein isothiocyanate-conjugated secondary antibody (1:100 dilution of FITC-goat anti-mouse IgG). After adequate washing with PBS, fluorescent image were captured with a SPOT digital camera mounted on an Olympus BX60 fluorescence microscope. Phase contrast images were captured using a SPOT digital camera mounted on an Olympus IX60 microscope.

RNA Isolation and Real Time qPCR

Total RNA extraction. Total RNA was isolated from the cultures using Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH) according to the manufacturer's protocol. RNA was extracted with phenol / 1-bromo-3-chloropropane (BCP), and

precipitated with ethanol, dried, and resuspended in H_2O. Contaminating genomic DNA was removed by treatment with DNase (DNA-free; Ambion).

Real-time qPCR. First strand cDNA synthesis and real time quantitative PCR were carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as previously described (Brown et al. 2003). Primer sets for perilipin and TATA binding protein (TBP) have previously been described (Brown et al. 2004). Primer sets for HSL were (accession # NM_005357) sense (5'aagtgggcgcaagtccc), antisense (5'gcgcatcggctctgctat), and for ADRP were (accession # NM_001122) sense (5'gctgagcacattgagtcacgtac), antisense (5'ctgagtcaggttgcgggc).

Statistical Analysis

Lipolysis data are expressed as the mean ± S.E. representing 16 independent observations from four different human subjects. Data were analyzed using one-way analysis of variance (ANOVA), followed by each pair student's t-tests for multiple comparisons. Differences were 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 Acutely Increases Lipolysis

To determine the isomer-specific influence of CLA on lipolysis, [14C]-oleic acid was preloaded into SV cultures containing newly differentiated human adipocytes, allowing esterification of radio-labeled oleic acid into TG. The release of [14C]-oleic acid

to the medium was measured after 3 h treatment with either 30 μM *trans*-10, *cis*-12 CLA or *cis*-9, *trans*-11 CLA, or BSA vehicle in the presence and absence of 10 μM isoproterenol, a β-adrenergic receptor agonist. As shown in Fig 2.1A, basal (e.g., in the absence of isoproterenol) [^{14}C]-oleic acid release was ~70% higher in *trans*-10, *cis*-12 CLA-treated cultures compared to the BSA controls. In contrast, in the presence of isoproterenol, lipolysis was not significantly affected by either CLA isomer.

Trans-10, *cis*-12 CLA Acutely Increases Cytosolic Accumulation of Perilipin

Lipolysis is regulated by the activity and location of lipid lipases (i.e., HSL) and perilipin, with the activity and localization of these proteins controlled by phosphorylation via PKA (Clifford et al. 2000). During basal lipolysis, perilipin surrounds lipid droplets, serving as a functional barrier to lipase access to neutral lipid substrates (Brasaemle et al. 2000b). Agents that increase intracellular cAMP levels such as isoproterenol or forskolin promote perilipin movement from the surface of lipid droplets to the cytosol by PKA-mediated phosphorylation of HSL and perilipin, leading to increased lipolysis. To determine the extent to which *trans*-10, *cis*-12 CLA-induced lipolysis was due to perilipin movement from the lipid droplet to the cytosol, we examined perilipin protein levels and changes in localization in the cultures. As seen in Fig 2.1B, *trans*-10, *cis*-12 CLA increased the levels of perilipin in total cell extracts after 12 of treatment. A subsequent time course study demonstrated that perilipin appeared in the cytosolic fractions as early as 3 h and peaked at 12 h following treatment with *trans*-10, *cis*-12 CLA (Fig 2.1C). Cultures treated for 12 h with 30 μM *trans*-10, *cis*-12 CLA

or for 30 min with 10 μM isoproterenol had appreciable amounts of perilipin in the cytosolic fractions (Fig 2.1D). In contrast, perilipin was not detected in cytosolic fractions of cultures treated with either BSA or *cis*-9, *trans*-11 CLA.

Supporting our immunoblotting data, a considerable amount of perilipin was found in the cytosol of cultures treated for 12 h with either 30 μM *trans*-10, *cis*-12 CLA or 10 μM forskolin (Fig 2.1E). CLA supplementation did not induce HSL movement from cytosol to lipid droplets, as measured by cytosolic fractionation followed by immunoblotting or immunostaining (data not shown). Collectively, these data demonstrate that *trans*-10, *cis*-12 CLA acutely stimulates basal lipolysis in human adipocytes, in part by inducing perilipin movement to the cytosol, thereby exposing TG to lipid hydrolases. However, our data suggest that HSL may not be the primary hydrolase/lipase that promotes CLA-induced lipolysis.

Trans-10, *cis*-12 CLA Chronically Alters Lipid Droplet Morphology, and the Expression and Location of Lipid Droplet-Associated Proteins

To investigate the isomer-specific regulation of adipocyte morphology and lipid droplet-associated protein expression by CLA, cultures were treated with either 30 μM *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, or vehicle for 2-8 days, at which time changes in morphology, and the expression and localization of perilipin and ADRP were measured. As seen in Fig 2.2, cultures treated with BSA and *cis*-9, *trans*-11 (9, 11) CLA for 7 days contained few, but relatively large lipid droplets within each adipocyte. In contrast, adipocytes in cultures treated with *trans*-10, *cis*-12 (10, 12) CLA had more, but

smaller lipid droplets compared to cultures supplemented with either BSA or *cis*-9, *trans*-11 CLA. The morphology of cells treated with *trans*-10, *cis*-12 CLA resembled that of a multilocular differentiating preadipocyte compared to a more unilocular adipocyte. In support of these data, we previously demonstrated that treatment of the cultures for 7 days with *trans*-10, *cis*-12 CLA, but not *cis*-9, *trans*-11 CLA, significantly reduced the TG content of the cultures (Brown et al. 2004). Consistent with these observations, cultures treated with *trans*-10, *cis*-12 CLA for 4-8 days had much higher protein levels of ADRP and lower levels of perilipin in total cell extracts compared to cultures supplemented with BSA or *cis*-9, *trans*-11 CLA (Fig 2.3). Levels of HSL protein decreased as the duration of trans-10, cis-12 CLA treatment increased.

It is generally accepted that ADRP associates with smaller neutral lipid droplets abundant in preadipocytes, whereas perilipin locates on the surface of larger lipid droplets of mature adipocytes (Londos et al. 1999). To determine whether the newly formed small lipid droplets occurring in *trans*-10, *cis*-12 CLA-treated cultures are covered with ADRP protein, immunostaining was conducted by using an ADRP-targeting antibody. As seen in Fig 2.4A (100X magnification), almost all lipid droplets were surrounded by ADRP protein in cultures treated for 7 days with *trans*-10, *cis*-12 CLA, whereas BSA controls had only background staining for ADRP. Strikingly, *trans*-10, *cis*-12 CLA treatment resulted in the accumulation of hundreds of distinct cytosolic lipid droplets within a single cell (Fig 2.4A), whereas control cells had much fewer, but relatively larger cytoplasmic droplets. To determine isomer-specific effects of CLA on the subcellular localization of lipid droplet coating proteins, cultures were treated with

either 30 μM *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, or BSA vehicle for 7 days, and then immunostaining with perilipin and ADRP was carried out. As seen in Fig 2.4B (40X magnification), perilipin, but not ADRP, was abundantly expressed around the lipid droplets in the BSA and *cis*-9, *trans*-11 CLA-treated cultures. In contrast, ADRP, but not perilipin, was abundantly expressed around the lipid droplets in cultures treated with *trans*-10, *cis*-12 CLA (Fig 2.4B). Taken together, these data suggest that *trans*-10, *cis*-12 CLA alters lipid droplet morphology from the perilipin-coated, large lipid droplets normally found in mature adipocytes to ADRP-coated, small lipid droplets which bear resemblance to differentiating preadipocytes.

Trans-10, *cis*-12 CLA Differentially Alters the Expression of ADRP and Perilipin

We previously demonstrated in cultures of SV cells containing newly differentiated adipocytes that *trans*-10, *cis*-12 CLA rapidly decreases perilipin gene expression prior to suppressing mRNA levels for PPARγ (Brown et al. 2004), a master regulator of adipocyte-specific genes including perilipin (Dalen et al. 2004). This *trans*-10, *cis*-12 CLA-mediated reduction of perilipin gene expression can be attenuated by pretreatment with the MEK inhibitor U0126 (Brown et al. 2004), implicating MEK/ERK signaling in CLA's ability to control perilipin gene expression. To determine the degree to which the *trans*-10, *cis*-12 CLA-mediated changes in lipid droplet morphology were associated with changes in gene and protein expression for lipid droplet-associated proteins, we treated cultures for 3 days with either 30 μM *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, or BSA vehicle, and compared the expression patterns for ADRP, perilipin, and HSL. The

extent to which *trans*-10, *cis*-12 CLA reduced protein expression of perilipin and HSL (Fig 2.5A) was equivalent to its attenuation of gene expression (Fig 2.5B). In contrast, ADRP protein expression (Fig 2.5A) was markedly increased whereas ADRP mRNA levels (Fig 2.5B) were increased by only ~50% in cultures treated with *trans*-10, *cis*-12 CLA compared to cultures treated with *cis*-9, *trans*-11 CLA and BSA vehicle. Furthermore, neither ADRP mRNA nor protein stability appeared to be influenced by the *trans*-10, *cis*-12 CLA treatment for up to 24 h in the presence of 5 μg /ml actinomycin D or 10 μM cyclohexamide, respectively (data not shown). This differential effect of CLA on mRNA and protein levels of adipocyte specific genes was accompanied by a robust increase in phospho-ERK (Fig 2.5A), suggesting a role of MEK-ERK signaling. Collectively, these data suggest that the *trans*-10, *cis*-12 CLA-mediated increase of ADRP protein expression is primarily regulated by a specific mechanism that increases ADRP protein synthesis.

Trans-10, *cis*-12 CLA Activates the mTOR/p70S6K/S6 Pathway

We examined signaling pathways regulating translation given the relative greater increase in the levels of ADRP protein compared to ADRP mRNA in cultures treated with *trans*-10, *cis*-12 CLA (Figs 2.5A and 2.5B, respectively). Mammalian cells possess an important nutrient-sensing pathway that controls protein synthesis at the level of translation (reviewed in Tokunaga et al. 2004; Hay et al. 2004). A central player in this pathway is mTOR, which is activated by growth factors, amino acids, and mitogenic signals via a mechanism that is not yet fully understood. We examined the time-

dependent effects of CLA on the phosphorylation status of key proteins known to regulate mTOR-dependent translation. As shown in Fig 2.6A, cultures treated with *trans*-10, *cis*-12 exhibited transiently activated S6 ribosomal protein (S6), a downstream target of mTOR, between 3 and 12 h compared to BSA controls. Furthermore, immunostaining using antibodies targeting to phospho-S6 ribosomal protein (p-S6) demonstrated that both 20% FBS (positive control) and 30 μM *trans*-10, *cis*-12 CLA treatment for 30 min and 3 h, respectively, increased the phosphorylation of S6 ribosomal protein compared to BSA controls (Fig 2.6B).

To determine if this early activation of the mTOR pathway was isomer-specific, cultures were treated for 3 h with either 30 μM *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA. In addition, a 30 min treatment was performed with two known potent activators of mTOR signaling (i.e., insulin [100 nM] or TNF-α [100 ng/ml]). As seen in Fig 2.6C, phosphorylated Akt was found only in insulin-treated cultures, whereas the levels of phosphorylated mTOR, p70S6 kinase, and S6 ribosomal protein were markedly higher in cultures treated with *trans*-10, *cis*-12 CLA, insulin, and TNF-α than in cultures treated with *cis*-9, *trans*-11 CLA or BSA vehicle. Similarly, phosphorylation of Mnk1, an ERK-activated protein that phosphorylates 40S ribosomal protein independent of mTOR, as well as phosphorylation of elongation initiation factor 4E (eIF4E), a protein possessing RNA helicase activity, was also increased in cultures treated with *trans*-10, *cis*-12 CLA and TNF-α. In contrast, neither *cis*-9, *trans*-11 CLA, BSA vehicle, nor insulin increased Mnk1 or eIF-4E phosphorylation.

To determine critical signaling steps involved in *trans*-10, *cis*-12 CLA-induced mTOR signaling pathway, we used several inhibitors to block upstream regulators of p70S6 kinase and S6 ribosomal proteins (Fig 2.7). Activation of P70S6 kinase and S6 kinase2 can be specifically blocked by the immunosuppressant rapamycin, a bacterial macrolide, without affecting kinases involved in mitogenic responses, resulting in attenuation of translational activation of 5'-TOP mRNA (Terada et al. 1994). In support of our concept that *trans*-10, *cis*-12 CLA-induced translational activation occurs via mTOR, phosphorylation of p70 S6 kinase and S6 ribosomal proteins by 3 h treatment with *trans*-10, *cis*-12 CLA were blocked by pretreatment with rapamycin (Fig 2.7). In addition, most inhibitors including the MEK/ERK inhibitor U0126, the G protein coupled receptor (GPCR)-$G_{i/o}$ coupling inhibitor pertussis toxin (PTX), the phosphatidyl inositol 3 kinase (PI3K) inhibitor LY-294002, the protein kinase C (PKC) inhibitor calphostin C, and the cSRC kinase protein inhibitor PP2 blocked or attenuated CLA's induction of phosphorylation of p70 S6 kinase and S6 ribosomal protein.

Taken together, these data suggest that: 1) CLA-mediated activation of mTOR signaling pathway is isomer-specific, and 2) its regulation is controlled by multiple factors that could potentially impact on 40S ribosomal protein activation of 5'-TOP mRNA translation, including MEK/ERK, PKC, protein kinase B, and GPCR-Gi protein. These data implicate a potential role of the mTOR pathway as a signal integrator of CLA's TG-lowering actions, and a potential mechanism by which *trans*-10, *cis*-12 CLA increases ADRP protein levels.

Rapamycin Blocks CLA's Increase in ADRP Protein Expression

Based on the isomer-specific activation of S6 ribosomal protein by CLA, we hypothesized that translational induction of ADRP by *trans*-10, *cis*-12 CLA is associated with activation of mTOR pathway. To test this hypothesis, we treated cultures for 24 h with either 30 μM *trans*-10, *cis*-12 CLA or BSA in the presence and absence of the mTOR-specific inhibitor rapamycin. As hypothesized, CLA's induction of ADRP protein was blocked by the pretreatment with rapamycin (Fig 2.8). In contrast, neither perilipin nor caveolin-1 expression was affected by rapamycin. It was also notable that 24 h treatment with *trans*-10, *cis*-12 CLA modestly attenuated perilipin-A and perilipin-B gene expression even in the presence of rapamycin, suggesting that a rapamycin-sensitive pathway may not be necessary for a reduction of perilipin expression by *trans*-10, *cis*-12 CLA (Fig 2.8). In fact, our data support the notion that *trans*-10, *cis*-12 CLA-mediated reduction of perilipin expression is mediated by a more chronic activation of cytokine-induced MEK/ERK signaling (Fig 2.5A), which we have described previously (Brown et al. 2004). Collectively, these data suggest that the *trans*-10, *cis*-12 CLA-mediated increase in the levels of the small lipid droplet associated protein ADRP, is partly due to a rapamycin-sensitive increase in ADRP protein synthesis.
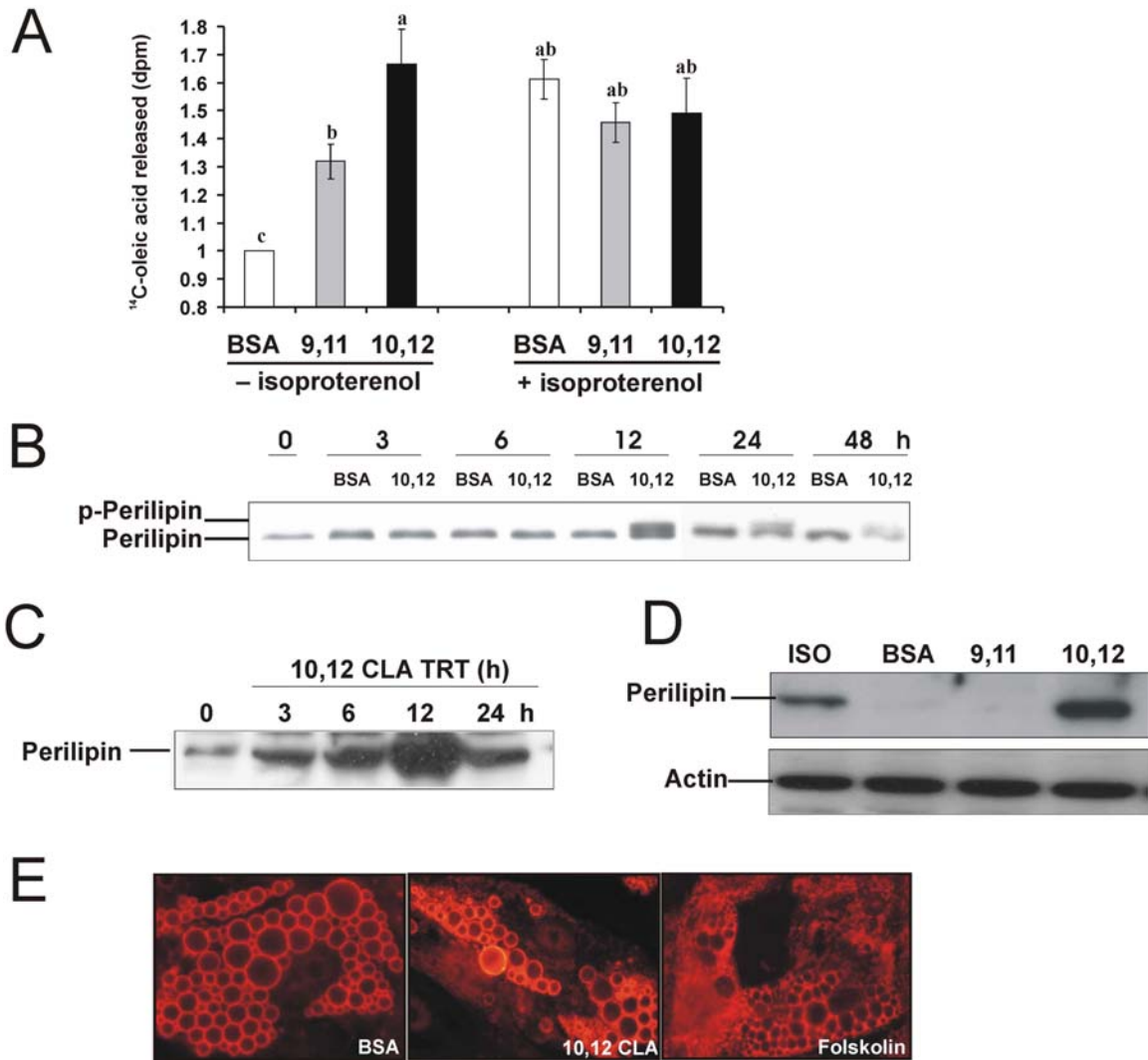
Figure 2. 1. ***Trans*-10, *cis*-12 CLA acutely increases basal lipolysis and perilipin accumulation in the cytosol.** A: Cultures of SV cells containing newly differentiated adipocyte were treated for 3 h with either a bovine serum albumin vehicle (BSA), 30 μM *cis*-9, *trans*-11 CLA (9,11) or 30 μM *trans*-10, *cis*-12 CLA (10,12) in the absence (-) or presence (+) of 10 μM isoproterenol. Lipolysis, expressed as the amount of [^{14}C]-oleic acid released into conditioned medium following treatment, was determined by scintillation counting. Data are expressed as a percentage of vehicle control (BSA, - isoproterenol) level. Means ($\pm$ SEM, n=16) not sharing a common superscript differ significantly (p<0.05). B: Cultures were treated for 0, 3, 6, 12, 24, or 48 h with either BSA (B) or 30 μM *trans*-10, *cis*-12 CLA (10) and total cell extracts were immunoblotted for perilipin. C: Cultures were treated for 0, 3, 6, 12, or 24 h with 30 μM *trans*-10, *cis*-12 CLA and cytosolic fractions were isolated and immunoblotted for perilipin. D: To determine isomer specificity of CLA, cultures were treated for 12 h with either BSA or 30 μM *trans*-10, *cis*-12 CLA or *cis*-9, *trans*-11 CLA and cytosolic fractions were isolated and immunoblotted for perilipin. A 30 min, 10 μM isoproterenol treatment was used as a positive control for perilipin movement to cytosol upon its phosphorylation. E**:** Cultures on grown on coverslips were pretreated for 72 h with either BSA or 30 μM *trans*-10, *cis*-12 CLA (10, 12) or for 12 h with 10 μM forskolin as a positive control. Cells were incubated with goat anti-perilipin antibody followed by Rhodamine-conjugated anti-goat IgG. Localization of perilipin was visualized by immunofluorescence microscopy. Data shown in all three panels are representative of three to four independent experiments.

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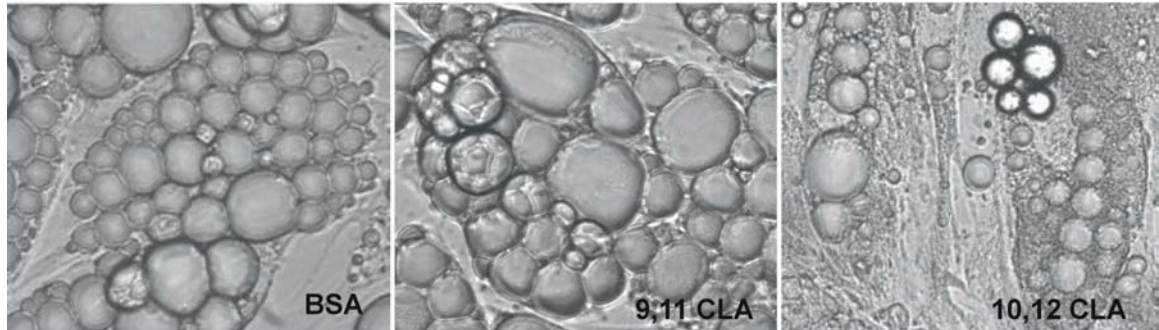
Figure 2. 2. *Trans*-10, *cis*-12 CLA alters lipid droplet morphology. Cultures of SV cells containing newly differentiated adipocytes were treated for 7 days with either BSA or 30 μM *trans*-10, *cis*-12 CLA (10,12) or *cis*-9, *trans*-11 CLA (9,11). Phase contrast images (100X) were taken to investigate isomer specific effects of CLA on changes in lipid droplet morphology. Data shown are representative of three independent experiments.
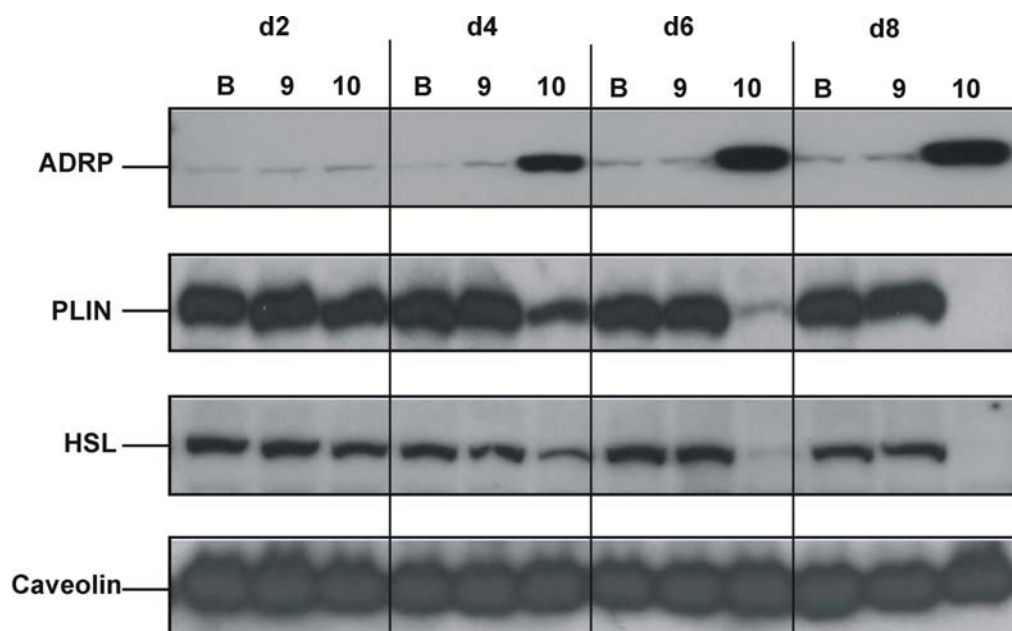
Figure 2. 3. T*rans*-10, *cis*-12 CLA changes protein expression of lipid droplet-associated proteins in a time-dependent manner. Cultures of SV cells containing newly differentiated adipocytes were treated for 2, 4, 6, or 8 days with either BSA (B) or 30 μM *trans*-10, *cis*-12 CLA (10,12) or *cis*-9, *trans*-11 CLA (9,11). Total cell extract extracts were immunoblotted for adipose differentiated-related protein (ADRP), perilipin, hormone sensitive lipase (HSL) and caveolin-1. Data shown are representative of three independent experiments.
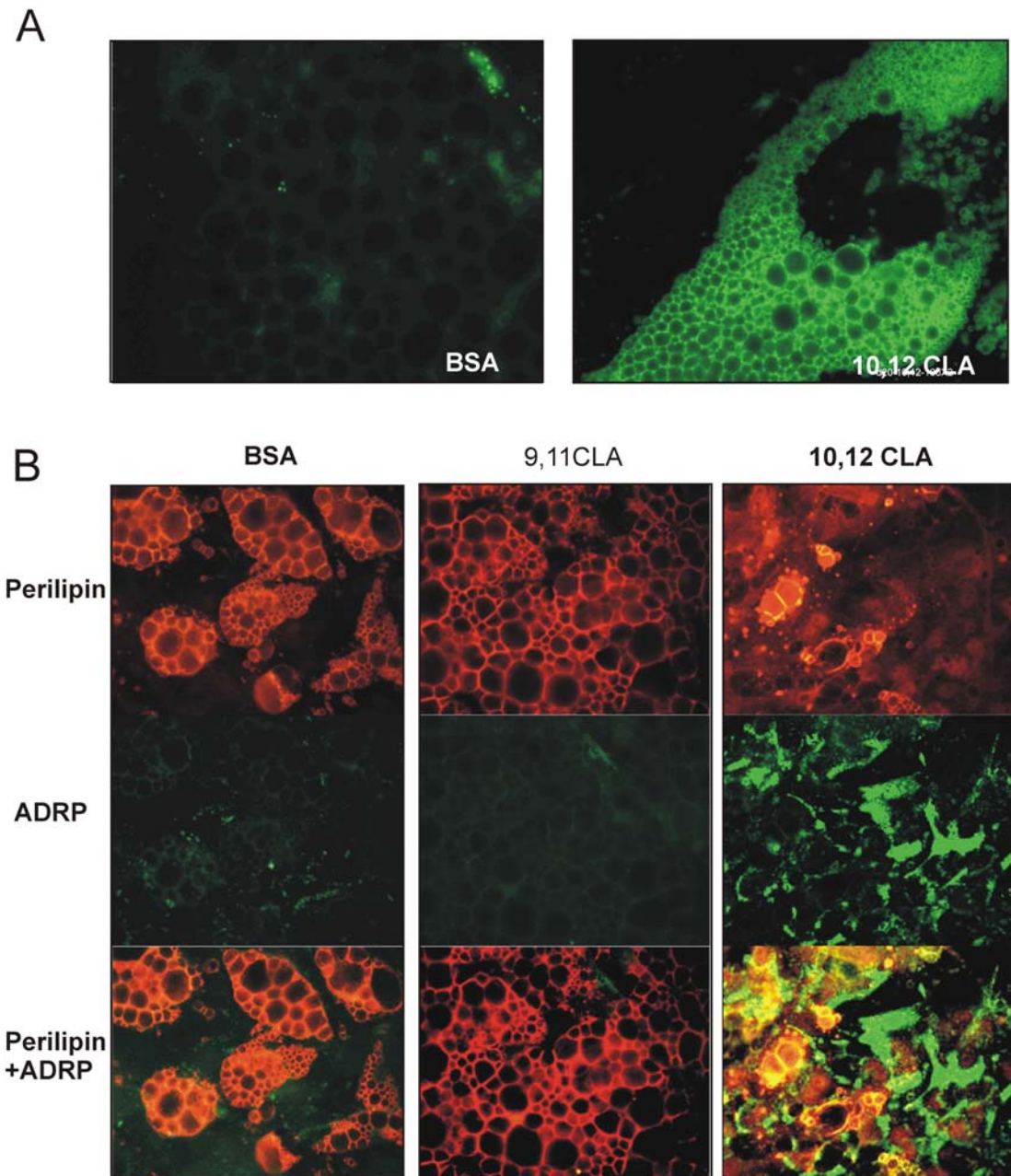
Figure 2. 4. ***Trans*-10, *cis*-12 CLA alters perilipin and ADRP localization.** Cultures of SV cells containing newly differentiated adipocytes grown on coverslips were treated for 7 days with either BSA (B) or 30 μM *trans*-10, *cis*-12 CLA (10,12) or *cis*-9, *trans*-11 CLA (9,11). A: An ADRP-immunofluorescence image was captured (100X magnification) in cultures treated with BSA vehicle or *trans*-10, *cis*-12 CLA (10,12). Results shown are representative for four separate experiments. B: Localization of perilipin and ADRP protein were detected by double-immunostaining with anti-perilipin antibody / rhodamine-conjugated anti-goat (red) and subsequent anti-ADRP antibody / FITC-conjugated anti-mouse (green). Fluorescent images were captured at the same spot in each column (40X magnification).

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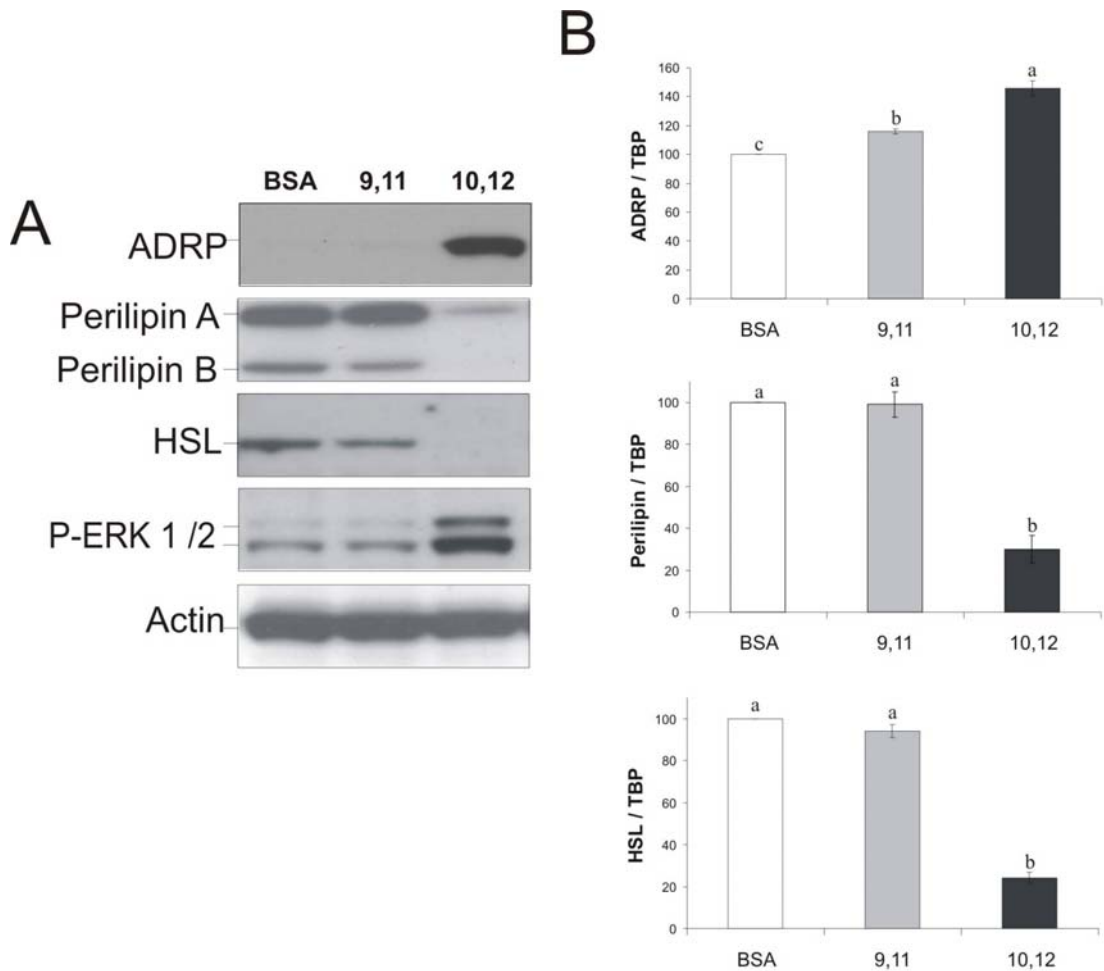
Figure 2. 5. *Trans*-**10,** *cis*-**12 CLA differentially affects gene and protein expression of lipid droplet-associated proteins.** Cultures of SV cells containing newly differentiated adipocytes were treated for 3 days with either BSA (B) or 30 μM *trans*-10, *cis*-12 CLA (10) or *cis*-9, *trans*-11 CLA (9). Cells were harvested on day 3 based on the data in Fig 2.3, indicating that between days 2 and 4, adipose differentiation-related protein (ADRP) protein increased, and perilipin and hormone sensitive lipase (HSL) proteins decreased. A: Total cell extracts were immunoblotted for adipose differentiated-related protein (ADRP), perilipin, and HSL. Actin was used as a loading control for the Western blots. Results shown are representative of three separate experiments using cells isolated from different human subjects each time. ERK phosphorylation was measured using a phospho-specific antibody for ERK. B: Total RNA was harvested using Tri-reagent and mRNA expression levels of ADRP, perilipin, and HSL were analyzed using real time quantitative (q) PCR. TATA binding protein (TBP) was used as a control for real time qPCR. Means ($\pm$ SEM, n=3 for ADRP, n=2 for perilipin and HSL) not sharing a common superscript differ significantly (p<0.05).
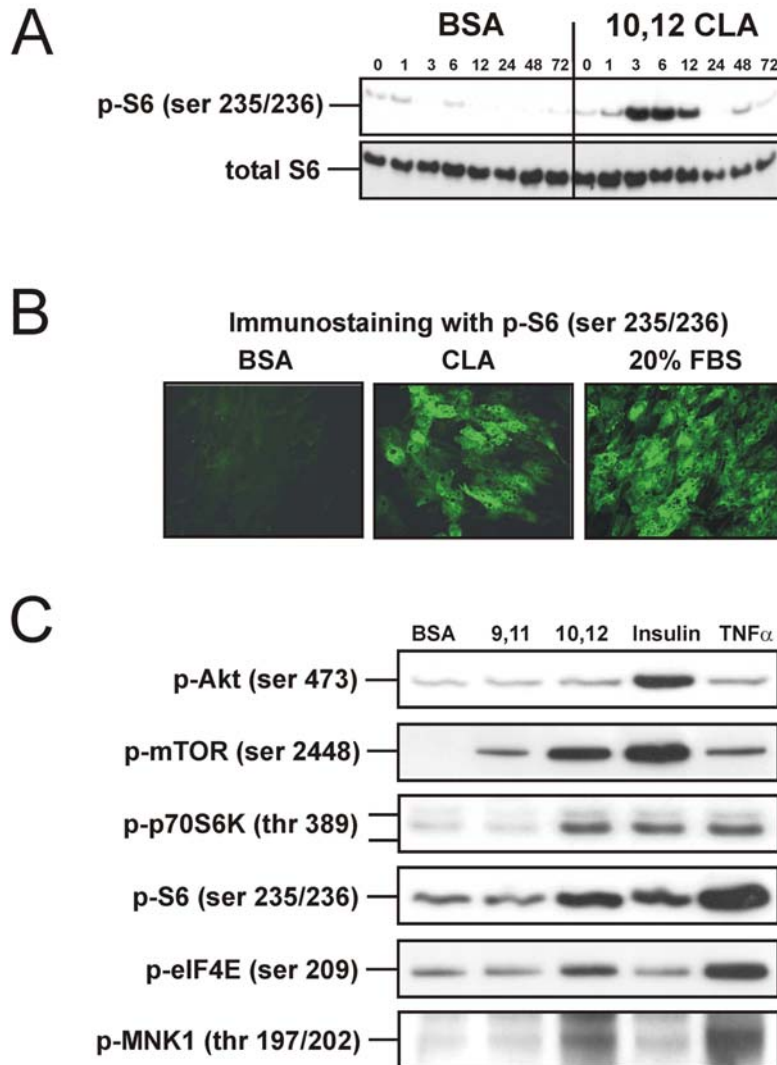
Figure 2. 6. *Trans*-10, *cis*-12 CLA induces mammalian target of rapamycin (mTOR) signaling. A: Cultures of SV cells containing newly differentiated adipocytes were treated for 0, 1, 3, 6, 12, 24, 48, or 72 h with either a bovine serum albumin vehicle (BSA) or 30 μM *trans*-10, *cis*-12 CLA (10,12 CLA). Total cell extracts were immunoblotted using phospho-specific or non-phosphospecific antibodies targeting the S6 ribosomal protein (S6). B: Cultures grown on coverslips were treated for 3 h with either a bovine serum albumin vehicle (BSA), or 30 μM *trans*-10, *cis*-12 CLA (10,12 CLA). A 30 min, 20% fetal bovine serum (FBS) treatment was used as a positive control. Following treatment, p-S6 was visualized by immunostaining using rabbit anti-pS6 ribosomal protein and FITC-conjugated anti rabbit IgG. C: Cultures were treated for 3 h with either a bovine serum albumin vehicle (BSA), 30 μM *cis*-9, *trans*-11 CLA (9,11), or 30 μM *trans*-10, *cis*-12 CLA (10,12). A 10 min human insulin (100 nM) treatment or 30 min TNF-α (100 ng/ml) treatment was used as a positive control. Total cell extracts were immunoblotted using phospho-specific antibodies targeting protein kinase B (Akt), mTOR, p70S6K, S6, eukaryotic initiation factor 4E (eIF4E), or MNK1. Results shown are representative of two independent experiments.

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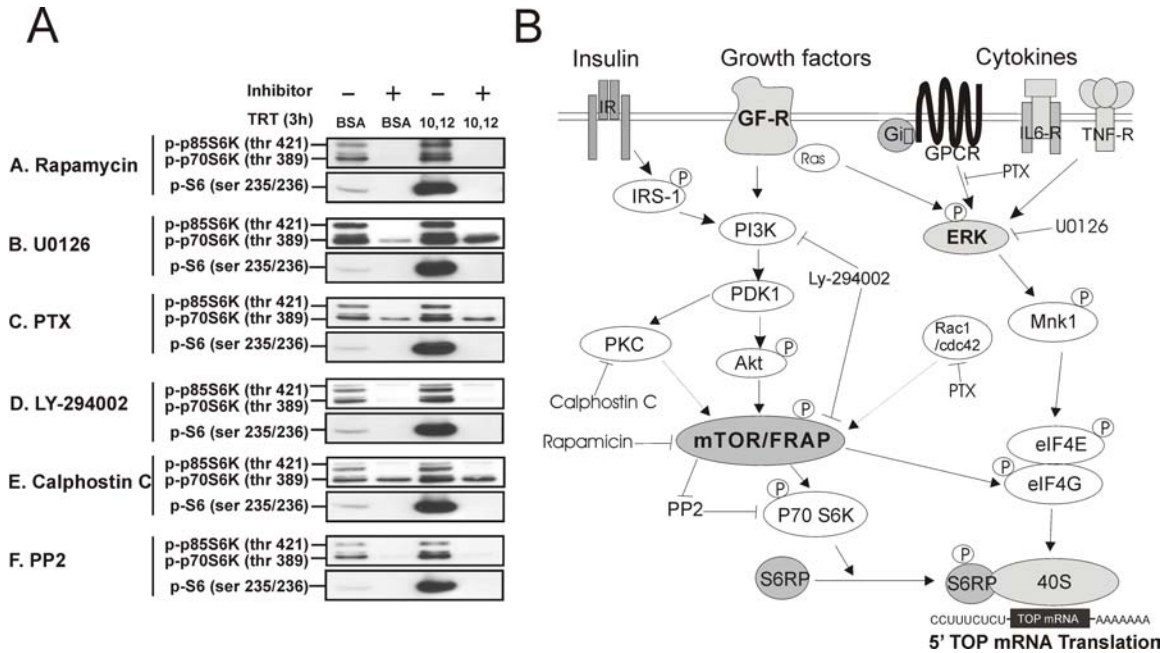
Figure 2. 7. Pharmacological inhibitors block or attenuate *trans*-10, *cis*-12 CLA activation of p70 S6 kinase (p70S6K) and S6 ribosomal protein (S6). A. Cultures of SV cells containing newly differentiated adipocyte were pretreated for 1 h with (+) or without (-) the following inhibitors, and then treated (TRT) for an additional 3 h with either a bovine serum albumin vehicle (BSA) or 30 μM *trans*-10, *cis*-12 CLA (10,12): 100 nM rapamycin (A), an inhibitor of mammalian target of rapamycin (mTOR); 10 μM U0126 (B), an inhibitor of mitogen-activated protein kinase kinase (MEK) phosphorylation; 100 ng/ml pertussis toxin (PTX) (C), an inhibitor of G-coupled protein receptor (GCPR) activation of Gi and Rac1/cdc42; 100 μM LY-294002 (D), an inhibitor of phosphatidyl inositol 3 kinase (PI3K) and mTOR/FRAP; 100 nM calphostin C (E), an inhibitor of protein kinase C (PKC); or 10 μM protein phosphatase 2 (PP2) (F), an inhibitor of SRC kinase and p70S6. Total cell extracts were then immunoblotted with phospho-specific antibodies targeting p70S6K, p85S6K, or S6. Results shown are representative of two independent experiments. **B**. Schematic diagrams of mTOR pathways.

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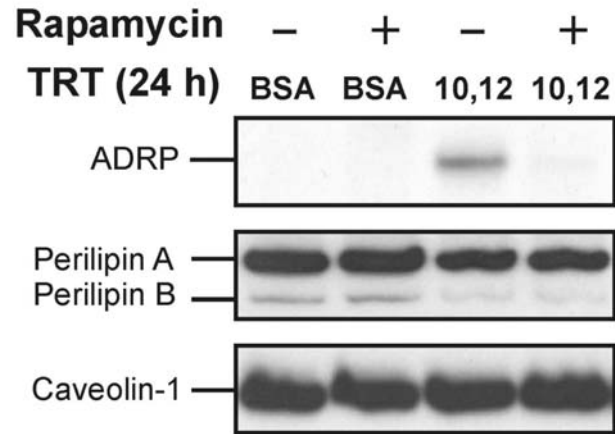
Figure 2. 8. ***Trans*-10, *cis*-12 CLA-induced ADRP protein levels are blocked by rapamycin.** Cultures of SV cells containing newly differentiated adipocytes were pretreated for 1 h with (+) or without (-) 100 nM rapamycin, and then treated (TRT) for an additional 24 h with either a bovine serum albumin vehicle (BSA) or 30 μM *trans*-10, *cis*-12 CLA (10,12). Total cell extracts were then immunoblotted with antibodies targeting adipocyte differentiation related protein (ADRP), perilipin A and B, and Caveolin-1. Results shown are representative of three independent experiments.

A

CLA induces changes in morphology of adipocytes

t10,c12 CLA

PLIN ● ADRP
HSL ○ TG

B

Perilipin ⟶ P P P Perilipin

ADRP

mTOR
translation

t10,c12 CLA

mRNA

transcription

HSL   Perilipin   ADRP

Nucleus
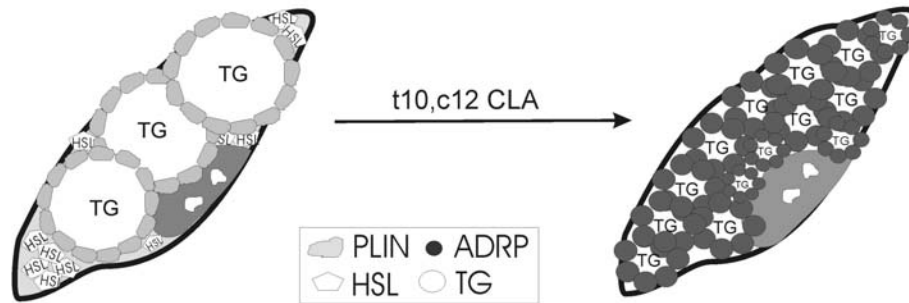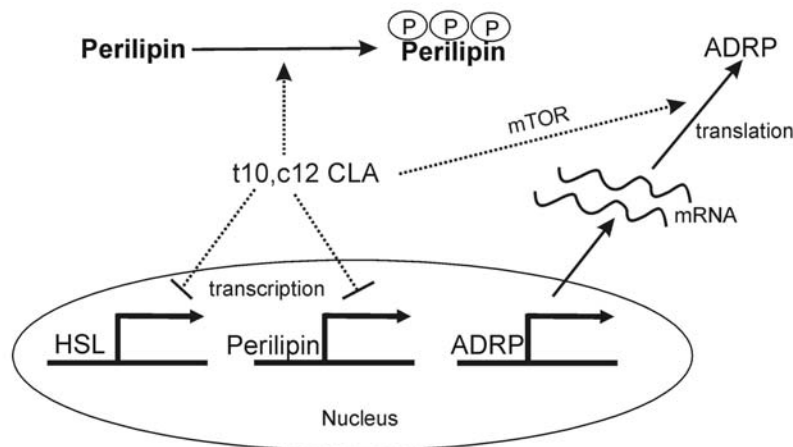
Figure 2. 9. Model of *trans*-10, *cis*-12 CLA-mediated changes of morphology in adipocytes: transcriptional, translational, and post-translational regulation of lipid droplet-associated proteins. Under normal, non-lipolyic conditions, perilipin is exclusively expressed in mature adipocytes, serving as a barrier to protect lipid droplets from hydrolysis by lipases such as hormone sensitive lipase (HSL). Chronic supplementation of *trans*-10, *cis*-12 CLA changes adipocyte morphology by promoting the development of numerous small lipid droplets coated with adipocyte differentiated related protein (ADRP). *Trans*-10, *cis*-12 CLA induces perilipin phosphorylation, which drives perilipin movement from the surface of lipid droplet to cytosol and thus increases the susceptibility to hydrolysis by lipase(s), resulting in increased basal lipolysis. As a physiological consequence of *trans*-10, *cis*-12 CLA supplementation, ADRP replaces perilipin as a lipid droplets coating protein due to CLA's combined action on transcriptional repression of perilipin and HSL, and translational activation of ADRP in a mammalian target of rapamycin (mTOR) pathway-dependent manner.

43

Discussion

Feeding mixed CLA isomers or trans-10, cis-12 CLA to humans decreases body fat (Blankson et al. 2000; Thom et al. 2001; Riserus et al. 2002), and supplementing cultures of human adipocytes with *trans*-10, *cis*-12 CLA reduces cell size and TG content (Brown et al. 2004). However, little is known about the mechanism by which CLA controls the flux of neutral lipids in and out of adipocyte lipid droplets. To our knowledge, the data presented here are the first to demonstrate that *trans*-10, *cis*-12 CLA alters adipocyte lipid droplet morphology from perilipin-associated large lipid droplets to ADRP-associated small lipid droplets. This process involves movement of perilipin to the cytosol, presumably by post translational regulation (e.g., perilipin phosphorylation), and differential regulation of lipid droplet-associated protein (ADRP, perilipin, and HSL) expression as shown in Fig 2.9. Furthermore, our data demonstrate for the first time that *trans*-10, *cis*-12 CLA induces the mTOR pathway, which could be responsible, in part, for the robust increase in the levels of ADRP protein. Alternatively, the CLA-mediated increase in perilipin movement away from large lipid droplets may provide greater access for ADRP binding on the lipid droplet surface, thereby increasing its stability. Taken together, we propose in our working model (Fig 2.9) that *trans*-10, *cis*-12 CLA increases basal lipolysis and reduces lipid droplet TG content by: 1) promoting perilipin dispersion to the cytosol, 2) downregulating perilipin gene expression, and 3) increasing the levels of ADRP protein bound to lipid droplets facilitated by migration of perilipin away from the large lipid droplets and/or by increased ADRP translation induced by the mTOR pathway.

We demonstrated that CLA acutely stimulates basal lipolysis in cultures of human adipocytes as previously shown in murine adipocytes (Evans et al. 2002; Park et al. 1997, 1999). However, it is not clear whether this CLA-induced lipolysis is mediated by HSL activation via classic cAMP and PKA signaling, because we did not observe an increase in isoproterenol-stimulated lipolysis in the CLA-treated cultures. Although we detected perilipin dispersion to cytosol within 3 hours of treatment with *trans*-10, *cis*-12 CLA, we failed to observe HSL phosphorylation or HSL translocation to lipid droplets in CLA-treated cultures. One possible explanation for these observations is that CLA may stimulate lipolysis via a HSL-independent mechanism. In support of this possibility, growing evidence suggests HSL is not the only lipase capable of TG hydrolysis in adipocytes (Okazaki et al. 2002; Soni et al. 2004). The fact that *trans*-10, *cis*-12 CLA stimulates basal, but not isoproterenol sensitive, lipolysis in our model (Fig 2.1A), supports the likelihood that *trans*-10, *cis*-12 CLA may increase basal lipolysis by a mechanism that is distinct from the classic cAMP driven, PKA-mediated phosphorylation of HSL and perilipin. However, this notion requires rigorous testing.

Alternatively, CLA may stimulate HSL activation without concomitant translocation to the lipid droplet. In support of this concept, it has recently been suggested that perilipin redistribution to the cytosol is not necessarily accompanied by HSL translocation to the lipid droplet surface (reviewed in Holm et al. 2003). It has also been shown in 3T3-L1 adipocytes that HSL can be phosphorylated via the ERK pathway (Greenberg et al. 2001). In agreement with these data, we previously reported that *trans*-10, *cis*-12 CLA induced ERK activation (Brown et al. 2004), supporting the idea that the CLA-mediated increase

in lipolysis may occur via an ERK-dependent activation of HSL or other candidate lipases such as adipose tissue triglyceride lipase (Zimmerman et al. 2004)/desnutrin (Villena et al. 2004).

Interestingly, *trans*-10, *cis*-12 CLA acutely (e.g., 3-12 h) induced perilipin accumulation in the cytosol (Fig 2.1C-2.1D), but chronically (e.g., > 24 h) decreased the levels of perilipin mRNA and protein (Figs 2.1B-2.1D, 2.3, and 2.5). Zhang et al. (2002) demonstrated in human adipocytes that TNF-α increased lipolysis and perilipin phosphorylation by an ERK-dependent pathway that increased PKA activity in a cAMP-dependent manner. In agreement with these data, we found that *trans*-10, *cis*-12 CLA increased ERK phosphorylation in an isomer-specific manner (Fig 2.5A). These data provide further support for the CLA-induced ERK activation as a possible cause for perilipin movement to the cytosol via phosphorylation. Furthermore, we found that treatment with IL-6 or IL-8, which increase MEK/ERK signaling (Brown et al. 2004), increased cytosolic perilipin (unpublished data), implicating CLA-induced adipokines may play an important role in TG efflux from lipid droplets. In contrast, the protein level of caveolin-1 (Fig 2.3), which plays a pivotal role in lipid droplet biogenesis and cholesterol metabolism (Cohen et al. 2004), was not affected by *trans*-10, *cis*-12 CLA.

ADRP, or its human analogue adipophilin, is a membrane-bound protein associated with lipid accumulation in diverse cell types (Heid et al. 1998; Imamura et al. 2002). ADRP is thought to act as a "shuttling protein" for lipids, particularly long chain fatty acids (Gao et al. 1999), from the plasma membrane to the lipid droplet. Long chain fatty acids induce ADRP gene transcription (Londos et al. 1999; Gao et al. 2000). ADRP is

46

highly upregulated during early stages of 3T3-L1 preadipocyte differentiation (Jiang et al. 1992), but is not expressed at appreciable levels in mature adipocytes (Brasaemle et al. 1997). In perilipin ablation studies, a compensatory ADRP coat is found on existing lipid droplets (Tansey et al. 2001).

In our cell model, CLA induced the appearance of small lipid droplets and ADRP expression both in the adipocyte portion and SV portion of the cultures based on immunostaining shown in Fig 2.4. Interestingly, Gatlin et al. (2002) reported the development of intramuscular microscopic lipid droplets in CLA-fed animals, which may have been due to increased ADRP expression. Given the fact that ADRP and perilipin share a competitive and exclusive relationship on the surface of lipid droplets, a CLA-mediated reduction in lipid droplet size and perilipin protein may increase ADRP binding to the lipid droplet surface, thereby increasing ADRP stability. However, cyclohexamide treatment did not appear to differentially affect ADRP abundance in cultures treated with *trans*-10, *cis*-12-12 CLA (data not shown), suggesting CLA does not increase ADRP stability.

Alternatively, *trans*-10, *cis*-12 CLA may increase ADRP translation by activating several protein kinase cascades, including the mTOR and ERK pathways that converge to increase translation efficiency. This hypothesis is based on: 1) data in Fig 2.5A and our previous data (Brown et al. 2004) demonstrating that *trans*-10, *cis*-12 CLA induces MEK/ERK signaling, 2) data in Fig 2.6 showing that *trans*-10, *cis*-12 CLA increases the phosphorylation of mTOR, p70S6, and S6, and 3) data in Fig 2.7 demonstrating that *trans*-10, *cis*-12 CLA's activation of p-p70S6K and p-S6 was inhibited or attenuated,

respectively, by the MEK inhibitor U0126 (Fig 2.7B) and other inhibitors of the mTOR pathway (Fig 2.7A, 2.7C-2.7F). Both mTOR and ERK can converge to activate p70S6K/S6 signaling which is known to regulate the translation of a specific type of mRNAs called 5' terminal oligopyrimidine (5'TOP) mRNAs (Hay et al. 2004). There are three important criteria for determining whether a transcript is under translational control of the mTOR pathway (Gingras et al. 2002). First, translation of 5'TOP mRNA is rapamycin sensitive (Fig 2.7A). Second, cis-acting elements in the 5' untranslated region (UTR) form extensive secondary structures prohibiting the access of translation machinery, which include an uninterrupted guanidine-cytosine (GC) rich region of 4-14 pyrimidines with a cytosine cap site or a long 5'UTR possessing multiple hairpins structures (Tuxworth et al. 2004). Thirdly, 5'TOP mRNAs are sequestered in translationally-inactive messenger ribonucleoprotein (mRNP) particles. Redistribution of 5'TOP mRNA from mRNP to large polysomes (40-60S) is obligatory for efficient translation upon growth stimuli including mitogens, hormones, growth factors, or branch chain amino acids such as leucine (Tokunaga et al. 2004).

Based on these three criteria, we speculate that ADRP might be a 5'TOP mRNA based on the following evidence. First, pretreatment of rapamycin efficiently inhibits CLA-induced ADRP protein expression (Fig 2.8). Second, even though human ADRP mRNA lacks the conventional structural 5'TOP moiety, it has 178 nucleotides in the 5' UTR region, which is rich in GC content (68%), and contains two independent oligopyrimidine stretches of six and eleven bases. This would potentially allow for at least 17 thermodynamically stable RNA secondary structures ($\Delta G \leq$ -70~-80 kcal/mol)

containing complicated hairpins, as we predicted using a computer simulation model (RNA structure, version 4.1; data not shown). Third, Brasaemle et al. (1997) suggested that ADRP mRNA may be recruited to polysomes for protein synthesis rather than to the endoplasmic reticulum, based on a discrepancy between mRNA and protein expression levels in differentiated 3T3L1 adipocytes. Fourth, leptin, a peptide that senses lipid status of adipocytes and is a 5'TOP mRNA (Roh et al. 2003), is also increased by *trans*-10, *cis*-12 CLA treatment (Brown et al. 2004). Based on these observations, we hypothesize that *trans*-10, *cis*-12 CLA increases ADRP protein levels by initiating translation of ADRP mRNA via activation of the mTOR/p70S6K/S6 signaling pathways that recruit ADRP mRNA into translational 40S ribosomal machinery.

In summary, these data demonstrate that acute treatment (within 3 h) of the cultures with *trans*-10, *cis*-12 CLA increases basal lipolysis and the appearance of perilipin in the cytosolic fraction. Chronic treatment ($\geq$ 48 h) of the cultures with *trans*-10, *cis*-12 CLA decreases perilipin and HSL gene and protein expression, and enhances ADRP protein expression and abundance on the lipid droplet. The robust increase in ADRP protein levels by *trans*-10, *cis*-12 CLA was accompanied by phosphorylation of ERK and acute activation of the mTOR pathway, including activation of p70S6 kinase and its downstream target S6 ribosomal protein, which was abolished by co-incubation of the CLA-treated cultures with specific inhibitors of the mTOR/p70S6K/S6 pathway. Collectively, these data suggest that *trans*-10, *cis*-12 CLA promotes adipocyte delipidation, in part, by promoting lipolysis, which may result from ERK-dependent alterations in perilipin and ADRP gene expression, protein activation, and localization.

49

Furthermore, the CLA-mediated increase in ADRP protein may be due to enhanced

ADRP translation induced by activation of mTOR/p70S6K/S6 signaling.

CHAPTER III

CONJUGATED LINOLEIC ACID PROMOTES HUMAN ADIPOCYTE INSULIN RESISTANCE THROUGH NFκB-DEPENDENT CYTOKINE PRODUCTION

Abstract

We previously demonstrated that *trans*-10, *cis*-12 conjugated linoleic acid (CLA) reduced the triglyceride (TG) content of human adipocytes by activating mitogen-activated protein kinase kinase/extracellular signal-related kinase (MEK/ERK) signaling via interleukins-6 (IL-6) and 8 (IL-8). However, the upstream mechanism is unknown. Here we show that CLA increased ($\geq$ 6 h) the secretion of IL-6 and IL-8 in cultures containing both differentiated adipocytes and stromal vascular (SV) cells, non-differentiated SV cells, and adipose tissue explants. CLA's isomer-specific induction of IL-6 and tumor necrosis factor-α (TNF-α) was associated with the activation of nuclear factor κB (NFκB) as evidenced by: 1) phosphorylation of IκBα, IκBα kinase (IKK), and NFκB p65; 2) IκBα degradation; and 3) nuclear translocation of NFκB. Pretreatment with selective NFκB inhibitors and the MEK/ERK inhibitor U0126 blocked CLA-mediated IL-6 gene expression. *Trans*-10, *cis*-12 CLA's suppression of insulin-stimulated glucose uptake at 24 h was associated with decreased total and plasma membrane glucose transporter 4 (Glut4)

proteins. Inhibition of NFκB activation or depletion of NFκB by RNA interference using siNFκB p65 attenuated CLA's suppression of Glut4 and peroxisome proliferator activated receptor gamma (PPARγ) proteins and glucose uptake. Collectively, these data demonstrate for the first time that *trans*-10, *cis-12* CLA promotes NFκB activation and subsequent induction of IL-6 which are, at least in part, responsible for *trans*-10, *cis*-12 CLA-mediated suppression of PPARγ target gene expression and insulin sensitivity in mature human adipocytes.

<u>Introduction</u>

Conjugated linoleic acid (CLA) is a collective term used to refer to positional and geometric isomers of linoleic acid (C18:2) with a conjugated double bond. The two predominant isomers of CLA, *cis*-9, *trans*-11 and *trans*-10, *cis*-12, are naturally found in dairy products and ruminant meats, with *cis*-9, *trans*-11 CLA being the most abundant isomer (e.g., 80% *cis*-9, *trans*-11 CLA, 10% *trans*-10, *cis*-12 CLA). CLA is also available commercially as a dietary supplement for weight loss, with both isomers reported to be present at in equal amounts (e.g., ~35% each). A great deal of attention has been centered on *trans*-10, *cis*-12 CLA due to its reported anti-obesity actions in animal models (reviewed in House et al. 2005) and some humans (reviewed in Larsen et al. 2003). We have reported that *trans*-10, *cis*-12 CLA, but not *cis*-9, *trans*-11 CLA, inhibited human preadipocyte differentiation (Brown et al. 2003) and caused delipidation of newly differentiated human adipocytes (Brown et al. 2004). CLA's isomer-specific delipidation of adipocytes was due largely to decreased glucose and fatty acid uptake and

TG synthesis as opposed to increased oxidation. Interestingly, CLA's suppression of glucose and fatty acid uptake was positively correlated with activation of mitogen-activated protein kinase kinase/extracellular signal-related kinase (MEK/ERK) and G protein coupled receptor (GPCR) signaling and robust secretion of the proinflammatory cytokines interleukin-6 (IL-6) and IL-8 after 24 h of treatment. However, the underlying mechanism(s) by which *trans*-10, *cis*-12 CLA triggers cytokine production and impairs glucose and fatty acid uptake is unclear.

There is growing evidence linking inflammatory cytokines with the development of obesity, insulin resistance (Wellen and Hotamisligil 2003; Xu et al 2003; Weisberg et al. 2003), and atherosclerosis (reviewed in Lau et al. 2005). Adipose tissue plays a central role in this relationship given its ability to both secrete cytokines and act as a substantial target for cytokines. A diverse array of cytokines such as tumor necrosis factor-α (TNF-α), IL-6, and IL-8 have been positively associated with obesity and the development of insulin resistance in muscle and adipose tissue.

IL-6 expression can be induced by many transcription factors such as nuclear factor κB (NFκB), NF-IL6 (a.k.a., C/EBPβ), activator protein-1 (AP-1), and cAMP response element (CRE)-binding protein (CREB) depending on cell type and stimulus (Akira et al. 1997; Vanden et al. 2000). Activation of NFκB is critical for fatty acid-induced IL-6 secretion and insulin resistance in myotubes *in vitro* (Sinha et al. 2004; Weigert et al. 2004) and muscle *in vivo* (Itani et al. 2002). Interestingly, several studies have shown that chronic exposure to IL-6 reduces adipogenic gene expression (Lagathu et al. 2003;

53

Sopasakis et al. 2004) and insulin signaling and glucose uptake in adipocytes (Rotter et al. 2003).

NFκB is a ubiquitous transcription factor regulating the expression of genes promoting inflammation and cell survival. NFκB-dependent transcription normally begins with the phosphorylation of the inhibitory κB proteins (IκBs) and their subsequent degradation. IκB proteins are key regulators of NFκB, sequestering the NFκB dimer in the cytoplasm. Phosphorylation of IκBα by IκBα kinase (IKK) triggers its polyubiquitination and proteosomal degradation, thereby releasing NFκB, especially p50/p65, to the nucleus (reviewed in Chen et al. 2004; Shoelson et al. 2003). Following phosphorylation by MAP kinases such as ERK1/2 and p38 or MSK1 (Reviewed in Dixit and Mak 2002; Jiang et al. 2001), phospho-p50/p65 binds to the NFκB response elements (NFκB RE) of target genes, thereby inducing their transcriptional activation (reviewed in Chen and Ghosh 1999; Camp et al. 1997).

CLA's TG-lowering actions in human adipocytes are consistent with NFκB activation based on our data (Brown et al. 2003, 2004) showing that *trans*-10, *cis*-12 CLA: 1) decreases adipogenic gene expression; 2) impairs glucose and fatty acid uptake and conversion to TG; and 3) increases the expression and secretion of several proinflammatory cytokines including IL-6 and IL-8. Therefore, the aim of the present study was to examine the extent to which NFκB activation was essential for *trans*-10, *cis*-12 CLA-induced cytokine expression and impaired glucose uptake in primary human adipocytes. Here we demonstrate for the first time that *trans*-10, *cis*-12 CLA promotes NFκB activation prior to inducing IL-6 expression and insulin resistance in cultures of

54

newly differentiated human adipocytes. The essential role of NFκB in mediating this effect of CLA was demonstrated by using either chemical inhibitors of NFκB or RNA interference (RNAi) targeting NFκB p65. Lastly, our data suggest that non-adipocytes or SV cells secrete a significant amount of cytokines in response to treatment with *trans*-10, *cis*-12 CLA.

Materials and Methods

Chemicals and Reagent

Isomers of CLA (>98% pure) were purchased from Matreya (Pleasant Gap, PA). Fetal bovine serum was purchased from Hyclone, Inc. (Logan, UT) and recombinant human TNF-α, and the ELISA kits for IL-6 and IL-8 were obtained from R & D Systems, Inc. (Minneapolis, MN). [^{3}H]-2-deoxy-glucose and Western Lightning Plus Chemiluminescence Substrate were purchased from Perkin Elmer Life Sciences (Boston, MA). NUPAGE precast gels, buffers for SDS-PAGE, and gene-specific primers of adipocyte fatty acid binding protein (aP2) and IL-6 for RT-PCR were purchased from Invitrogen (Carlsbad, CA). One-step RT-PCR kits were purchased from Qiagen Inc. (Valencia, CA) and ribosomal 18S competimer technology internal standards, and DNA-free, gene-specific TNF-α primers were purchased from Ambion (Austin, TX). Polyclonal insulin-dependent glucose transporter 4 (Glut4) antibody was a generous gift from Drs. S. Cushman and X. Chen (NIH-NIDDK, Bethesda, MD). aP2 antibody was a generous gift from Dr. D. Bernlohr (Univ. of Minnesota). Monoclonal antibodies for anti-lamin A/C (sc7293), PPARγ (sc7273), and NFκB p65 (sc8008), and polyclonal

antibodies for anti-glyceraldehydre-3-phosphate dehydrogenase (GAPDH) (sc20357), β-actin (sc1616), and caveolin-1 (sc894) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho (Ser 307)- and total-IRS-1, anti-phospho (Thr 202, 204)- and total-ERK, anti-phospho (Ser32)- and total-IκBα, anti-phospho (Tyr 707)- and total-signal transducer and activator of transcription 3 (STAT3), anti-phospho (Ser 536)-NFκB p65, anti-phospho (Ser 473)-Akt, anti-phospho (Ser176/180, Ser177/181)-IKK, and anti-IκBβ antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Polyclonal anti-phospho (Tyr891)-IRS-1 was purchased from Oncogene Science (Cambridge, MA). Monoclonal anti-nucleoporin was obtained from BD Transduction Laboratories (Franklin Lakes, NJ). Polyclonal anti-NFκB p50 and the Trans-AM DNA Binding ELISA NFκB kit were purchased from Active Motif (Carlsbad, CA). Cy3- and FITC-conjugated IgG were purchased from Jackson Immunoresearch (West Grove, PA). U0126, Proteasome inhibitor I (PSI), Bay11-7082, Pertussis toxin (PTX), Kamebakaurin (KA), and NEMO binding domain binding peptide (NEMO BP) were purchased from Calbiochem/EMD Biosciences Inc. (San Diego, CA). *Trans*IT-TKO was purchased from Mirus Corp. (Madison, WI). siRNA SMART pool of NFκB p65 (Rel A), non-specific control pool (mock), and siGLO-RISC- free were purchased from Dharmacon (Lafayette, CO). All the other chemicals and reagents were purchased from Sigma Chemical Co. unless otherwise stated.

Cell Culture of Primary Human SV Cell

Primary human stromal vascular (SV) cells were obtained from subcutaneous adipose tissue of non-obese (BMI < 30) females undergoing abdominoplasty with the consent from the Institutional Review Board at the University of North Carolina at Greensboro. Isolation, culture of SV cells from adipose tissue, and differentiation into adipocytes were performed as previously described (Brown et al. 2004) except for the condition that SV cells were pooled from 3~5 independent human subjects for most experiments. Experimental treatment of cultures containing adipocytes began on ~day 12 of differentiation unless otherwise indicated. For the preparation of cultures of non-differentiated SV cells, cells were seeded at ~80% confluent.

Fatty Acid Preparation

Both isomers of CLA were complexed to FA-free (>98%) bovine serum albumin (BSA) at a 4:1 molar ratio using 1 mM BSA stocks.

[^{3}H]-2-deoxy-glucose Uptake

For the 8 and 24 h treatments, newly-differentiated cultures of adipocytes were incubated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA in serum-free, low glucose DMEM [1,000 mg/liter D-(+)-glucose] in the presence or absence of 20 pM insulin. For the 72 h treatment, BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA were added to adipocyte medium for 48 h on day 12. Then, for an additional 24 h, cultures were incubated in 1 ml of serum-free basal DMEM containing 1,000 mg/liter D-(+)-glucose with or without 20

pM of human insulin in the presence of BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA, giving a total of 72 h of exposure to the treatments. Following the experimental treatments, [^{3}H]-2-deoxy-glucose uptake was measured as described previously (Brown et al. 2004).

PM Membrane Isolation and Glut4 Levels

For the detection of Glut4 translocation, plasma membrane (PM) was fractionated according to Carvalho et al. (2004). Briefly, cultures were washed once with TES buffer (20 mM Tris-HCl, 225 mM sucrose, 1 mM EDTA, pH 7.4 at 20°C) and then homogenized in ice-chilled TES using a 1 ml dounce homogenizer. The homogenate was centrifuged at 16,000 g for 20 min at 4°C and solidified surface fat was removed. The resulting pellets (crude membrane) were resuspended in TES and layered on a 1.12 M sucrose cushion in 20 mM Tris-HCl, 1 mM EDTA, and centrifuged at 100,000 g for 30 min. PMs at the interface were collected, resuspended in TES, and centrifuged at 100,000 g for 30 min. PM pellets were resuspended in TES and immunoblotted for Glut4. The abundance of Glut4 was quantified from exposed x-ray film using the KODAK image station 440 (Eastman Kodak, Rochester, NY).

IL-6 and IL-8 Secretion

Differentiated cultures of adipocytes and cultures of non-differentiated SV cells were serum starved for 20 h before fatty acid treatment. Fatty acid treatment was initiated by adding either BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA to the medium directly.

Conditioned medium from above the cell monolayer was collected at 0, 3, 6, 12, or 24 h, centrifuged at 12,000 g for 20 min to remove cell debris, and kept at -80°C before analysis. Cultures were washed twice with ice-chilled HBSS and cells harvested and dedicated for protein determination. IL-6 and IL-8 secretion to the medium was quantified using a commercial ELISA (R&D System) following the manufacturer's protocol and normalized to the protein content of the monolayer.

To measure IL-6 and IL-8 secretion from human subcutaneous adipose tissue explants, 500 mg pieces of adipose tissue obtained per subject were each minced into ~100 fragments as described by Fried et al. (Fried et al. 1993). Each minced explant was then preincubated in DMEM-Ham's F12 (1:1) medium containing 100 U/ml penicillin, 100 U/ml streptomycin, 50 ug/ml gentamicin, and 0.25 mg/ml amphotercin-B at 37°C overnight. Then, explants were transferred to 10 ml of fresh medium containing either BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA in culture tubes. Subsequently, conditioned medium was collected after 8, 24, or 72 h of incubation with treatments and stored at -80°C until analysis. IL-6 and IL-8 secretion were quantified by ELISA.

Relative RT-PCR

Total RNA was isolated from the cultures using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) following the manufacturer's protocol. Relative (semi-quantitative) RT-PCR was carried out using One-step RT-PCR kit (Qiagen Inc) as we described previously (Brown et al. 2003). Primer sets for adipose fatty acid binding protein (aP2) were previously described (Brown et al. 2003). Primer sequences for IL-6

were (accession #NM000600) sense (5'CCAGCTATGAACTCCTTCTC), antisense (5'GCTTGTTCCTCACATCTCTC), and running conditions for IL-6 were 26 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The running conditions for gene-specific primers for TNF-α (Ambion #5345) were 40 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min.

Immunofluorescence Microscopy and Phase Contrast Image

Cells were cultured on coverslips for immunofluorescence microscopy and stained as described previously (Brown et al. 2004; Chung et al. 2005). For phospho-IKK immunostaining, cells were permeablized with 0.1% saponin and then incubated with a 1:10 dilution of rabbit-anti-phospho-IKK overnight at 4°C. After three vigorous washes, coverslips were incubated with 1:200 dilutions of FITC-conjugated anti-rabbit IgG for 1 h (Fig 3.4C). For NFκB p65 and aP2 double staining (Fig 3.5), cells were initially incubated with a 1:10 dilution of NFκB p65 for 12 h at 4°C followed by incubation with a 1:500 dilution of Cy3-conjugated anti-mouse IgG. After adequate washing, coverslips were incubated with a 1:200 dilution of aP2 for 2 h at room temperature followed by incubation with a 1:400 dilution of FITC-conjugated anti-rabbit IgG for 1 h. Fluorescent images were captured with a SPOT digital camera mounted on an Olympus BX160 fluorescence microscope. Cy3-labelled siGLO fluorescent and phase contrast images were captured without fixation under the Olympus IX60 microscope equipped with a SPOT digital camera (Fig 3.8B). For PPARγ immunostaining (Fig 3.8E), transfected cells grown on coverslips were permeablized with 0.1% Triton X-100 on ice for 10 min

followed by incubating with a 1:10 dilution of mouse-anti-human PPARγ (sc7273) for 16 h at 4°C and a 1:200 dilution of FITC-conjugated anti-mouse IgG for 1 h. 1 ug/ml of Hoechst dye was used for nuclei staining

Nuclear and Cytoplasmic Separation and Assessment of NFκB p65 DNA Binding

Nuclear and cytosolic cellular fractions were prepared using a commercially available kit from Active Motif, with the following minor modifications. Cells were directly lifted in a 1X hypotonic buffer, gently scraped from the plate, and then treated according to the manufacturer's recommendations. *Trans*-10, *cis*-12 CLA-treated nuclear extract was used to assess CLA induced-NFκB p65 DNA binding by using the ELISA-based TransAM^TM NFκB family transcription factor assay kit (Active Motif) following the manufacturer's instructions.

Transfection with NFκB p65 siRNA

Transfection of newly-differentiated human adipocytes with NFκB p65 siRNA was conducted on day 6 of differentiation in 35 mm cell culture plates without detaching the cells. Cells were seeded and differentiated as previously described. On ~day 6 of differentiation, cultures containing fresh adipocyte medium (AM-1, Zen Bio Inc, RTP, NC) were supplemented with either 25 nM NFκB p65 siRNA or non-targeting siRNA complexed with *Trans*IT-TKO (6 ul/ml), a non-lipid based transfection reagent from Mirus Corp. Transfection reagent and undelivered siRNA were removed 24 h post-transfection by removing the medium and washing the cells twice with HBSS.

Transfection efficiency was examined by transfecting the cells with a fluorescent-tagged, RISC-free siRNA obtained from the company.

Statistical Analysis

Unless otherwise indicated, data are expressed as means ± S.E. Data were analyzed using one-way analysis of variance followed by student's *t* tests for each pair for multiple comparisons. Differences were considered significant if $p < 0.05$. All analyses were performed using JMP IN version 4.04 (SAS Institute; Cary, NC) software.

Results

Trans-10, *Cis*-12 CLA Reduces Glucose Uptake and the Abundance of Glut4 and IRS-1

We previously demonstrated that *trans*-10, *cis*-12 CLA decreased glucose uptake and Glut4 gene expression after 24 or 72 h of treatment (Brown et al. 2004). However, we did not examine the time course of CLA-induced insulin resistance and the extent to which dysregulation of insulin signaling and Glut4 expression played a role in impaired glucose uptake. Therefore, we measured [^{3}H]-2-deoxy-glucose uptake, phosphorylation of downstream targets of insulin, and Glut4 abundance in cultures of differentiated human adipocytes. As shown in Fig 3.1A, insulin-stimulated [^{3}H]-2-deoxy-glucose uptake was significantly lower in cultures treated with 30 μM *trans*-10, *cis*-12 CLA for either 24 or 72 h compared to vehicle (BSA) controls. However, *trans*-10, *cis*-12 CLA had minimal effects on insulin signaling as determined by basal- and insulin-stimulated phosphorylation of insulin receptor substrate-1 (IRS-1) at Ser-307 or Tyr-891 and on Ser-

473 of Akt/protein kinase B (Fig 3.1B). Interestingly, *trans*-10, *cis*-12 CLA modestly

decreased the abundance of total IRS-1 in the absence and presence of insulin as early as

24 h, but more robustly after 72 h (data not shown), which is similar to that reported for

IL-6 induced-insulin resistance in 3T3-L1 adipocytes (Rotter et al. 2003). The basal

levels of Glut4 were markedly lower in all three cellular fractions from cultures treated

with *trans*-10, *cis*-12 CLA (Fig 3.1C) compared to BSA controls, consistent with CLA-

induced reductions in Glut4 gene expression (Brown et al. 2004). More importantly, the

abundance of Glut4 in the PM fractions of cultures treated with both insulin and *trans*-10,

cis-12 CLA was lower than in cultures treated with insulin and vehicle. The degree to

which *trans*-10, *cis*-12 CLA decreased [^{3}H]-2-deoxy-glucose uptake after 24 h of

treatment (~30%, Fig. 1A) was relatively similar to the degree to which it attenuated

Glut4 abundance in both membrane fractions (~35%, Fig 3.1C). Taken together, these

data suggest that *trans*-10, *cis*-12 CLA decreases insulin-stimulated glucose uptake by

decreasing intracellular pools of key proteins involved in insulin-stimulated glucose

uptake (i.e., IRS-1, Glut4) rather than impairing insulin signaling *per se*.

<u>*Trans*-10, *Cis*-12 CLA Induces Cytokine Secretion and/or Expression in SV Cells,
Adipocytes, and Tissue Explants</u>

Recent reports, including our own data using human primary cultures (Brown et

al. 2004), suggest that adipocytes are molecular targets for IL-6, resulting in insulin

resistance (Rotter et al. 2003). Although we previously demonstrated that *trans*-10, *cis*-12

CLA robustly increased IL-6 and IL-8 protein secretion and gene expression after 24 h of

treatment (Brown et al. 2004), we did not know how early this occurred, which cells in the cultures were secreting these cytokines, or if CLA's induction of cytokine gene expression was isomer-specific. Therefore, we examined CLA-induced changes in cytokine secretion and gene expression over time. IL-6 and IL-8 secretion in cultures treated with 30 μM *trans*-10, *cis*-12 CLA increased in a time-dependent manner in differentiated cultures of adipocytes (Fig 3.2A), whereas IL-6 and IL-8 secretion in control cultures increased only marginally with time (Fig 3.2A). Accumulations of IL-6 and IL-8 in the medium of cultures treated with *trans*-10, *cis*-12 CLA were apparent after 6 h treatment, reaching a plateau after 24 h (Fig 3.2A). Interestingly, *trans*-10, *cis*-12 CLA induced IL-6 and IL-8 secretion in non-differentiated cultures of SV cells (Fig 3.2B) and adipose tissue explants (Fig 3.2C) as well. In fact, IL-6 and IL-8 secretion was 10- and 7-fold higher, respectively, in the non-differentiated cultures of SV cells treated with CLA for 24 h (Fig 3.2B) compared to differentiated cultures of adipocytes treated with CLA (Fig 3.2A). This supports our previous observation that SV cells are the predominant source of IL-6 and IL-8 secretion in our cultures treated with *trans*-10, *cis*-12 CLA (Brown et al. 2004).

To determine whether CLA-mediated IL-6 secretion was due to increased IL-6 gene expression, differentiated cultures of adipocytes were treated with either BSA vehicle or 30 μM *trans*-10, *cis*-12 for 1, 3, 6, 12 or 24 h. As seen in Fig 3.3A, *trans*-10, *cis*-12 induced IL-6 gene expression beginning at 3 h, which was consistent with the IL-6 protein secretion shown in Fig 3.2A. Intriguingly, *trans*-10, *cis*-12 CLA treatment induced transient TNF-α gene expression, which peaked at 3 h. However, TNF-α protein

secretion was not detectable (data not shown, < 0.5 pg/ml). The mRNA levels of IL-6 and IL-8 in adipose tissue explants treated with CLA were also higher compared to those receiving the BSA vehicle (data not shown).

To determine the extent to which CLA's induction of IL-6 and TNF-α gene expression was isomer-specific, differentiated cultures of adipocytes were treated with either BSA vehicle, 30 μM *cis*-9, *trans*-11 CLA, or 30 μM *trans*-10, *cis*-12 CLA for 3 h and IL-6 and TNF-α mRNA levels were measured. As a positive control, another set of cultures was treated with 100 ng/ml of human recombinant TNF-α for 30 min. As seen in Fig 3.3B, *trans*-10, *cis*-12 CLA, but not *cis*-9, *trans*-11 CLA or BSA, induced IL-6 and TNF-α gene expression in cultures of differentiated adipocytes (Fig 3.3B) as well as in cultures of non-differentiated SV cells (data not shown). Collectively, these data demonstrate for the first time that cells isolated from human adipose tissue secrete proinflammatory cytokines *ex vivo* when treated with *trans*-10, *cis*-12 CLA.

Trans-10, *Cis*-12 CLA Promotes NFκB Activation-

We hypothesized that *trans*-10, *cis*-12 CLA treatment would lead to NFκB activation in cultures of newly-differentiated adipocytes. This is supported by the following evidence:1) both IL-6 and TNF-α possess a nuclear factor κB response element (κBRE) (Chen et al. 1999); 2) transient TNF-α gene expression is positively linked to NFκB activation (Bouwmeester et al. 2004; Ruan et al. 2002); 3) ERK1/2 phosphorylation of nuclear p50/p65 is important for its activation (Jiang et al. 2001); 4) NFκB-mediated IL-6 and TNF-α production are associated with insulin resistance in

myotubes (Sinha et al. 2004; Weigert et al. 2004; Jove et al. 2005); and 5) human adipose

tissue is a target for IL-6-mediated insulin resistance (Rotter et al. 2003). To test this

hypothesis, cultures were treated with either BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA

and IκBα protein level was measured by immunoblotting. In support of our hypothesis,

IκBα was lower in cultures treated for 3 h with *trans*-10, *cis*-12 CLA than in the controls

(Fig 3.4A), implying NFκB is activated by CLA. CLA-mediated NFκB activation

appeared to be isomer-specific as evidenced by phosphorylation and subsequent

degradation of IκBα and ERK1/2 phosphorylation in *trans*-10, *cis*-12 CLA-treated

cultures compared to cultures treated with *cis*-9, *trans*-11 CLA or BSA (Fig 3.4B). In

contrast to TNF-α treatment, *trans*-10, *cis*-12 CLA had no effect on IκBβ degradation.

To determine the upstream regulator of IκBα phosphorylation by CLA, cultures

treated with either BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA were immunostained

with an antibody that recognizes active phospho-IKKα/β (Ser176/180, Ser177/181).

Trans-10, *cis*-12 CLA treatment for 2 h increased the phosphorylation of IKKα/β

compared to the vehicle controls (Fig 3.4C), suggesting that *trans*-10, *cis*-12 CLA

activates the IKKs-NFκB-IκBα cascade in differentiated cultures of human adipocytes.

To determine the specificity of CLA induced-NFκB DNA binding among five

NFκB families (i.e., p50, p52, p65, c-Rel, Rel B) (reviewed in Chen et al. 2004),

differentiated cultures of adipocytes were treated for 3 h with BSA vehicle or 30 μM

trans-10, *cis*-12 CLA. Subsequently, nuclear extracts were added to 96 well plates to

which   an   oligonucleotide   containing   an   NFκB   consensus   binding   site

(5'GGGACTTTCC3') had been immobilized as provided in the Trans-AM NFκB Kit

(Active Motif). Thereafter, the NFκB complex bound to the oligonucleotide was detected by using an antibody that was specifically recognized by each NFκB subunit. As shown in Fig 3.4D, NFκB p50- and p65-DNA binding were increased ~120 and 80%, respectively, in cultures treated with *trans-10, cis-*12 CLA compared with the BSA controls. Binding of DNA to the other NFκB subunits due to CLA treatment was not detectable (data not shown). As shown in Fig 3.4E, NFκB p50 and p65 translocation from cytosol to nucleus were also increased in cultures treated with *trans-10, cis-*12 CLA compared with BSA-treated cultures. As expected, our positive control (100 ng/ml TNF-α for 1 h) increased nuclear accumulation of NFκB p50 and p65. Furthermore, phosphorylation of nuclear NFκB p65 (Ser-536) was higher in CLA-treated cultures compared to the BSA-treated cultures. Fractionation efficiency was verified using the nuclear protein nucleoporin and cytosolic protein GAPDH as markers. These data support our hypothesis that *trans-*10, *cis-*12 CLA promotes NFκB activation and its translocation to the nucleus in differentiated cultures of human adipocytes.

These data raised the question as to the extent to which NFκB activation occurs in adipocytes and SV cells. As our differentiated cell model is a heterogeneous model consisting of ~50% adipocytes and ~50% SV cells that do not differentiate into adipocytes, we used double-immunostaining of NFκB p65 and aP2, which is expressed in adipocytes but not SV cells, to determine which cell type exhibited increased NFκB activity in response to CLA. Supporting our data in Fig 3.4E showing that *trans-*10, *cis-*12 CLA induced NFκB translocation to nucleus, more NFκB p65 (red) staining was found in nuclei of CLA-treated cultures whereas most NFκB p65 staining in the BSA

controls was found in cytosol (Fig 3.5). The staining pattern of the TNF-α positive control was similar to that of the CLA treatment. As seen in the merged image in Fig 3.5, nuclear NFκB p65 appeared to be localized in both SV cells (e.g., aP2 negative cells staining red) and adipocytes (e.g., aP2 positive cells staining yellow) in CLA-treated cultures. These data suggest that *trans*-10, *cis*-12 CLA induces NFκB activation both in human adipocytes and SV cells.

Inhibitors of NFκB Block Trans-10, Cis-12 CLA-Induced IL-6 and TNF-α Gene Expression

To determine the extent to which CLA-induced cytokine gene expression is dependent on the activation of MEK/ERK, GPCR, and/or NFκB, the effects of selective chemical inhibitors of MEK/ERK, GPCR, and NFκB activation on cytokine mRNA levels were investigated. The MEK/ERK inhibitor U0126 blocked CLA-induced IL-6 gene expression, but not CLA's induction of TNF-α (Fig 3.6). Similarly, the GPCR-$G_{i/o}$ inhibitor PTX attenuated CLA-induced IL-6 gene expression without affecting CLA-induced TNF-α gene expression. Chemical inhibition of NFκB activation was performed using: 1) PSI, which blocks proteasomal degradation of IκBα; 2) Bay11-7082, which inhibits IκBα phosphorylation (Pierce et al. 1997; Lappas et al. 2005); and 3) KA, which prevents NFκB p50 DNA binding (Fig 3.6). Chemical inhibition of NFκB activation abolished CLA-inducible IL-6 and TNF-α gene expression. However, pretreatment with antioxidant N-acetyl-cysteine (NAC) did not block CLA's induction of either TNF-α or IL-6 (data not shown), suggesting CLA is not activating NFκB or inducing cytokines by

68

producing proxidants. These data show that CLA-induced IL-6 expression is dependent on both NFκB and ERK1/2 activation. In contrast, CLA induced TNF-α gene expression is dependent on NFκB activation, but not on the activation of ERK1/2 or GPCR.

Blocking IKK Complex Formation Reverses CLA's Suppression of Glut4 and PPARγ
Protein Levels

Given the reported antagonistic interaction between PPARγ and NFκB (Ruan et al. 2003), we investigated the role of NFκB in mediating *trans*-10, *cis*-12 CLA's suppression of insulin-stimulated glucose uptake via down regulation of PPARγ. Differentiated cultures of adipocytes were treated with either BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for 24 h in the presence and absence of NEMO BP, a synthetic peptide which blocks the regulatory site of the active IKK complex, thereby inhibiting IκBα degradation (May et al. 2000; Li et al. 2002). As shown in Fig 3.7, NEMO BP prevented or attenuated CLA-mediated suppression of PPARγ and Glut4 protein levels, respectively, without altering caveolin-1 protein expression. Collectively, these data demonstrate that *trans*-10, *cis*-12 CLA promotes NFκB activation via the IKK-IκB-NFκB axis, thereby repressing the expression of PPARγ and its target genes that are required for insulin-stimulated glucose uptake and TG synthesis.

Depletion of NFκB p65 by RNAi Attenuates CLA's Suppression of Adipogenic Protein
Expression and Glucose Uptake and Activation of ERK1/2

We demonstrated that the perturbation of NFκB activation was sufficient to
prevent or attenuate CLA's suppression of adipogenic protein expression (Fig 3.7). Using
a second approach, selective depletion of NFκB p65 using siRNA was performed prior to
the treatment with *trans*-10, *cis*-12 CLA. As shown in Fig 3.8A, control (non targeting
siRNA) or NFκB p65 siRNA were introduced to the cultures of differentiating adipocytes
on day 6. 72 h post-transfection, cultures were serum starved for 20 h and treated with
either BSA control or 30 μM *trans*-10, *cis*-12 CLA for 24 h (Fig 3.8A). To monitor the
transfection efficiency, cultures were transfected with siGLO-RISC-free, a fluorescent
labeled, non-targeting siRNA with impaired ability for RISC formation. Almost all cells
($\geq$ 90%) were positive to Cy3-fluoresence of siGLO 24 h post- transfection (Fig 3.8B),
indicating that both adipocytes and non-adipocytes were efficiently transfected (Fig 3.8B).
Specific depletion of NFκB p65 was examined using immunoblotting as shown in Fig 3.
8C. We consistently obtained $\geq$ 50% NFκB depletion. NFκB p65 protein expression was
severely blunted in the NFκB p65 siRNA transfected cultures compared to untreated or
non-targeting control or lamin-siRNA transfected cultures (Fig 3.8C). In contrast, protein
levels of: 1) NFκB p50, a heterodimeric partner of active NFκB; 2) GAPDH, a
constitutive cytoplasmic protein; 3) β-actin, a cytoskeleton protein; and 4) aP2, a specific
adipocyte marker protein, were unchanged. siRNA-mediated specific knockdown was
validated using lamin siRNA as a positive control (Fig 3.8C).

To investigate the impact of CLA's regulation on adipocytes under conditions of NFκB depletion, we examined PPARγ expression and the activation of ERK1/2 and STAT3. As shown in Fig 3.8D, depletion of NFκB p65 modestly attenuated CLA's suppression of PPARγ2 (also confirmed by immunostaining in Fig 3.8E) and its activation of ERK1/2, but had no marked effect on STAT3 phosphorylation. These data further support our hypothesis that NFκB p65 plays a central role in CLA-induced suppression of adipogenic gene expression, activation of MEK/ERK signaling, and subsequent secretion of cytokines.

Because CLA's suppression of PPARγ was attenuated by knocking down NFκB activity using siRNA, we hypothesized that silencing NFκB would block or attenuate CLA's suppression of insulin-stimulated glucose uptake demonstrated in Fig 3.1. To test this hypothesis, we measured insulin-stimulated [^{3}H]-2-deoxy-glucose uptake in NFκB-depleted cultures of adipocytes treated for 24 h with BSA vehicle or *trans*-10, *cis*-12 CLA. CLA's suppression of glucose uptake was attenuated in siP65-transfected cultures compared to CLA-treated cultures not receiving siP65 (Fig 3.9A). The degree of rescue of insulin-stimulated glucose uptake (~45%) and Glut4 protein levels (~55%) by siP65 for NFκB in the CLA-treated group was nearly similar to the degree of knockdown achieved for NFκB-p65 in this experiment (~54%; densitometry not shown) (Fig 3.9B). Collectively, these data demonstrate for the first time that specific depletion of NFκB p65 in CLA-treated cultures partially rescued PPARγ and Glut4 protein levels and insulin-stimulated glucose uptake. This isomer-specific, CLA-mediated activation of cytokines and suppression of PPARγ and Glut4 protein levels shows striking similarity with other

reports of cytokine-mediated insulin resistance reported in myotubes (Sinha et al. 2004; Jove et al. 2005), and adipocytes (Rotter et al. 2003).
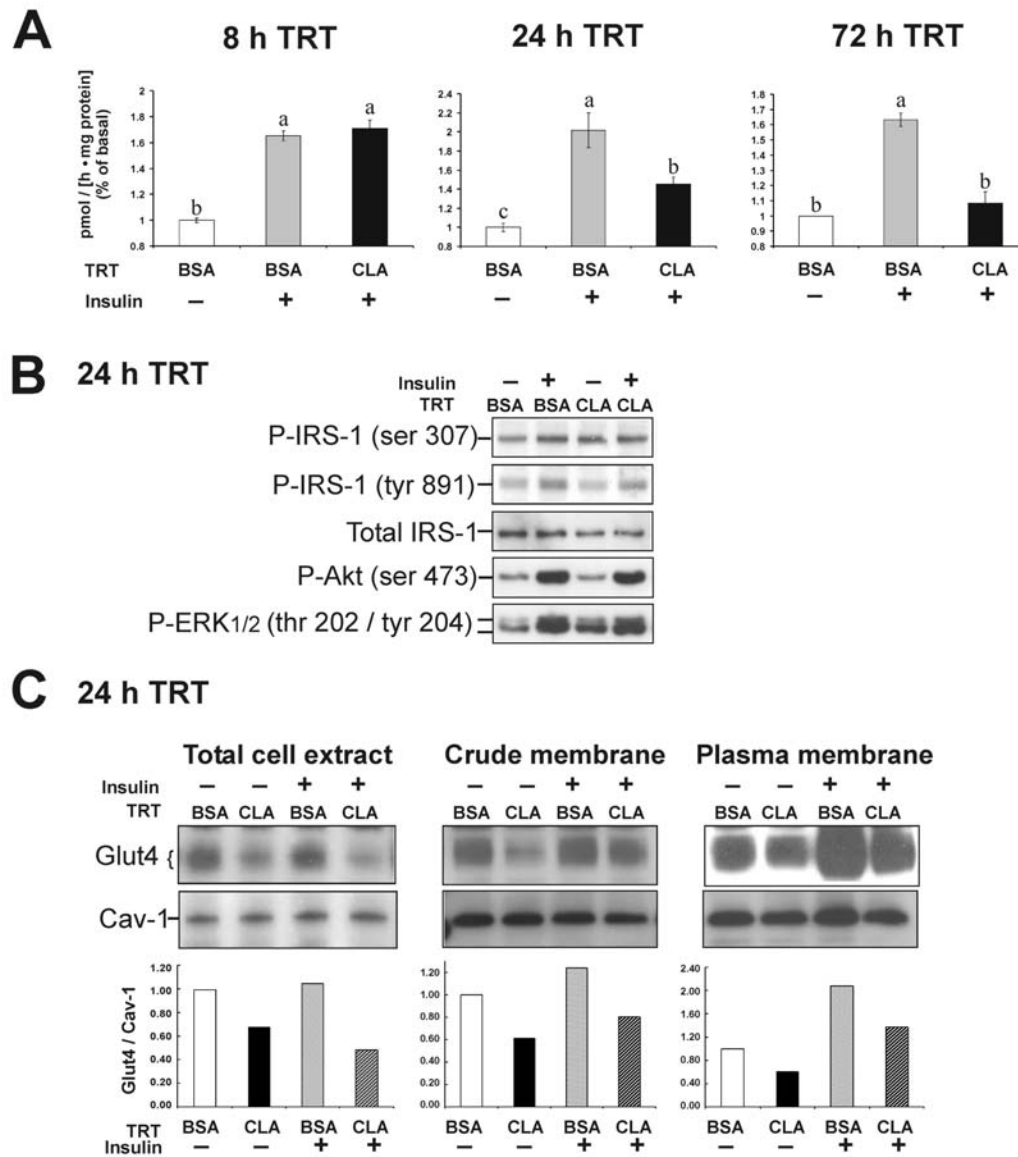
Figure 3. 1. *Trans*-10, *cis*-12 reduces insulin-stimulated glucose uptake and Glut4 protein expression. Differentiated cultures of human adipocytes were treated for 8, 24, or 72 h with either a bovine serum albumin (BSA) vehicle or 30 μM *trans*-10, *cis*-12 CLA (CLA). **A:** Basal and insulin-stimulated uptake of 4 nmol [³H]-2-deoxy-glucose were measured for 90 min after treatment in the absence (-) or presence (+) of 100 nM human insulin. The control rate of uptake was ~111 pmol/(h·mg of protein). Data are normalized to the basal vehicle control's (BSA, - insulin) rate. Means ($\pm$ S.E.; n = 6) not sharing common *superscript* differ, p<0.05. **B:** Following treatment with either BSA or *trans*-10, *cis*-12 CLA for 24 h, cells were cultured in the absence (-) or presence (+) of 100 nM insulin for 10 min, and total cell extracts were immunoblotted with phospho (p)-specific antibodies targeting p-IRS-1 (ser307), p-IRS-1 (tyr891), p-Akt (ser473), p-ERK1/2 (thr202,204), and total-insulin receptor substrate 1 (IRS-1). **C:** Cultures were treated with either BSA vehicle or *trans*-10, *cis*-12 CLA (CLA) for 24 h prior to incubation in the absence (-) or presence (+) of 100 nM human insulin for 30 min. Total cell extracts were harvested and crude and plasma membrane fractions were isolated by differential centrifugation. Each fraction was immunoblotted for Glut4 and caveolin-1 (Cav-1). Blots were quantified by densitometry and amount of Glut4 relative to Cav-1 was expressed as a bar graph under the each blot.

A **Differentiated Cultures of Adipocytes**

B **Non-differentiated Cultures of SV cells**

C **Tissue Explants**
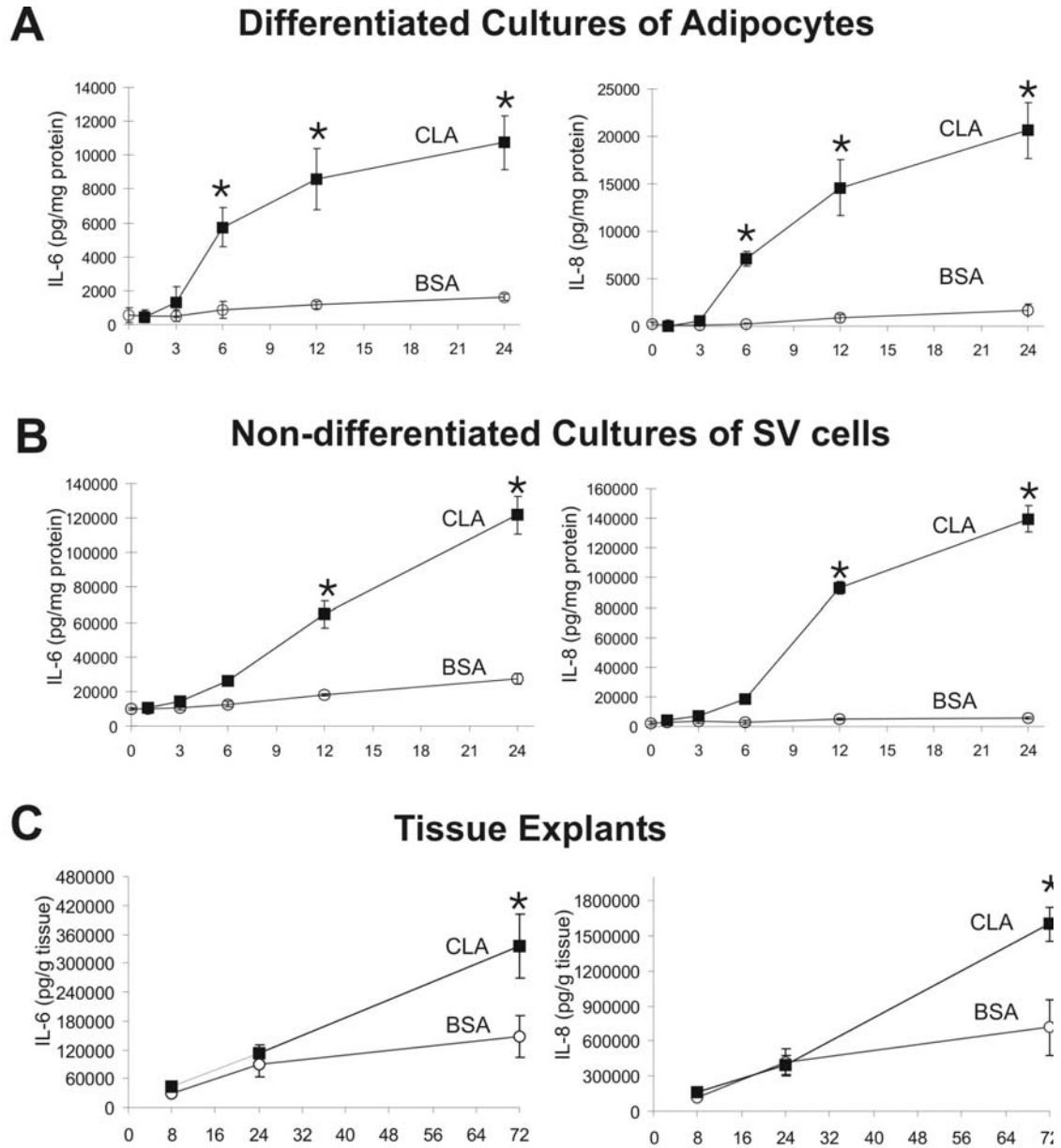
Figure 3. 2. ***Trans*-10, *cis*-12 CLA increases IL-6 and IL-8 secretion.** Cultures were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA (CLA) for the indicated times (0-72 h) and IL-6 and IL-8 in the media were determined at each time point by ELISA from cultures of human differentiated adipocytes (**A**), non-differentiated SV cells (**B**), or adipose tissue explants (**C**). Means (±S.E.) for IL-6 and IL-8 were obtained from three independent experiments using either a pool of cells obtained from three to six different human subjects (A, B, n=9) or from adipose tissue from three different subjects (C, n=8). Data are expressed as pg cytokine/mg protein (A, B) for the cell cultures studies and as pg cytokine/g tissue for the tissue explants studies. Means with *asterisks* differ significantly (p<0.05) from the BSA controls at each time point.

74
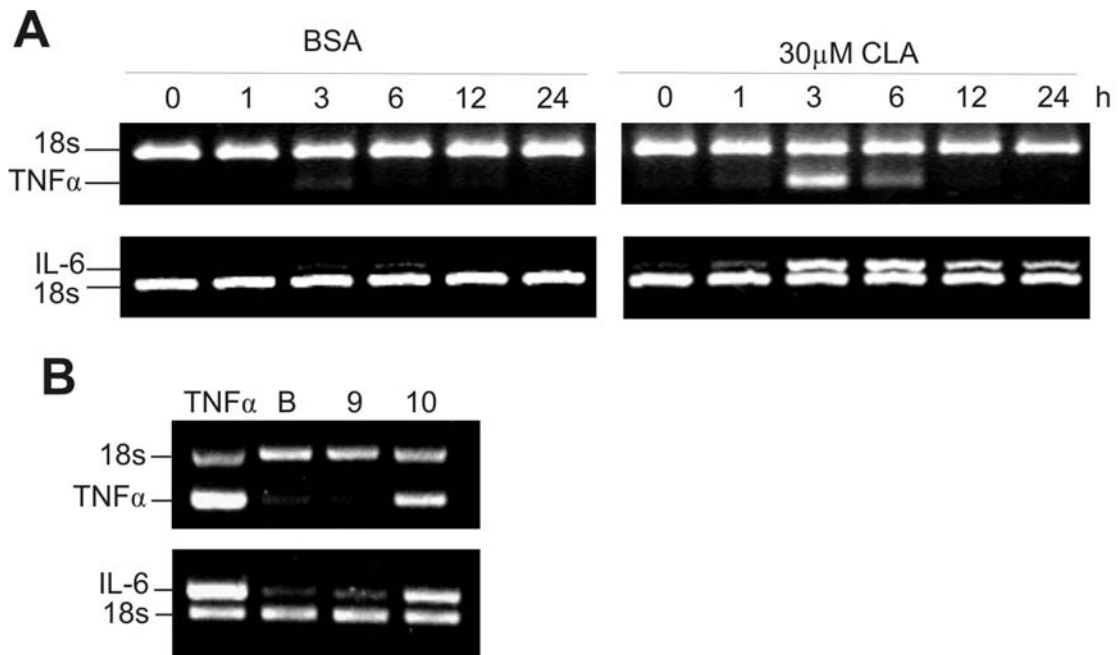
Figure 3. 3. *Trans*-10, *cis*-12 CLA increases TNF-α and IL-6 gene expression. A: Differentiated cultures of human adipocytes were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA (CLA) for the indicated times (0-24 h). mRNA levels of TNF-α and IL-6 were analyzed using semi-quantitative RT-PCR. **B**: Cultures were treated with BSA (B), 30 μM *cis*-9, *trans*-11 CLA (9), or 30 μM *trans*-10, *cis*-12 CLA (10) for 3 h. TNF-α (100 ng/ml) treatment for 30 min was used as a positive control. mRNA levels of TNF-α and IL-6 were examined using semi-quantitative RT-PCR. Results shown are representative of three (A) or four (B) separate experiments from independent human subjects.
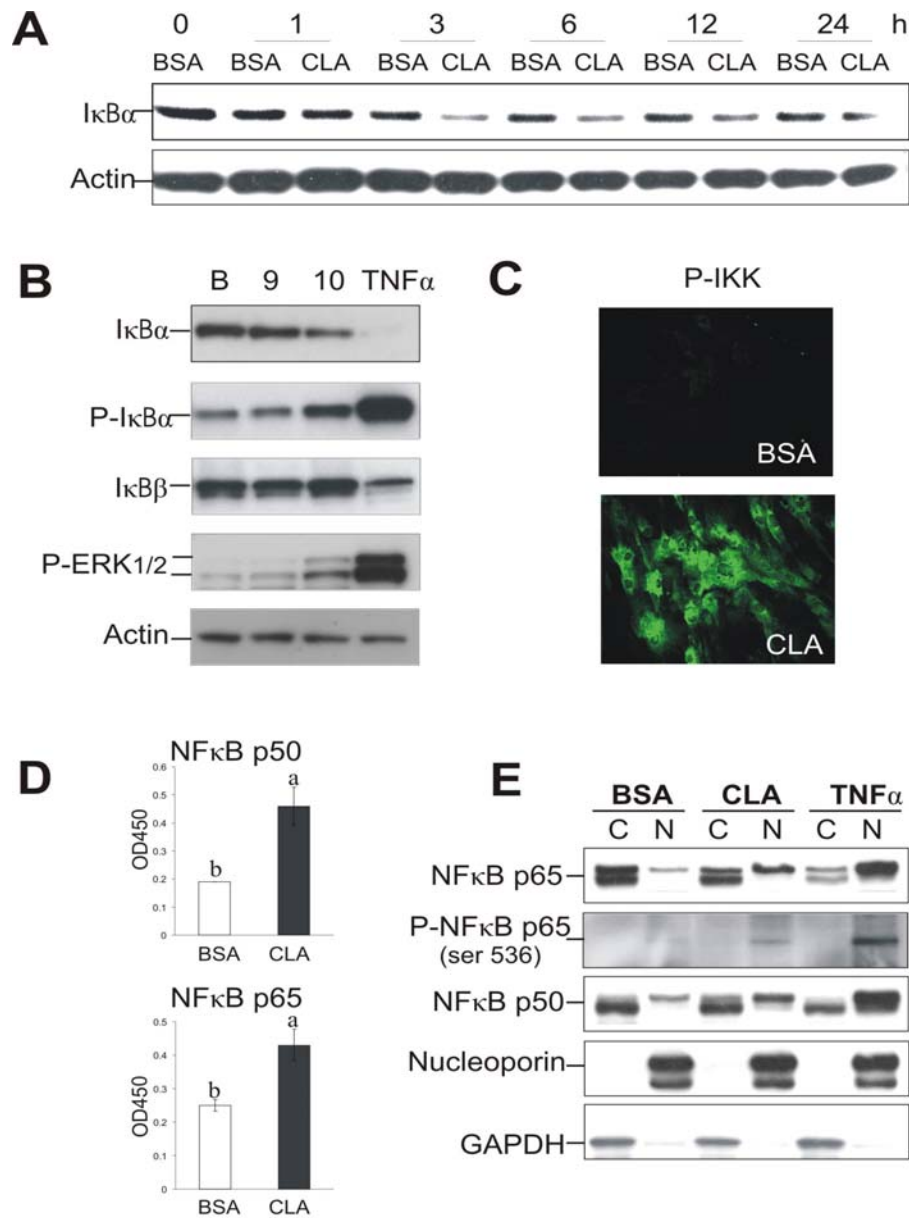
Figure 3. 4. *Trans*-**10,** *cis*-**12 CLA activates NFκB. A:** Differentiated cultures of human adipocytes were treated with BSA vehicle or 30 µM *trans*-10, *cis*-12 CLA (CLA) for 0, 1, 3, 6, 12, or 24 h and the abundance of IκB was determined using immunoblotting. **B:** Cultures were treated with BSA, 30 µM *cis*-9, *trans*-11 CLA (9), or 30 µM *trans*-10, *cis*-12 CLA (10) for 3 h and IκBα, p-IκBα, IκBβ, p-ERK1/2, actin levels were analyzed by immunoblotting. 100 ng/ml TNF-α for 30 min was used as a positive control. **C:** Cultures were treated BSA or 30 µM *trans*-10, *cis*-12 CLA (CLA) for 2 h and immunostained with p-IKK (Ser177/180, Ser177/181). Active IKK was then detected using immunofluorescence microscopy. **D:** Cultures were treated with BSA or 30 µM *trans*-10, *cis*-12 CLA for 3 h and nuclear extracts were used for detecting binding affinity of NFκB families to the κB response element consensus oligonucleotide on 96 well plates (Trans-AM NFκB kit). Means ($\pm$ S.E.; n=2) not sharing a common *superscript* differ (p<0.05). **E:** Nuclear (N) and cytosol (C) extracts were immunoblotted with antibodies targeting NFκB p65, p-NFκB p65 (ser536), and NFκB p50. Fractionation was validated by immunoblotting nucleoporin and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH).

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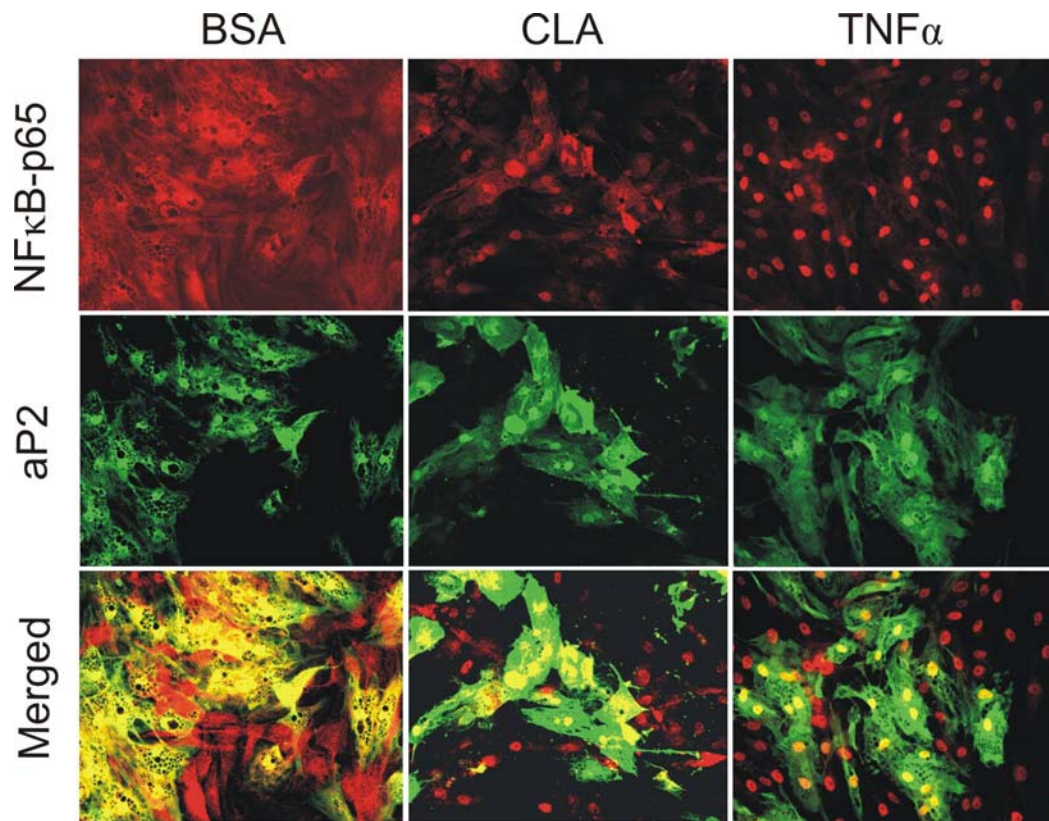
Figure 3. 5. *Trans*-10, *cis*-12 CLA promotes NFκB localization to the nucleus in adipocytes and nonadipocytes. Differentiated cultures of human adipocytes were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA (CLA) for 24 h and then cells were double-stained for NFκB p65 (cy3 conjugated anti-mouse IgG = red) and adipocyte fatty acid-binding protein (aP2; FITC-conjugated anti-rabbit IgG = green). TNF-α (100 ng/ml for 1 h) treatment was used as a positive control. Results shown are representative of two separate experiments from pools of cells obtained from three to five different human subjects.
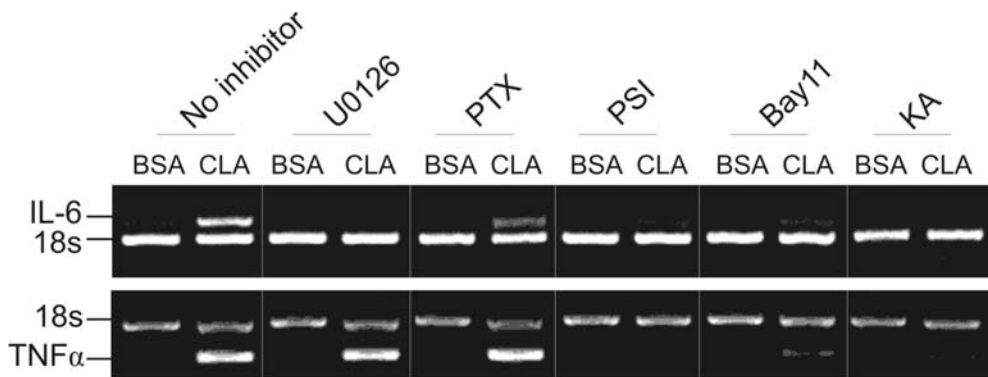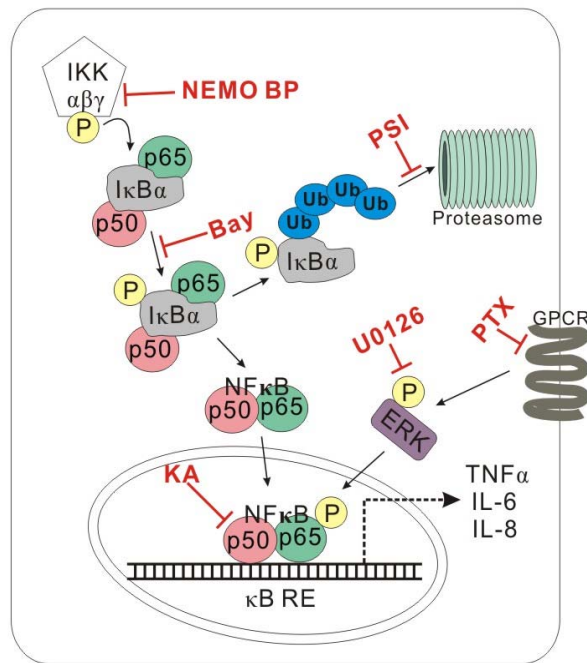
Figure 3. 6. *Trans*-10, *cis*-12 CLA-induction of IL-6 and TNF-α gene expression is blocked by NFκB inhibitors. Differentiated cultures of human adipocytes were serum starved for 20 h and then pretreated for 1 h with either with the MEK/ERK inhibitor U0126 (10 µM), the GCPR-Gi/o inhibitor Pertussis toxin (PTX; 100 ng/ml), the NFκB inhibitors proteasome inhibitor I (PSI; 50 µM), Bay11-7082 (Bay11; 2.5 uM), or kamebakaurin (KA; 26 uM), and subsequently treated with BSA vehicle or 30 µM *trans*-10, *cis*-12 CLA (CLA) for additional 3 h. Total RNA was harvested and semi-quantitative RT-PCR analyses were performed to examine the expression of TNF-α, IL-6, and aP2. Data shown are representative of three independent experiments from different human subjects.
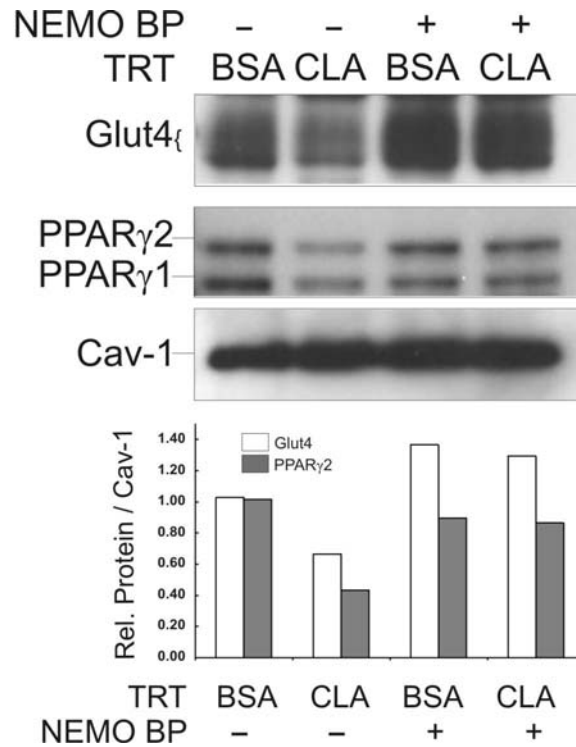
Figure 3. 7. Inhibiting IKK complex formation by NEMO-binding peptide (BP) blocks CLA's suppression of Glut4 and PPARγ protein levels. Differentiated cultures of human adipocytes were serum-starved for 20 h and pretreated with 100 ug/ml NEMO BP for 1 h. Subsequently, cultures were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA (CLA) for 24 h. Cellular fractions containing nuclei and membranes were collected and immunoblotted for the Glut4, peroxisome proliferator activated receptor gamma (PPARγ), and caveolin-1. Data shown are representative of three independent experiments using a pool of cells obtained from three to four different human subjects. Blots were quantified by densitometry and the amounts of Glut4 and PPARγ relative to Cav-1 were expressed as bar graphs under the blot.
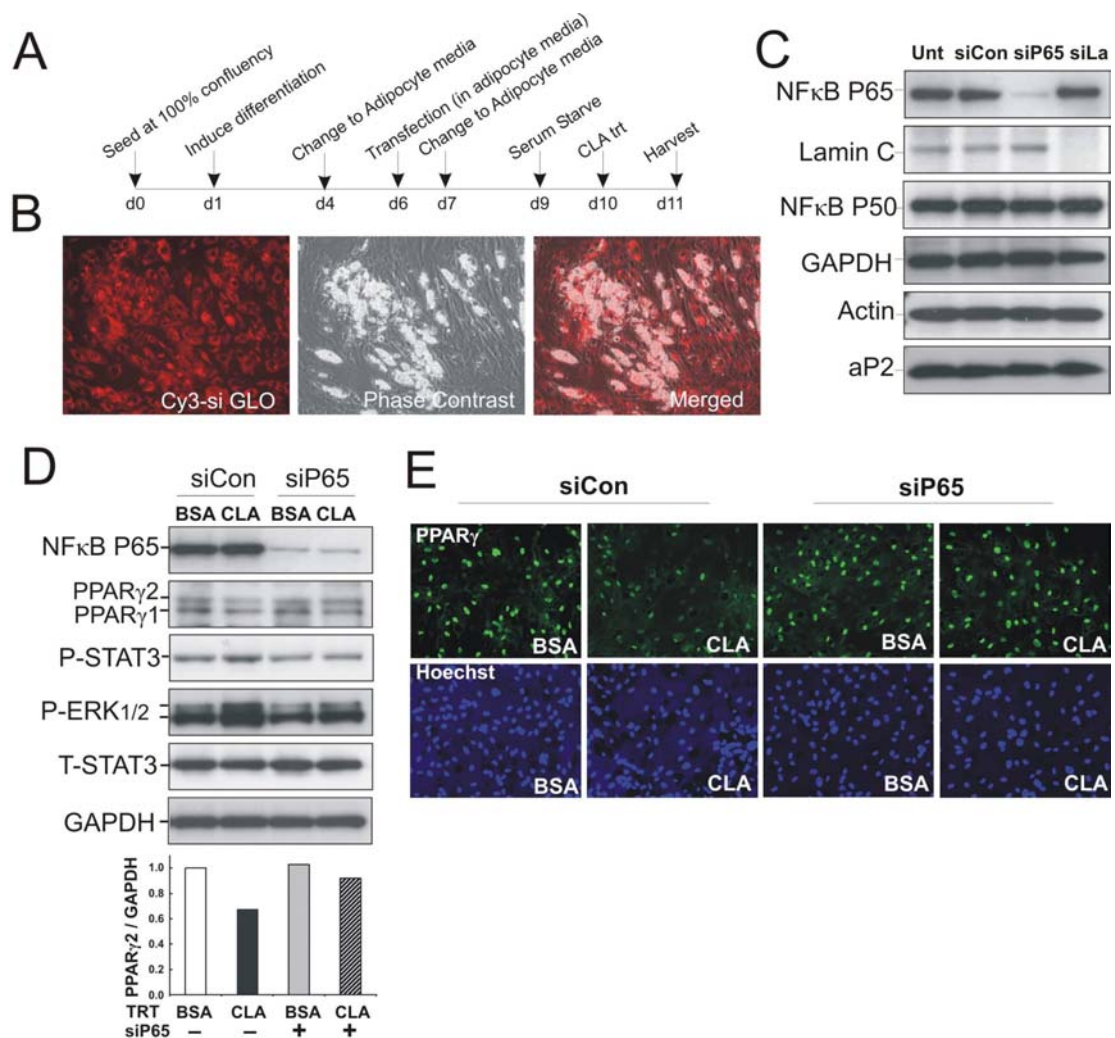
Figure 3. 8. Specific depletion of NFκB p65 attenuates CLA's suppression of PPARγ and activation of MEK/ERK signaling in cultures of primary human adipocytes. A: Experimental design of transfection protocol with siRNA NFκB p65 in primary cultures of human adipocytes. **B**: Transfection efficiency was evaluated by transfection with siGLO, fluorescence (Cy3)-tagged non-targeting siRNA. **C:** Knockdown specificity was analyzed using immunoblotting. Total cell extracts from either untreated (Unt) samples or samples transfected with non-targeting control siRNA (siCon), NFκB p65 siRNA (siP65), and positive control Lamin siRNA (siLa) were immunoblotted with the antibodies targeting NFκB p65, Lamin C, NFκB p50, GAPDH, actin and aP2. **D**: The impact of CLA on NFκB p65-depleted cultures were examined using immunoblotting. 72 h post-transfection with either siCon or siP65, cultures were treated with BSA vehicle or a 30 μM *trans*-10, *cis*-12 CLA (CLA) for additional 24 h. Total cell extracts were immunoblotted with antibodies targeting NFκB p65, PPARγ, Glut4, p- signal transducer and activators of transcription 3 (STAT3), p-ERK1/2, total-STAT3, and GAPDH. The blots for PPARγ and GAPDH were quantified by densitometry and the amount of PPARγ relative to GAPDH was expressed as a bar graph under the blot. **E**: Cultures were transfected, 72 h later treated with either BSA or 30 μM *trans*-10, *cis*-12 CLA (CLA) for 24 h, and then immunostained for PPARγ. Hoechst staining was conducted to identify nuclei.

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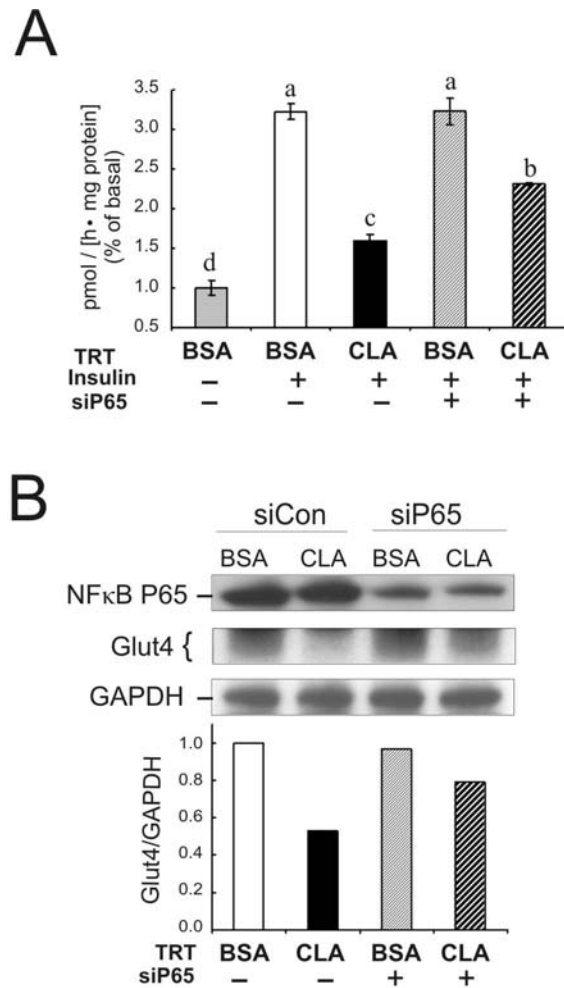
Figure 3. 9. Depletion of NFκB p65 attenuates CLA's suppression of Glut4 levels and insulin-stimulated glucose uptake. A: Cultures of differentiated human adipocytes were transfected with either control siRNA (siCon) or NFκB p65 siRNA (siP65) as described in Fig 3.8, and 72 h later were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA (CLA) for 24 h. Basal and insulin-stimulated uptake of 4 nmol [^{3}H]-2-deoxy-glucose were measured for 90 min in the presence or absence of 100 nM human insulin. The basal control rate was ~100 pmol/(h·mg of protein). Data are normalized to the basal vehicle control's (BSA, - insulin) rate. Means ($\pm$ S.E.; n=4) not sharing common *superscript* differ, p<0.05. **B:** Immunoblotting for NFκB p65, Glut4, and GAPDH were carried out as described in Fig.3.8. The blots for Glut4 and GAPDH were quantified by densitometry and the amount of Glut4 relative to GAPDH was expressed as a bar graph under the blot.
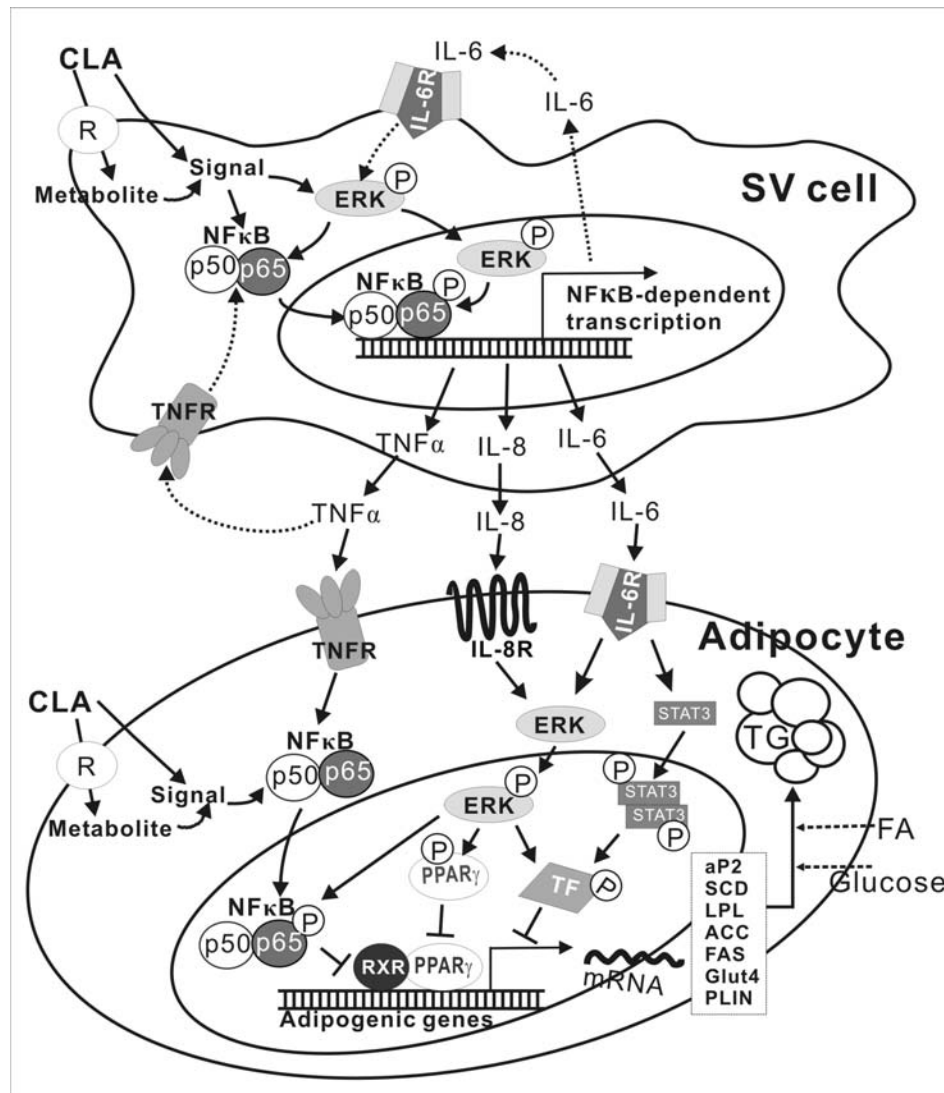
Figure 3. 10. Working model: *Trans*-10, *cis*-12 CLA reduces glucose and fatty acid uptake and TG synthesis via activation of NFκB and ERK1/2 signaling and cytokine production. Upon entry into SV cells, *trans*-10, *cis*-12 CLA generates an unidentified signal, thereby activating NFκB and ERK1/2. NFκB p65/p50 then translocates to the nucleus where it is activated by P-ERK. Activated NFκB initiates the transcription of specific cytokines (i.e., TNF-α, IL-6, IL-8). These secreted cytokines, in turn, activate their cognate cell surface receptors on adipocytes, leading to NFκB and ERK1/2 activation. NFκB p65/p50 translocates into the nucleus where P-ERK activates NFκB p65 and other transcription factors (TF), leading to suppression of PPARγ activity and target gene expression including aP2, sterol-CoA desaturase (SCD), lipoprotein lipase (LPL), fatty acid synthase (FAS), glucose transporter 4 (Glut4) and perilipin (PLIN). CLA may also generate an unidentified signal in adipocytes that activates NFκB and ERK1/2, which further suppresses PPARγ target gene expression. Together, this leads to adipocyte delipidation via decreased glucose and fatty acid uptake and TG synthesis.

82

Discussion

We demonstrate for the first time that *trans*-10, *cis*-12 CLA promotes NFκB activation that induces cytokine production, leading to decreased glucose uptake in primary cultures of human adipocytes. Based upon these data and our previously published data (Brown et al. 2003, 2004; Chung et al. 2005), we propose in our working model (Fig 3.10) that *trans*-10, *cis*-12 CLA first enters SV cells and activates a membrane protein or enters the cell by diffusion and is converted to a metabolite, which triggers a signal that activates NFκB and ERK1/2, leading to cytokine synthesis, specifically TNF-α, IL-6, and IL-8. These, and possibly other cytokines, activate their respective cell surface receptors on adipocytes, activating NFκB and ERK1/2, leading to p65 and phosphorylated (P) ERK1/2 localization in the nucleus. P-ERK1/2 then phosphorylates specific nuclear transcription factors including p65, ELK-1, and PPARγ. Together, these transcription factors acutely decrease PPARγ activity by phosphorylating PPARγ and/or by interfering with its ability to transactivate adipogenic target genes such as aP2, Glut4, fatty acid synthase (FAS), lipoprotein lipase (LPL), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase (SCD). Chronically, this leads to decreased PPARγ gene expression. Collectively, this causes decreased expression of adipogenic genes that promote glucose and fatty acid uptake and synthesis to TG, leading to insulin resistance and delipidation.

Using either chemical inhibition or depletion of NFκB, we demonstrated that NFκB activation was essential for CLA-induced IL-6, IL-8, and TNF-α production, impaired adipogenic gene expression and glucose uptake, and activation of ERK1/2 signaling.

Based on our working model (Fig 3.10) and data showing that NFκB is activated by CLA in both SV cells and adipocytes (Fig 3.5), we propose a differential role of NFκB activation in SV cells vs. adipocytes. In SV cells, we postulate that CLA activates NFκB by an unidentified upstream signal. Upon activation and nuclear localization, ERK1/2 phosphorylates NFκB-p65, which then induces cytokine production as we demonstrated in non-differentiated SV cells (Fig 3.2B) and in differentiated cultures of adipocytes (Fig 3.2A). Quantitatively, the non-differentiated SV cells (Fig 3.2B) produced 10-fold and 7-fold more IL-6 and IL-8, respectively, in response to 24 h treatment with *trans*-10, *cis*-12 CLA than did the differentiated cultures of adipocytes (Fig 3.2A). In agreement with these data, Harkins et al. (2004) showed that 3T3-L1 preadipocytes stimulated with LPS express more IL-6 than adipocytes. Consistent with these data, IL-6, IL-8, and TNF-α possess a κBRE (Chen et al. 1999). Furthermore: 1) cytokine secretion was preceded by IκBα degradation (Figs. 3.4A and B), IKK phosphorylation (Fig 3.4C), and increased p50 and p65 nuclear translocation (Figs. 3.4E and 3.5) and binding to a consensus NFκB oligomer (Fig 3.4D) compared to controls; and 2) selective chemical inhibitors of NFκB acutely blocked CLA-mediated increases in IL-6 and TNF-α gene expression (Fig 3.6).

Once these cytokines are secreted into the medium, we propose that they bind to their cognate receptors on adipocytes, activating NFκB. Upon activation and nuclear localization, ERK1/2 phosphorylates NFκB and other transcription factors, which together suppress PPARγ activity leading to decreased adipogenic gene expression, glucose and fatty acid uptake, and TG synthesis (Brown et al. 2004). Consistent with these data, cytokines secreted from adipose tissue regulate glucose and lipid metabolism

locally and peripherally (Rotter et al. 2003; Bruun et al. 2003, 2004; de Mora et al. 1997). Several cytokines have been reported to signal through NFκB (i.e., TNF-α via TNF-α receptors) and MEK/ERK (i.e., IL-6 via gp130-JAK, IL-8 via GPCR-Gi/o). In support of our working model proposing that *trans*-10, *cis*-12 CLA induces the activation of NFκB in adipocytes, we found that selective inhibition of NFκB using chemical inhibitors (Fig 3.7) or depletion of NFκB (Fig 3.8 and 3.9) attenuated CLA's suppression of PPARγ and Glut4 levels and glucose uptake, and activation of ERK1/2 signaling.

IL-6 is a major paracrine regulator whose expression is controlled by NFκB. Certain saturated fatty acids such as palmitate at high concentrations have been reported to activate NFκB and IL-6 resulting in systemic insulin resistance in adipocytes (Rotter et al. 2003), myotubes (Weigert et al. 2004; Jove et al. 2005), and hepatocytes (Cai et al. 2005). We demonstrated that *trans*-10, *cis*-12 CLA activates the IKK-IκB-NFκB axis (Figs 3.4 and 3.5) and NFκB inhibitors block or attenuate CLA-induced IL-6 gene expression (Fig 3.4, 3.6), Glut4 protein levels (Figs 3.7 and 3.9), and suppression of glucose uptake (Fig. 3.9), suggesting that the production of IL-6 is due to the CLA-mediated activation of NFκB. To support our hypothesis, we found that differentiated human adipocytes treated with IL-6 (20 ng/ml) for 48 h had decreased Glut4 and adiponectin gene expression compared to controls (unpublished data). Furthermore, we previously demonstrated that neutralization of IL-6 blocked CLA's activation of ERK1/2 (Brown et al. 2004), supporting the important role of IL-6 in mediating CLA's anti-adipogenic actions.

Unlike IL-6, TNF-α secretion to the medium was not observed in CLA-treated human adipocytes (data not shown), even though there was a transient increase in the

mRNA levels of TNF-α (Fig. 3). However, TNF-α may undergo altered processing in the plasma membrane (mTNF-α) rather than being released in its soluble form. Xu et al. (2002) reported that mTNF-α is capable of exerting diverse biological functions through cell contact-dependent signaling rather than through a receptor-mediated mechanism. Because bypassing the TNF-α receptor may result in activation of different mechanisms compared to the classic downstream signaling of TNF-α receptor, we can not rule out the possibility that TNF-α plays an important role in mediating CLA's induction of insulin resistance and delipidation (Brown et al. 2004) in adipocytes.

The term adipokines has been used to identify cytokines or chemokines originating from adipocytes (e.g., IL-6, IL-8, TNF-α, leptin, resistin, adiponectin). However, this term is misleading based on data suggesting that non-fat cells from adipose tissue are the major site for cytokine production rather than adipocytes (Bruun et al. 2004; Fain et al. 2004 a,b). These data are consistent with ours, suggesting that human SV cells or non-adipocytes have a greater capacity for cytokine production than adipocytes, at least in response to CLA. Considering the intimate communication between non-adipocytes and adipocytes within adipose tissue, this may explain why our primary human adipocytes cultures, which consist of ~50% SV cells and ~50% adipocytes, respond differently to CLA treatment compared to 3T3-L1 adipocytes which are almost exclusively adipocytes. Studies are underway in our lab examining the effects of CLA on purified cultures of adipocytes devoid of SV cells to accurately address this issue.

We reported that *trans*-10*, cis*-12 CLA decreased insulin-stimulated glucose uptake in human differentiating preadipocytes (Brown et al. 2003) and newly differentiated

adipocytes (Brown et al. 2004). Herein, we demonstrated that this CLA-mediated suppression of insulin-stimulated glucose uptake (Fig 3.1A) was due primarily to decreased levels of Glut4 (Fig 3.1C), particularly plasma membrane Glut4, an indicator of insulin sensitivity. CLA had no effects on phosphorylation of IRS-1 (ser 307 or tyr 891) or Akt (Ser 437) (Fig 3.1B), suggesting CLA has no significant effects on insulin signal transduction *per se*. Our hypothesis that CLA impaired insulin-stimulated glucose uptake via activation of NFκB was confirmed by our NFκB p65 siRNA transfection study demonstrating the depletion of NFκB attenuates CLA's suppression of glucose uptake (Fig 3.9). To our knowledge, this is the first published report documenting depletion of NFκB p65 using RNAi technique in primary human adipocytes. In addition, there is evidence that phosphorylation of IKK is a potential inhibitor of insulin-stimulated glucose uptake (Gao et al. 2005; Yuan et al. 2001). Based on these reports, our data showing *trans*-10, *cis*-12 CLA activates IKK (Fig 3.4C) reinforces our working model demonstrating that CLA promotes the IKK-IκB-NFκB cascade, which is critical for suppression of PPARγ and Glut4 proteins (Fig 3.7).

Collectively, these data and our previously published data (Brown et al. 2003, 2004; Chung et al. 2005) demonstrate that *trans*-10, *cis*-12 CLA impairs glucose and fatty acid uptake in adipocytes, which is important for *de novo* TG synthesis and adipocyte hypertrophy. These data support human (Riserus et al. 2004 a,b) and animal (Park et al. 1999) studies showing that *trans-10, cis*-12 CLA, but not *cis*-9, *trans*-11 CLA, reduces adiposity. This isomer-specific effect of CLA on suppressing adipocyte glucose and fatty acid uptake may contribute to the hyperglycemia and hyperinsulinemia observed in obese

humans (Riserus et al. 2004a), and the insulin resistance (Clement et al. 2002; Tsuboyama-kasaoka et al. 2003), and/or lipodystrophy (Houseknecht et al. 1998) observed in some animal models supplemented with CLA. However, future clinical trials examining the isomer-specific effects of CLA on human adipose tissue metabolism, gene expression, and cell signaling are needed to validate this theory.

The intricate relationship between PPARγ, NFκB, and MAP-kinase is not yet fully understood. P-ERK1/2 is an important regulator of PPARγ as demonstrated by its inhibition of adipogenesis (Hu et al. 1996). Furthermore, cytokine expression suppressed PPARγ activity in mesenchymal stem cells, showing direct interaction between PPARγ and NFκB (Suzawa et al. 2003). Similar reports demonstrating that activation of NFκB (Ruan et al. 2002, 2003; Nie et al. 2005) and MAPK (Adams et al. 1997) hinders PPARγ DNA binding affinity or transcriptional activation provides a potential mechanism by which *trans*-10, *cis*-12 CLA suppresses the expression of PPARγ target genes prior to that of PPARγ itself, leading to insulin resistance and delipidation (Brown et al. 2004). In our primary cultures of human adipocytes, *trans*-10, *cis*-12 CLA treatment activated NFκB and ERK1/2. Furthermore, our results showed that chemical inhibition of NFκB or targeted depletion of NFκB p65 by siRNA not only attenuated CLA's suppression of PPARγ2 (Figs 3.7, 3.8D, and 3.8E) and Glut4 (Figs 3.7 and 3.9B), but also prevented CLA-mediated ERK1/2 phosphorylation (Fig 3.8D). Based on these findings, we hypothesize that mutual interactions between NFκB and ERK1/2 are required for CLA to inhibit PPARγ activity acutely, and PPARγ expression chronically. Studies are currently underway in our lab to test this hypothesis.

CLA's suppression of insulin sensitivity and TG accumulation (Brown et al. 2003, 2004; Chung et al. 2005) in human adipocytes could be due to de-differentiation or apoptosis, or blocking new differentiation. However, 30 μM *trans*-10, *cis*-12 CLA does not cause apoptosis in our cultures based on: 1) NFκB activation antagonizes apoptosis and promotes cells survival (Dixit et al. 2002); 2) caspase 3 is not activated by 30 μM CLA for up to 72 h (unpublished data); 3) Hoechst and DAPI staining indicate similar numbers of nuclei and no differences in nuclear condensation or fragmentation in CLA-treated cultures compared to controls (Fig 3.8E) and (Brown et al. 2004); 4) protein and RNA levels are not reduced after CLA treatment; and 5) CLA reduces the TG content of adipocytes without reducing the number of adipocytes (Brown et al. 2004; Chung et al. 2005). Similarly, CLA does not appear to cause de-differentiation *per se* because: 1) adipocytes still contain small lipid droplets after 3 wk of treatment, even though CLA suppresses adipogenic gene expression and protein levels and impairs glucose uptake within 24 h of treatment (Brown et al. 2004); 2) CLA increases adipose differentiation-related protein (ADRP) and leptin expression (Brown et al. 2004; Chung et al.2005), suggesting that CLA-treated cultures still contain adipocytes; and 3) Pref-1 gene expression, which is abundant in our non-differentiated SV cells, is absent in CLA-treated cultures containing adipocytes (unpublished data), suggesting these cells do not de-differentiate back to preadipocytes. Finally, we do not observe new differentiation of adipocytes after ~day 6 of differentiation. Instead, the increased TG content of the cultures after day 6 is due primarily to increased size of the lipid droplets within adipocytes. Thus, because our experiments began on ~ day 12 of differentiation, CLA is

most likely not impairing the differentiation of new adipocytes. Instead, we postulate that CLA is causing delipidation, primarily by blocking *de novo* TG synthesis (Brown et al. 2003, 2004), and to a lesser extent, by increasing lipolysis (Chung et al. 2005).

In summary, our *in vitro* data demonstrate that a physiological level of *trans*-10, *cis*-12 CLA activates NFκB- and ERK1/2-dependent cytokine production, which together suppress PPARγ and Glut4 levels, and lead to impaired glucose uptake. Studies are currently underway examining: 1) how CLA regulates PPARγ and the expression of its target genes; 2) the specific signaling role of SV cells and adipocytes in mediating the TG-lowering actions of CLA; and 3) the CLA-induced, upstream signal that activates NFκB and ERK1/2.

CHAPTER IV

PREADIPOCYTES MEDIATE LPS-INDUCED INFLAMMATION AND INSULIN RESISTANCE IN PRIMARY CULTURES OF NEWLY DIFFERENTIATED HUMAN ADIPOCYTES

Absract

Recent data suggest that proinflammatory cytokines secreted from adipose tissue contribute to the morbidity associated with obesity. However, characterization of the cell types involved in inflammation and how these cells promote insulin resistance in human adipocytes are unclear. We simulated acute inflammation using endotoxin lipopolysaccharide (LPS) to define the roles of non-adipocytes in primary cultures of human adipocytes. LPS induction of the mRNA levels of proinflammatory cytokines (e.g., IL-6, IL-8, TNF-α, IL-1β) occurred primarily in the non-adipocyte fraction of newly differentiated human adipocytes. Non-adipocytes were characterized as preadipocytes based on their abundant mRNA levels of preadipocyte markers preadipocyte factor-1 (Pref-1) and adipocyte enhancer protein-1 (AEBP-1) and only trace levels of markers for macrophages and myocytes. The essential role of preadipocytes in inflammation was confirmed by modulating the degree of differentiation in the cultures from ~0-90%. LPS induced-proinflammatory cytokine expression and nuclear factor kappa B (NFκB) and mitogen activated protein kinase (MAPK) signaling decreased as differentiation increased. LPS-induced cytokine expression in preadipocytes was associated with

1) decreased adipogenic gene expression, 2) decreased ligand-induced activation of a peroxisome proliferator activated receptor (PPAR) γ reporter construct and increased phosphorylation of PPARγ, and 3) decreased insulin-stimulated glucose uptake. Collectively, these data demonstrate that LPS induces NFκB- and MAPK-dependent proinflammatory cytokine expression primarily in preadipocytes, which triggers the suppression of PPARγ activity and insulin sensitivity in human adipocytes.

Introduction

Obesity and its associated metabolic pathologies are the most common metabolic diseases in the U.S., affecting over 50% of the adult population. One emerging feature of obesity is the linkage between obesity and chronic inflammation characterized by increased cytokine production and acute-phase inflammatory signaling in adipose tissue (reviewed in Wellen and Hotamisligil 2005; Trayhurn 2005). Thus, white adipose tissue (WAT) is no longer considered an inert depot of stored energy, but also an active endocrine organ secreting a diverse array of proinflammatory "adipokines" such leptin, interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, all of which have been linked to insulin resistance. However, the exact role of cells comprising adipose tissue in mediating inflammation and causing insulin resistance is still unclear.

Human WAT has been reported to be composed of ~50-70% adipocytes, ~20-40% stromal vascular (SV) cells (*i.e.*, preadipocytes, fibroblasts, nondifferentiated mesenchymal cells), and ~1-30% infiltrated macrophages (Hauner 2005). However, less

is known about the localization and secretory pattern of cytokines in adipose tissue. It has been suggested that non-adipocytes (e.g., SV cells and/or cells from the supporting matrix) in human WAT are the major producers of IL-6 and TNF-α rather than adipocytes (Fain et al. 2004). Similarly, preadipocytes have been reported to act as 'macrophage-like cells' and secrete an array of cytokines (Cousin et al. 1999). Conversely, it has been proposed that macrophages residing in adipose tissue are responsible for most of the secreted cytokines (Xu et al. 2003). Weisberg et al. (2003) reported that adipose tissue recruits circulating monocytes/macrophages from bone. Intriguingly, Charrière et al. (2003) reported plasticity of preadipocytes showing evidence that 3T3-L1 cells have the ability to acquire phagocytic phenotypes and properties in the presence of macrophages.

Given these emerging data linking crosstalk between non-adipocytes and adipocytes with the development of obesity and insulin resistance, using primary cultures of newly differentiated human adipocytes as a cell model to investigate this linkage is timely. In accordance with the situation *in vivo* in adipose tissue, these heterogeneous cultures contain various percentages of non-adipocytes and adipocytes, depending on the differentiation protocol used. However, data on the types of cells in these cultures, and their role in triggering inflammation and insulin resistance are lacking.

Based upon our previous findings demonstrating that human adipocyte cultures robustly secrete cytokines that impair insulin sensitivity (Brown et al. 2004; Chung et al. 2005), and reports showing that preadipocytes are targets of inflammatory stimuli (Cousin et al.1999; Harkins et al. 2005), we focused on delineating the role of non-

adipocytes from WAT in inflammation and insulin resistance. To simulate acute inflammation, we treated the cultures with the bacterial endotoxin lipopolysaccharide (LPS). LPS has been reported to induce NFκB signaling through toll-like-receptors (TRL) in macrophages (Lee et al. 2003) and adipocytes (Lin et al. 2000; Berg et al. 2004), and has been linked to insulin resistance. However, the mechanism by which LPS induces inflammation and insulin resistance in human WAT is less clear.

To this end, we tested the hypothesis that LPS triggers proinflammatory cytokine expression predominately in non-adipocytes that triggers insulin resistance in adipocytes. We found that cytokine expression was predominately in the non-adipocyte fraction, which was primarily preadipocytes based on marker analyses. We also demonstrated that LPS-stimulated endotoxemia activated proinflammatory cytokine production via NFκB and MAPK signaling, predominately in preadipocytes, and decreased PPARγ activity and insulin sensitivity in adipocytes. These data demonstrate that human preadipocytes play a pivotal role in the development of insulin resistance in human adipocytes via increasing proinflammatory cytokine expression involving NFκB-and MAPK signaling.

Materials and Methods

Chemicals and Reagent

All cell cultureware and scintillation cocktail (ScintiSafe) were purchased from Fisher Scientific (Norcross, GA). Fetal bovine serum (FBS) was purchased from Cambrex/BioWhittaker (Walkersville, MD). Monoclonal antibody for CD68 was purchased from Research Diagnostics Inc. (Boston) and CD11b/Mac-1 was purchased

from BD Pharmingen (San Jose, CA). Rodamine red and fluorescein isothiocyanate-conjugated IgGs were purchased from Jackson Immunoresearch (West Grove, GA). Gene specific primers for real time PCR were purchased from IDT (Coralville, IA). DMAP and MG132 were purchased from Calbiochem (San Diego, CA). LY294002, U0126, and primary antibodies for p-IκBα kinase (IKK) α/β (Ser 180/ser181, rabbit), p-stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK) (Thr183/Tyr185, mouse), p-AKT (protein kinase B)(Ser473, rabbit), p-extracellular signal-related kinase (ERK) 1/2 (Thr 202/Tyr204 rabbit) were purchased from Cell Signaling Technology (Beverly, MA). Pref-1 monoclonal antibody was obtained from R&D Systems (Minneapolis, MN). All the other chemicals reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Cell Cultures

Abdominal adipose tissue was obtained from females with a body mass index (BMI) $\leq$ 30 during elective surgery with approval from the Institutional Review Board at the University of North Carolina-Greensboro. SV cells were isolated and cultured as previously described with minor modification (Brown et al. 2003). To induce ~50% differentiation (e.g., 50% of cells containing visible lipid droplets), confluent cultures of SV cells were supplemented with differentiation media-1 (DM1; 97% DMEM/Ham F-12, 3% FBS, 1 μM Rosiglitazone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, 33 μM biotin, 17 μM panthothenate, 100 nM insulin) for the first 3 days (referred to as DM1 in Fig 4.1 or AD50 in the other figures). To obtain maximum

differentiation, cultures of SV cells were exposed to DM1 for 6 days, which resulted in ~90% differentiation (AD90). To generate cultures that did not differentiate into adipocytes (referred to as –DM1 in Fig 4.1 or AD0 in the other figures), cultures of SV cells were supplemented with adipocyte media (AM1; 97% DMEM/Ham F-12, 3% FBS, 1 μM dexamethasone, 33 μM biotin, 17 μM panthothenate, 100 nM insulin) beginning on day 1 of differentiation until the assays were performed.

For the co-culture experiment (Fig 4.5C), SV cells were initially seeded as a mono-culture in individual cell culture inserts (Falcon 0.4 μm pores cat#3090, Fisher Scientific, Norcross, GA) and suspended above 6-well Multiwell^{TM} plates containing AM1 for 12 d (AD0). On day14, inserts containing the AD0 cultures were transferred to 6-well Multiwell^{TM} plates and co-cultured above AD50 cultures for 8 h, thereby allowing the AD0 and AD50 cultures to communicate with one another by sharing the same medium during treatment with LPS.

For the positive controls of macrophages, the U937 human monocyte line (ATCC CRL1593, Rockville, MD) was used. U937 cells were supplemented with RPMI 1640 medium containing 10% FBS and induced to differentiation by adding 10 nM phorbol 12-myristate 13-acetate (PMA) for 72 h.

Fractionation of SV Cells and Adipocytes Using Density Gradient

For fractionation of lipid-laden adipocytes from the non-differentiated SV cells, cultures grown in 100 mm plates (~3 million cells) were washed with ice cold HBSS/0.5mM EDTA and trypsinized with Trypsin-like enzyme. Cells were layered onto

the 6% iodixanol (Optiprep^{TM}, Oslo, Norway) in 0.5% BSA/HBSS (~1.03 g/ml) in the 15 ml centrifuge tube and centrifuged at 650 X g for 20 min at 4ºC. SV cells were collected from the pellet. The floating adipocytes were harvested from the top and delivered to microfuge tubes. To remove SV cell contamination and dead cell debris, adipocytes were resuspended with ice cold HBSS and centrifuge 5,000 X g for 5 min. Adipocytes were collected from the top of the microfuge tube where fat cells formed a fat film. Tri Reagent (Molecular Research Center Inc, Cincinnati, OH) was added to each fraction for RNA extraction.

Immunostaining

Cells were cultured on coverslips for immunofluorescence microscopy and stained as described previously (Brown et al. 2004). For double staining of Pref-1 and adipose tissue fatty acid binding protein (aP2), coverslips were first incubated with mouse-anti Pref-1 (1:10) overnight and stained with FITC-conjugated secondary antibody (1:500). Then, coverslips were blocked again and incubated with rabbit-anti aP2 for 2 h and stained with Rodamine red-conjugated secondary antibodies (1:500). For Mac-1 and CD68 immunostaining, 1:10 diluted antibodies were incubated overnight at 4ºC. Fluorescent images were captured with a SPOT digital camera mounted on an Olympus BX60 fluorescence microscope.

Immunoblotting and 4MUrea-SDS-PAGE

Immunoblotting was conducted as we previously described (Brown et al. 2004) using NuPage precasted gels (Invitrogen, Carlsbad, CA). To resolve PPARγ phospho-proteins, total cells extracts (75 ug protein) were subjected to 10% SDS-PAGE (acrylamide:bisacrylamide ratio was 100:1) containing 4 M urea and to electrophoresis at 80 V for 20 h. Separated proteins were subsequently transferred to PVDF membranes and immunoblotted with a monoclonal PPARγ antibody (Santa Cruz Inc, Santa Cruz, CA). The abundance of PPARγ was quantified from exposed X-ray flim using the KODAK image station 440 (Eastman Kodak Co).

$[2\text{-}^3H$ deoxyglucose] Uptake

Basal and insulin-stimulated glucose uptakes were measured as we described previously (Chung et al. 2005).

RNA Isolation and PCR

Total RNA was isolated from the cultures using Tri Reagent according to manufacturer's protocol for RT-PCR. 0.5 ug of total RNA from each RNA sample was used with the One-Step RT-PCR kit (Qiagen, Valencia, CA). Primer sets for aP2 were previously described (Brown et al. 2003). Primer sequences for Pref-1 (accession # NM_003836) were forward (5'TACGAGTGTCTGTGCAAGC), reverse (5' ACACAAGAGATAGCGAACACC) and running conditions were 37 cycles of $95^{\circ}C$ for 30 sec, $56^{\circ}C$ for 30 sec, $72^{\circ}C$ for 30 sec.

For real time qPCR, 1 ug total RNA was converted into first strand cDNA using Omniscript RT kit (Qiagen Inc). qPCR was performed in a Smartcycler (Cepheid, Sunnyvale, CA) using the QuantiTect SYBR Green PCR kit (Qiagen) for 40 cycles. To account for possible variation related to cDNA input amounts or the presence of PCR inhibitors, the endogenous reference gene glycerol aldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously quantified in a separate tube for each sample. Initial real-time amplifications were examined by agarose gel electrophoresis to confirm the sizes of the products. After PCR amplification, a melting curve was generated for every PCR product to check the specificity of the PCR. Primer sequences and running conditions are summarized in Table 1.

Transient Transfection and PPAR Activity

For measuring PPARγ activity, primary human adipocytes were transiently transfected with a 3X PPAR responsive luciferase reporter construct pTK-PPRE3x-Luc (Kliewer et al. 1992; generously provided by Dr. Susanne Mandrup) using the Amaxa Nucleofactor (Amaxa, Cologne, Germany) according to the manufacturer's protocol. On day 6 of differentiation, 1×10^6 cells from a 60 mm plate were trypsinized and resuspended in 100 µl of nucleofector solution (Amaxa) and mixed with 2 µg of pTK-PPRE3x-luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the V-33 nucleofector program (Amaxa). Cells were replated in 96-well plates after 10 minutes recovery in calcium-free RPMI medium. Two hours later, cultures were supplemented with charcoal-stripped AM1 before LPS stimulation. Firefly luciferase

activity was measured using the Dual-Glo luciferase kit (Promega, Madison, WI) and normalized to *Renilla* luciferase activity from the co-transfected control pRL-CMV vector. All luciferase data are presented as a ratio of firefly luciferase to *Renilla* luciferase activity.

<u>Statistical Analysis</u>

Unless otherwise indicated, data are expressed as the mean ± S.E. (n = 3-6) obtained from cultures containing cells isolated from 3-4 different subjects. Data were analyzed using one-way analysis of variance (ANOVA), followed by student's t-tests for each pair for multiple comparisons. Differences were considered significant if $p < 0.05$. All analyses were performed using JMP IN 4.04 (SAS Institute; Cary, NC) software.

Results

<u>Primary Cultures of Newly Differentiated Adipocytes Contain Preadipocytes and Adipocytes</u>

Our normal differentiation protocol using DM1 for the first 3 days of differentiation resulted in a cell population on day 12 containing ~50% adipocytes and ~50% non-adipocytes (cells without visible lipid droplets). Based on our findings that the non-adipocyte fraction of our cultures robustly express and/or secrete cytokines (e.g., IL-6, IL-8, TNF-α) in response to *trans*-10, *cis*-12 CLA treatment (Brown et al. 2004; Chung et al. 2005), we wanted to know if preadipocytes were present in this non-adipocyte or SV fraction. To answer this question, we first cultured the cells in the absence or presence of

DM1 for the first 3 days of differentiation, followed by 9 days of exposure to AM1 used to maintain the adipocyte phenotype. As shown in Fig 4.1A, about ~50% of the cells were differentiated into lipid-containing adipocytes by day 12 when exposed to DM1. In contrast, cells not supplemented with DM1 had few lipid-containing cells. Expression analyses and/or immunolocalization of gene markers associated with preadipocytes [Pref-1; a trans-membrane protein containing an epidermal growth factor (EGF)-like domain exclusively expressed in preadipocytes] and adipocytes (e.g., aP2, PPARγ) on day 12 revealed that cultures receiving DM1 had less mRNA (Fig 4.1B) or protein (Fig 4.1C, D) levels for Pref-1 and greater mRNA (Fig 4.1B) or protein (Fig 4.1C, D) levels of aP2 and PPARγ compared to cultures not receiving DM1. Taken together, these data demonstrate that cultures exposed to DM1 for 3 days and then AM1 for 9 more days contain both adipocytes and preadipocytes. In contrast, cultures receiving only AM1 for 12 days contained primarily preadipocytes.

Primary Cultures of Newly Differentiated Adipocytes do not Express Markers of Macrophages or Myocytes.

Next, we wanted to determine which cell types (other than preadipocytes) reported to produce cytokines were present in our cultures. To answer this question, we measured the expression and/or localization of markers of human macrophages (e.g., CD68, MAC-1) and myocytes (e.g., MyoD), cells known to secrete cytokines, in our differentiated cultures (+DM1). We measured CD68/MAC-1 and MyoD in differentiated human macrophages (U937 cells) and in RNA obtained from muscle as positive controls for

macrophages and myocytes, respectively. We measured CD68 and MyoD in RNA from muscle and hepatocytes (generously provided by Zen Bio Inc, RTP, NC) as negative controls for macrophages and myocytes, respectively. Very little mRNA for CD68 (Fig 4.2A, B) or MAC-1 (Fig 4.2A) was detected in our newly differentiated cultures of human adipocytes. mRNA levels of the myocyte marker MyoD were not detectable in our differentiated cultures (Fig 4.2C). Interestingly, RNA obtained from freshly isolated floating adipocytes (generously provided by Zen Bio Inc.) expressed significant amounts of mRNA for CD68, suggesting the presence of monocytes or lipid-laden macrophages in this fraction (Fig 4.2B). Collectively, these data suggest that our cultures of newly differentiated adipocytes contain negligible amounts of macrophages or myocytes.

Preadipocytes Play an Essential Role in LPS-Induced Cytokine Gene Expression and Insulin Resistance

In order to determine the capacity of preadipocytes and adipocytes in our differentiated cultures to express cytokine genes (and secrete cytokines) reported to cause insulin resistance, we first developed a procedure to separate SV cells (preadipocytes) from adipocytes obtained from our differentiated cultures (Fig 4.3A). Next, we treated the cultures with LPS, separated the SV cells from the adipocytes, and measured the mRNA levels for several cytokines, preadipocyte markers, and adipogenic genes in these two fractions (Fig 4.3B). As shown in Fig 4.3A, our fractionation procedure using 6% Iodixanol yielded an SV fraction (SVF) in the pellet containing cells with little mRNA for the adipocyte marker aP2 and significantly more mRNA for the preadipocyte markers

adipocyte-enhancer binding protein (AEBP-1) and Pref-1 compared to the buoyant adipocyte fraction (ADF), which had more aP2 and less AEBP-1 and Pref-1. LPS robustly induced the expression of IL-6, IL-8, TNF-α, IL-1β, and cyclooxygenase (COX)-2, genes positively associated with inflammation and NFκB activation, in the SVF compared to the ADF (Fig 4.3B). Conversely, the expression levels of adiponectin (APM-1) and PPARγ, almost exclusively expressed in the ADF, were attenuated by LPS treatment. These data demonstrate the capacity of preadipocytes to generate inflammatory signals and their association with the suppression of markers of insulin sensitivity in human adipocytes.

To further investigate the role of preadipocytes in inflammation, we established three human (pre)adipocyte models by manipulating the duration and exposure to DM1. Using this protocol, cultures on day 14 had ~0 (AD0), ~50 (AD50), or ~90% (AD90) adipocytes (Fig 4.4A). Intriguingly, LPS-stimulated TNF-α, IL-6, and IL-8 expression decreased as the degree of differentiation increased to 90% (Fig 4.4B). A similar trend was observed for TNF-α and IL-6 mRNA levels under basal conditions. These data provide further support for the notion that preadipocytes are the major inducers of inflammation in primary cultures of human adipocytes.

To determine the impact of LPS-induced cytokine production in preadipocytes on insulin sensitivity in adipocytes, we measured [2-^{3}H] deoxyglucose uptake in our three models (Fig 4.5). As expected, AD0 cultures, which are primarily preadipocytes, showed a blunted response to insulin-stimulated glucose uptake (Fig 4.5A, B). However, even this small increase in insulin-stimulated glucose uptake was attenuated by LPS. In AD50

cultures, insulin's stimulation of glucose uptake was suppressed ~30% by LPS (Fig 4.5A), which was consistent with its attenuation of adiponectin gene expression shown in Fig 4.3B. Intriguingly, in our AD90 model where insulin-stimulated glucose uptake was 3-fold higher than in the AD50 model (Fig 4.5B), LPS had no adverse effect on glucose uptake (Fig 4.5A). Consistent with these data, co-culturing preadipocytes (AD0) using inserts (AD0-insert) with the AD50 cultures suppressed LPS-mediated glucose uptake by another 30% compared to LPS-treated cultures without inserts (Fig 4.5C). Collectively, these data demonstrate that preadipocytes are required for LPS suppression of insulin-stimulated glucose uptake, and suggest that proinflammatory cytokines originating in preadipocytes mediate insulin resistance in adipocytes.

LPS Decreases the Activity and Increases the Phosphorylation of PPARγ

LPS suppression of adipogenic gene expression (Fig 4.3B) and insulin-stimulated glucose uptake (Fig 4.5) suggested that LPS may decrease the activity of PPARγ, which is essential for insulin-stimulated glucose uptake and TG synthesis in adipocytes. To answer this question, basal and ligand-induced activation of PPARγ activity were measured in AD50 cultures transfected transiently with a luciferase reporter construct containing 3XPPRE. We consistently obtained ~65% transfection efficiency revealed by parallel transfections with a green fluorescent protein (GFP) reporter construct (Fig 4.6A). Both adipocytes and preadipocytes were equally transfectable using this protocol, based on aP2 immunostaining and DAPI nuclear staining (Fig 4.6A). Although basal levels of

PPARγ activity were not affected by LPS, it decreased Rosiglitizone-stimulated PPARγ activity in a dose-dependent manner (Fig 4.6B).

To determine the extent to which LPS decreased PPARγ activity by increasing PPARγ phosphorylation, AD50 cultures were treated with and without LPS for 3 h and the isolated cell proteins were separated by SDS PAGE-Urea gel electrophoresis to detect band shifts in PPARγ. As seen in Fig 4.6C, LPS caused a band shift of PPARγ 1 and 2, which was attenuated or lowered by treatment with alkaline phosphatase (AP). These data indicate that LPS may decreases the activity of PPARγ by increasing its degree of phosphorylation, and suggests a mechanism by which LPS impairs insulin sensitivity. However, the upstream signaling mechanism linking LPS-induced cytokine production in preadipocytes to decreased PPARγ activity and insulin sensitivity in adipocytes remains unknown.

LPS-Induced Cytokine Gene Expression Depends on NFκB Signaling in Preadipocytes

Although well-characterized in macrophages, the regulation of NFκB activation and signaling in adipose tissue is less clear. Berg et al. (2004) reported altered NFκB sensitivity to LPS-induced signaling in adipocytes during differentiation using the murine 3T3-L1 cell line. However, NFκB sensitivity to LPS in primary cultures of human adipocytes has not yet been established. To determine the extent to which preadipocytes and adipocytes contributes to NFκB and MAPK activation and subsequent cytokine expression in mixed culture (AD50), we measured protein phosphorylation kinetics associated with NFκB and MAPK (e.g., JNK, ERK1/2) signaling in AD0, AD50 and

AD90 cultures treated with LPS. Consistent with the inflammatory cytokine expression profile in Fig 4.4B, the degree to which LPS induced the phosphorylation of IκBα kinase (IKK), JNK, and ERK, and the degradation of IκB decreased as the degree of differentiation increased from ~0–90% (Fig 4.7). NFκB and MAPK activation reached its maximum after 1 h of LPS treatment in all three models, albeit at much lower levels in the AD50 and AD90 cultures compared to the AD0 cultures. These data provide additional evidence that the presence of preadipocytes in cultures of human adipocytes modulates the susceptibility of LPS-induced NFκB and MAPK activation that trigger cytokine production.

Next, we determined the extent to NFκB and MAPK activation contributed to the LPS-inducted cytokine expression using the selective chemical inhibitors of NFκB and MAPK. LPS is known to act as TLR4/2, in (pre)adipocytes (Lin et al. 2000), which triggers NFκB activation through MAPK and phosphatidyl 3-kinase (PI3K)/AKT pathway (Guha et al. 2002; Fang et al. 2004). As seen in Fig 4.8A, the proteasome inhibitor MG132 abolished LPS-induced TNF-α gene expression, implicating proteasomal degradation of IκBα is crucial for NFκB activation and subsequent induction of TNF-α gene expression in human adipocytes. MG132 treatment also decreased the expression of IL-6 and IL-8, genes also regulated by NFκB, but not to the extent of TNF-α. The MEK/ERK inhibitor U0126, and the JNK inhibitor DMAP also attenuated LPS-induced cytokine production. The PI3K inhibitor LY-294002 blocked LPS-induced TNF-α gene expression by ~50%, but had only minimal effects on IL-6 or IL-8. Collectively, these results suggest that NFκB and MAPK activation and signaling

106

play an essential role in LPS-mediated proinflammatory cytokine expression in preadipocytes and insulin resistance in human adipocytes.

Table 4.1. List of human-gene specific primers for qPCR

Target mRNA	Sense primer	Antisense primer	GeneBank	Anneal Tm	Size (bp)
AEBP-1	f-atg ggt gat gta cac caa cgg cta	r-agt ggg tag atg cgg atg aaa cga	NM_001129	61	123
aP2	f- cct ggt aca tgt gca gaa at	r- aga gtt caa tgc gaa ctt ca	NM_001442	56	169
CD68	f-gct ggc tgt gct ttt ctc g	r-gtc acc gtg aag gat ggc a	NM_001251	60	111
Mac-1	f-act tgc agt gag aac acg tat g	r-aga gcc atc aat caa gaa ggc	NM_000632	58	141
MyoD	f-cgg cgg aac tgc tac gaa g	r-gcg act cag aag gca cgt c	NM_002478	60	172
IL-6	f- aaa tgc cag cct gct gac gaa	r- aac aac aat ctg agg tgc cca tgc tac	NM_000600	63	150
IL-8	f- gaa tgg gtt tgc tag aat gtg ata	r- cag act agg gtt gcc aga ttt aac	NM_000584	60	129
IL-1β	f- cgc caa tga ctc aga gga aga	r- agg gcg tca ttc agg atg aa	NM_000576	60	144
TNF-α	f- tct tct cga acc ccg agt ga	r- cct ctg atg gca cca cca g	NM_000592	60	151
Cox-2	f- agt ccc tga gca tct acg gtt t	r- ccc att cag gat gct cct gtt	NM_00963	58	123
APM-1	f- gca gag atg gca ccc ctg	r-ggt ttc acc gat gtc tcc ct	NM_004797	60	80
GAPDH	f- gag aag gct ggg gct cat	r- tgc tga tga tct tga ggc tg	NM_002046	59	130
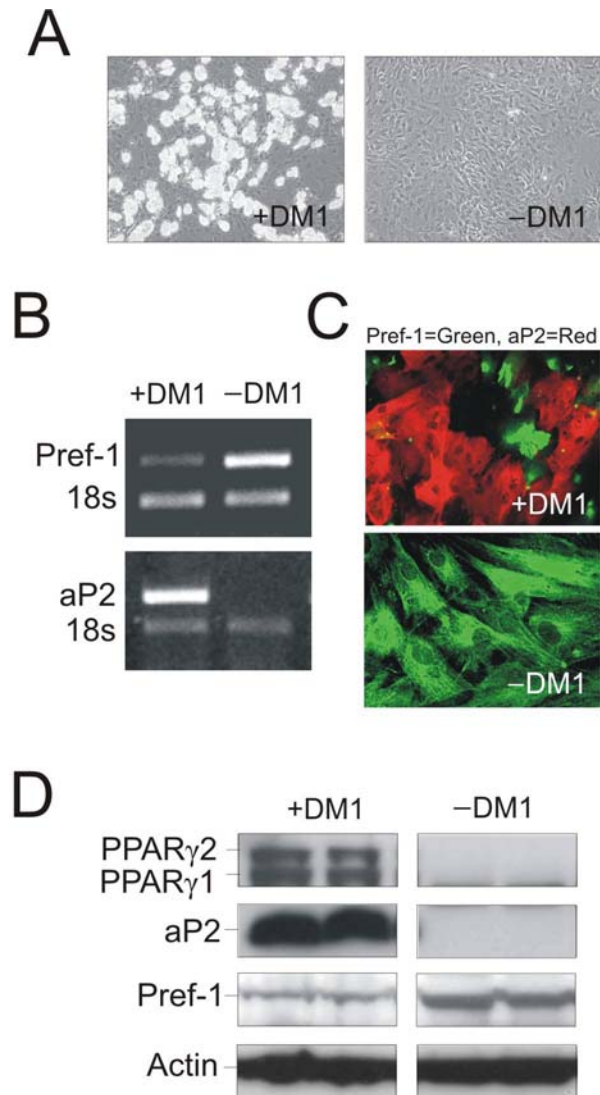
Figure 4.1. Primary cultures of newly differentiated human adipocytes are composed of adipocytes and preadipocytes. Stromal vascular (SV) cells were isolated from human subcutaneous adipose tissue. Confluent SV cells were either induced to differentiation (+DM1) or kept in adipocyte medium (-DM1) for 12 days. **A:** Morphological changes observed using phase contrast microscopy (10X), **B:** Gene expression of preadipocyte factor-1 (Pref-1) and adipocyte specific fatty acid binding protein (aP2) using RT-PCR, **C:** Immunolocalization of Pref-1(green) and aP2 (red), and **D**: Protein expression of peroxisome proliferator activated receptor gamma (PPARγ), aP2, Pref-1, and actin using western blotting. Data shown in all panels are representative of three or four independent experiments.
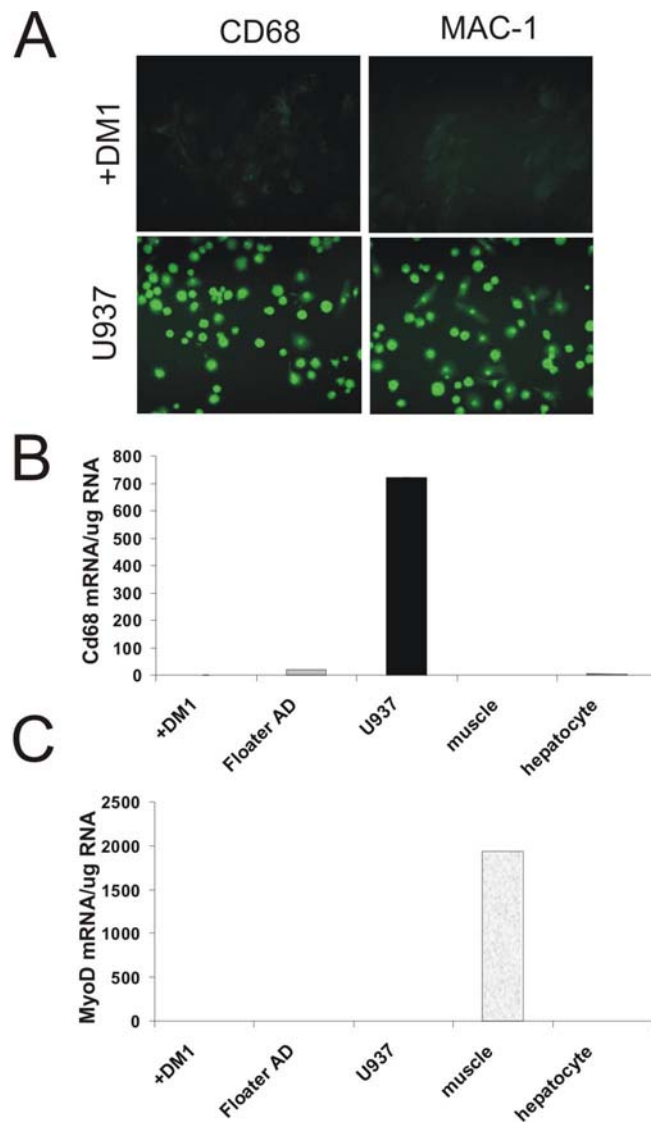
Figure 4.2. Primary cultures of newly differentiated human adipocytes do not express markers of macrophages or myocytes. A: Differentiated cultures of human adipocytes (+DM1) were immunostained with the macrophage-specific markers CD68 or Mac-1. Differentiated U937 cells (human macrophage cell line) were used as positive controls, **B:** Gene expression profile of CD68 from RNA from our differentiated cultures (+DM1) compared to RNA from freshly isolated, floating human adipocytes (Floater AD), differentiated human U937 cells (U937), human muscle, and human primary hepatocytes by real time qPCR, **C:** Gene expression profile of the muscle marker MyoD in our differentiated cultures (+DM1) were compared to RNA from the cultures or tissues described in Panel B. Data shown in all panels are representative of at least two independent experiments.
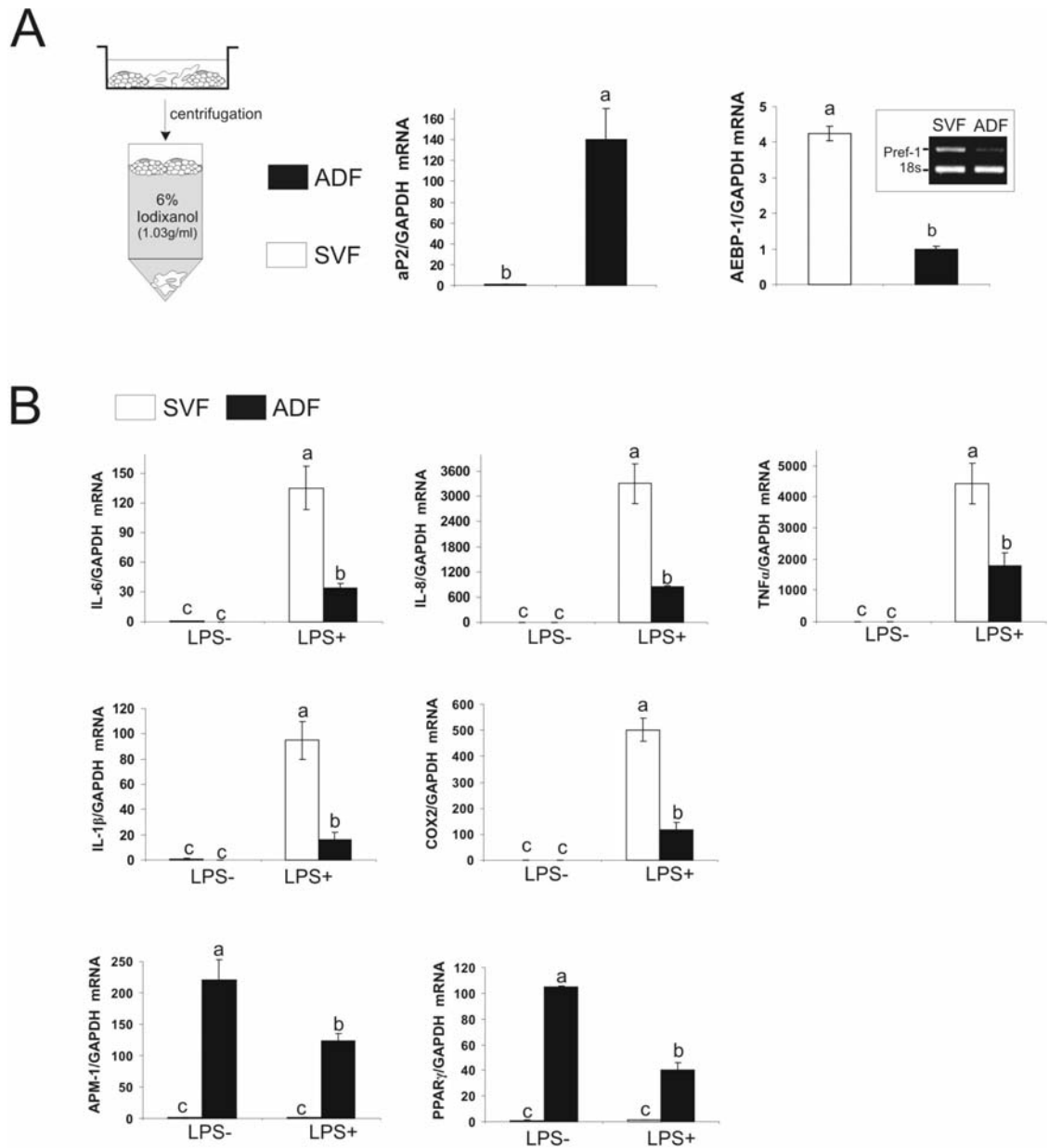
Figure 4.3. LPS stimulates inflammatory cytokine gene expression predominantly in the stromal vascular fraction (SVF) obtained from primary cultures of newly differentiated human adipocytes. Differentiated cultures of human adipocytes were fractionated using 6% Iodixanol (1.03g/ml). Lipid-laden adipocytes fraction (ADF ■) were floated, leaving the stromal vascular fraction (SVF □) as pellets. **A:** Fractionations were verified by measuring the gene expression of aP2 and adipocyte enhancer-binding protein1 (AEBP-1) using real time qPCR and Pref-1 using RT-PCR, **B:** Cultures of differentiated human adipocytes (day 14) were incubated in the presence or absence of LPS (10 ng/ml) for 3 h before fractionation. Relative mRNA expression of interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), cyclooxygenase 2 (COX2), adiponectin (APM-1), and PPARγ were investigated using qPCR. Means ($\pm$ SEM, n=4) not sharing a common superscript differ significantly (p<0.05). Data are representative of two separate experiments using a mixture of cells obtained from 4-5 human subjects.
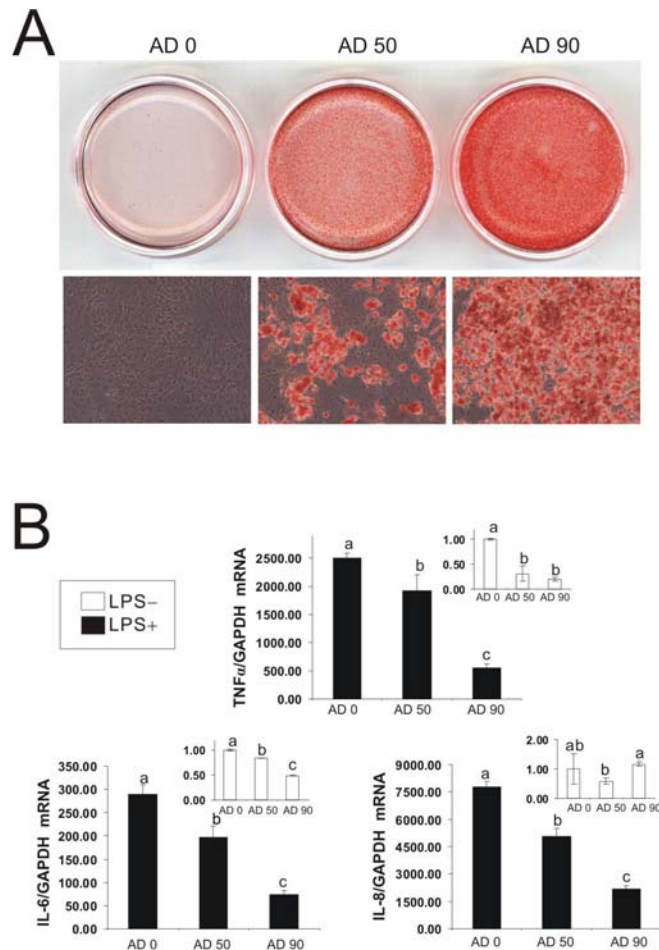
Figure 4.4 LPS induction of cytokine gene expression decreases as the degree of adipocyte differentiation increases. Three human (pre)adipocyte cell models containing ~0 (AD0), ~50% (AD50) or ~90% (AD 90) adipocytes were established by modulating exposure to differentiation medium (DM1; see Methods for details). **A**: On day 14, each culture was stained with Oil-red-O to show neutral lipid accumulation, **B**: On day 14, cultures of human (pre)adipocyts were incubated in the absence (□) or presence(■) with LPS for 3 h and total RNA were harvested for the analyses of TNF-α, IL-6, IL-8 mRNA expression using qPCR in each cell model. Means ($\pm$ SEM, n=4) not sharing a common superscript differ significantly (p<0.05). Data are representative of at least three separate experiments using a mixture of cells obtained from 4-5 human subjects.
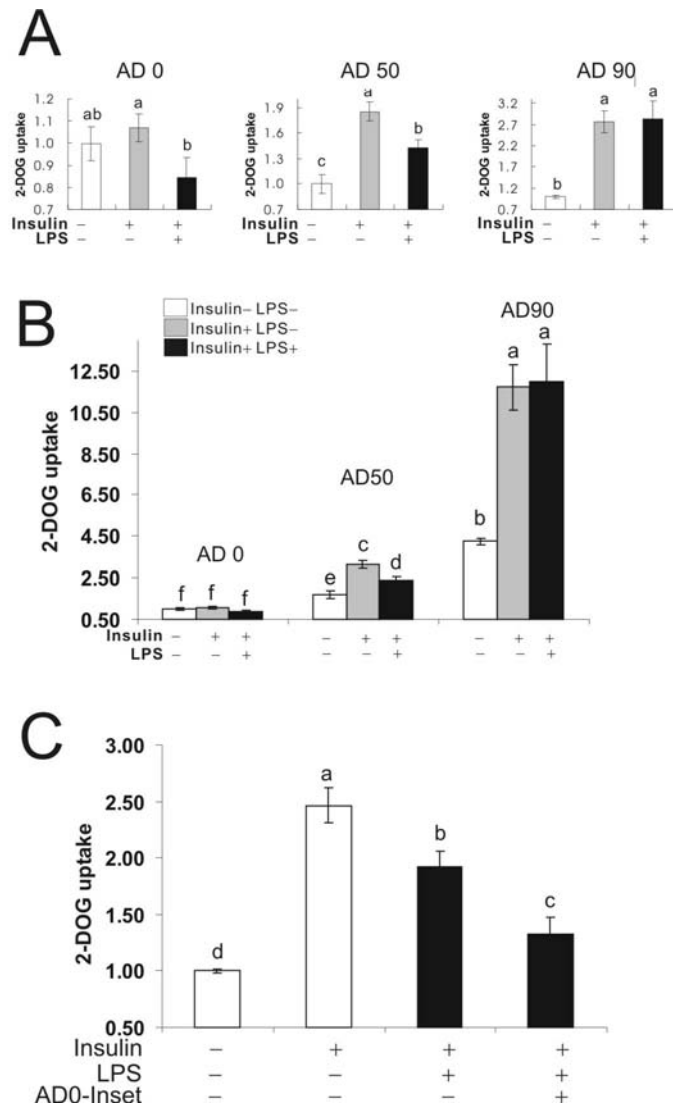
Figure 4.5. LPS suppression of insulin-stimulated glucose uptake and adipogenic gene expression decreases as the degree of adipocyte differentiation increases. Three human (pre)adipocyte cell models containing ~0 (AD0), ~50% (AD50) or ~90% (AD 90) adipocytes were established by modulating exposure to differentiation medium (DM1; see Methods for details). **A.** Culture were incubated on day 12 with low glucose medium (1000 mg/ml) for additional 48 h and stimulated with LPS for 8 h. Basal and insulin (100 nM)-stimulated glucose uptake of 4 nmol of [2-^{3}H] deoxyglucose (2-DOG) were measured for 90 min. Data are normalized to the basal glucose uptake level (-Insulin, -LPS) in each cell model. Means ($\pm$ SEM, n=4) not sharing a common superscript differ significantly (p<0.05). **B.** Data from panel **A** were normalized to the AD0 basal glucose uptake level to demonstrate the differences in absolute amounts of glucose uptake in each model. **C**. Cultures were grown and treated as in panel **A** with the following exceptions. Preadipocytes (AD0) were grown independently on inserts (AD0-inserts) for 12 days and then suspended about the AD50 cultures during the 8 h of LPS treatment.

113
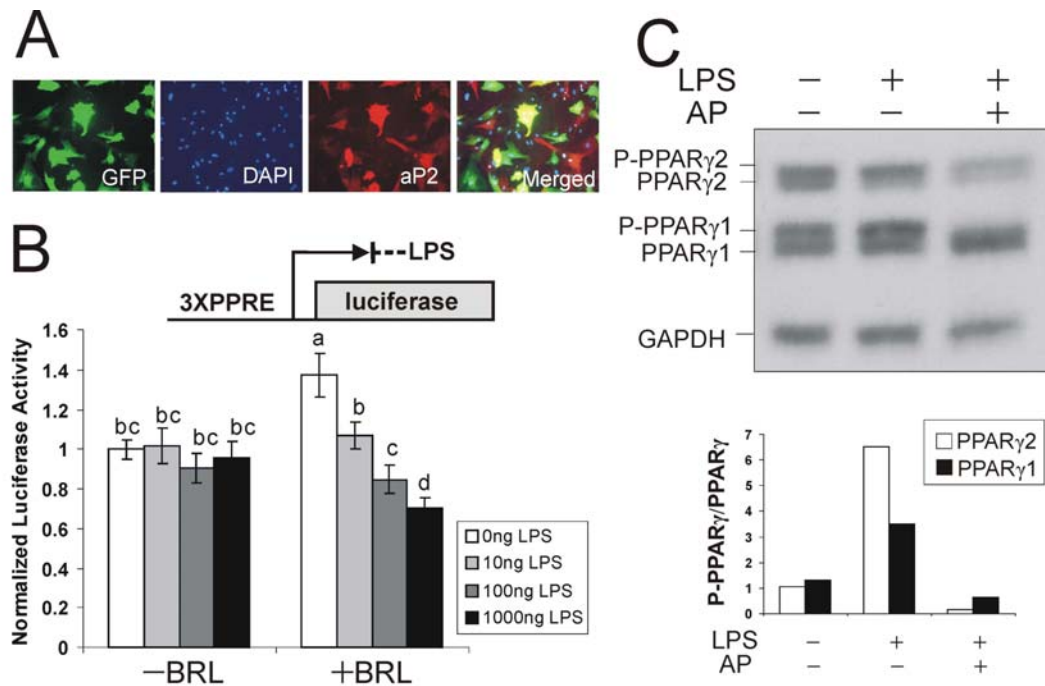
Figure 4. 6. LPS suppresses PPARγ activity and induces its phosphorylation. Cultures of human SV cells were induced differentiation for 3 days and then kept in adipocyte medium (AM1) for 3 more days. On day 6 of differentiation, cultures were transfected using Amaxa's nucleofactor. **A**: Cultures were transfected with a green fluorescent protein (GFP) reporter construct, and transfection efficiency was examined with DAPI and aP2 immunostaining. Data are representative of at least four different transfections. **B**: Cultures were transiently transfected with 3XPPRE reporter construct and stimulated with 0, 10, 100 and 1000 ng/ml LPS in the absence or presence of BRL (Rosiglitazone; 0.1 μM) for 3 h before the luciferase assay. Means ($\pm$ SEM, n=8) not sharing a common superscript differ significantly ($p<0.05$), **C**: Cultures of human adipocytes (day 12-14) were stimulated with either LPS (10 ng/ml) for 3 h. To remove phosphorylated protein, 75 ug of total protein was incubated with alkaline phosphatase (AP; 20unit/50ul) for 30 min at 37ºC and for 15 min at 55 ºC, and then subjected to 4M Urea-SDS-PAGE for the separation of phsophorylated PPARγ bands. Blot was quantified by densitometry, and the ratio of P-PPARγ/PPARγ was expressed as a bar graph under each lane. Results shown are representative of two separate experiments.

114
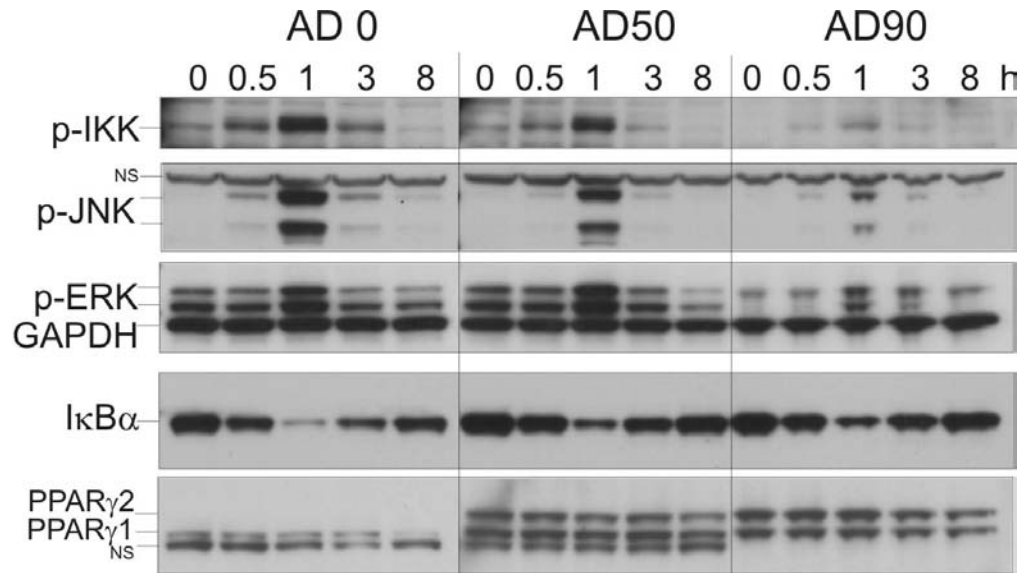
Figure 4. 7. LPS-induced NFκB activation decreases as the degree of differentiation increases. Three human (pre)adipocyte cell models containing ~0 (AD0), ~50% (AD50) or ~90% (AD 90) adipocytes were established by modulating exposure to differentiation medium (DM1; see Methods for details). Each culture was stimulated with LPS (10 ng/ml) for 0, 0.5, 1, 3, or 8 h and 15 ug of total proteins used for immunoblotting. Transferred PVDF membranes were probed with antibodies targeting to P-IKK, P-JNK, P-ERK, IκBα, PPARγ and GAPDH (loading control).
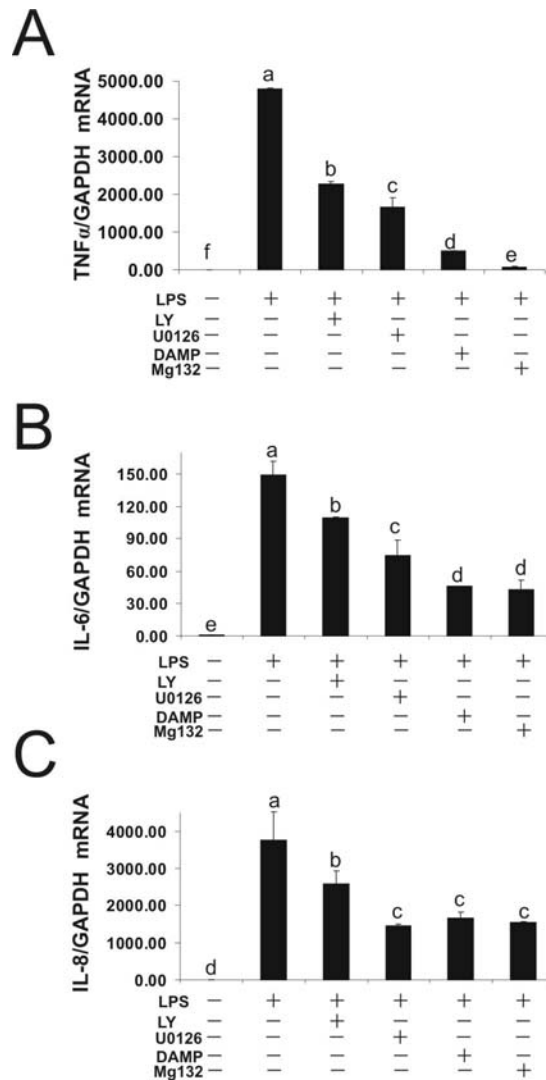
Figure 4. 8. Inhibitors of NFκB attenuate LPS-induction of cytokine gene expression. Cultures of newly differentiated human adipocytes on day 14 were preincubated for 1h in the absence or presence of the Akt inhibitor of LY-294002 (LY, 10 μM), the MEK-ERK inhibitor U0126 (10 μM), the JNK inhibitor DMAP (250 μM), or the proteasome inhibitor MG132 (10 μM) before LPS treatment (10 ng/ml, 3h). Total RNA was harvested and mRNA levels of TNF-α (**A**), IL-6 (**B**) and IL-8 (**C**) were analyzed using qPCR. Means ($\pm$ SEM, n=4) not sharing a common superscript differ significantly (p<0.05). Data are representative of one experiment using a mixture of cells obtained from 4-5 human subjects.
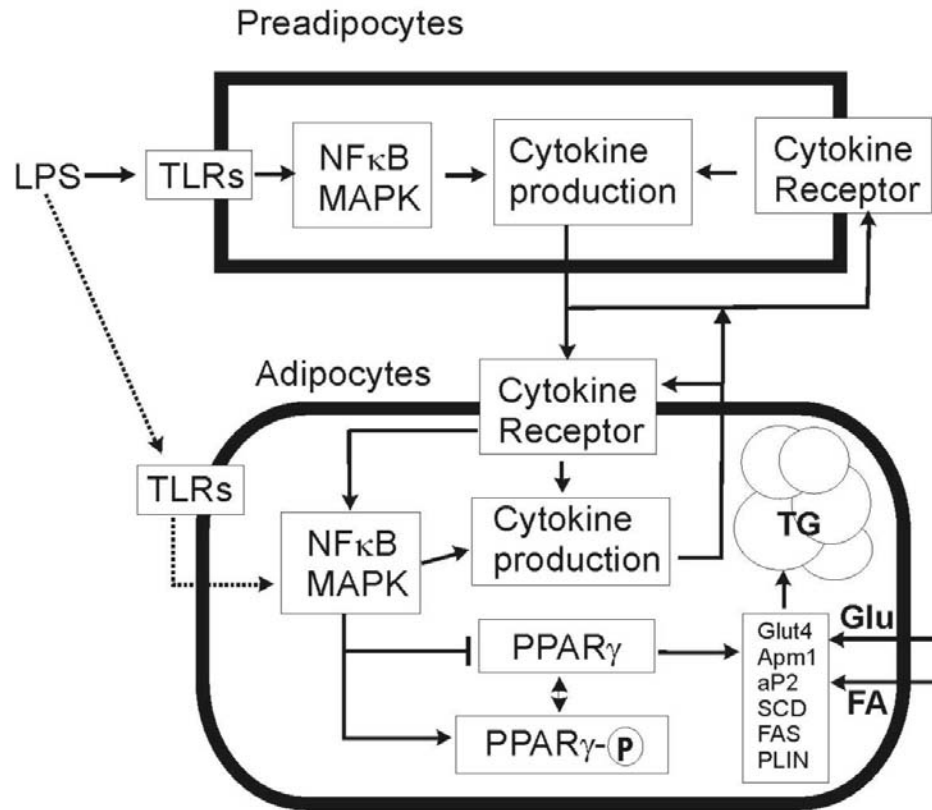
Figure 4. 9. LPS-induced inflammation in preadipocytes suppresses PPARγ activity and insulin sensitivity in adipocytes. Our results suggest that preadipocytes are more susceptible targets to LPS, increasing cytokine expression though NFκB and MAPK pathway. In turn, secreted cytokines activate NFκB and MAPK signaling in adipocytes leading to suppression of PPARγ activity, target gene expression (Glut4, glucose transporter 4; APM-1, adiponectin; aP2, adipocyte-specific fatty acid binding protein; SCD, stearoyl-coA desaturase; FAS, fatty acid syntase; PLIN, perilipin), and insulin sensitivity.

Discussion

Adipose tissue is a source of mediators of inflammation and insulin resistance. We previously demonstrated that a dietary trans fatty acid (*i.e.*, *trans*-10, *cis*-12 conjugated linoleic acid) induced IL-6 and IL-8 production predominantly from non-adipocytes, which was associated with insulin resistance in cultures of newly differentiated human adipocytes (Brown et al. 2004; Chung et al. 2005). By inducing acute inflammation with LPS in this study, we demonstrate for the first time that preadipocytes are the primary source of proinflammatory cytokines in these cultures (Fig 4.1-4.4), transmitting paracrine signals to neighboring adipocytes that suppress glucose uptake (Fig 4.5) and PPARγ activity (Fig 4.6) via NFκB and MAPK signaling (Fig 4.7-4.8). Based on these data, we propose a working model in Fig 4.9 showing that LPS initiates proinflammatory signaling through TLRs primarily in preadipocytes, which triggers activation of NFκB, MAPK, and PI3K pathways resulting in cytokine (i.e., TNF-α, IL-6, IL-8) production in preadipocytes. These cytokines, in turn, activate their cognate cell surface receptors on both adipocytes and preadipocytes, further augmenting cytokine production. In adipocytes, cytokine activation of NFκB, MEK/ERK, and JNK leads to decreased PPARγ activity, possibly by increasing PPARγ phosphorylation, thereby attenuating PPARγ target gene expression and insulin-stimulated glucose uptake.

Characterization of Non-Adipocytes in the Cultures

WAT is composed of several cell types. Of the cells residing in WAT, mature adipocytes are by far the largest in size, but their abundance depends on the specific

adipose depot and species. For example, Hauner (2005) reported that human adipocytes represent ~50-70% of the cells in human WAT. In contrast, Fain et al. (2002) reported that ~70% of the protein from human WAT digests is associated with tissue matrix, and the remaining 30% was equally divided between SV cells and floating adipocytes. Interestingly, non-adipocytes from human WAT, especially from the tissue matrix, accounted for ~90% of the cytokine secretion (Fain et al. 2004). Furthermore, the more robust cytokine secretion from WAT obtained from morbidly obese subjects [e.g., body mass index (BMI) = 45] compared to obese subjects (BMI = 32) was from the non-adipocytes. In contrast, Granneman et al. (2004) reported that mature adipocytes account for only 16% of murine epididymal WAT cells. Thus, the relative abundance of adipocytes compared to non-adipocytes in human WAT has yet to be resolved, and the type and function of these non-adipocytes in human WAT are poorly understood.

Based on these gaps, we first characterized the cells in our cultures, and then examined the role that the non-adipocytes play in inflammation and their impact on signaling, gene expression, and glucose metabolism in neighboring adipocytes. Initially, we characterized the cultures using DM1 during the first 3 days of differentiation (+DM1) compared to cultures not receiving DM1 (–DM1). Cultures receiving DM1 had about ~50% of their cells (Fig 4.1A) filled with lipid. In contrast, few of the cells lacking DM1 had visible lipid droplets. As shown in Fig 4.1B-D, cultures receiving DM1 abundantly expressed markers of adipocytes (e.g., PPARγ, aP2) and lesser amounts of the preadipocyte marker Pref-1 compared to cultures lacking DM1. Thus, our differentiated cultures contain both adipocytes and preadipocytes.

To determine if non-adipocytes other than preadipocytes were present in our cultures receiving DM1, we measured the expression levels of markers of macrophages and myocytes using CD68/MAC-1 and Myo-D, respectively. As shown in Fig 4.2, neither CD68/MAC-1 nor Myo-D mRNA were detectable in our cultures. Thus, we could find no evidence that macrophages or myocytes are present in our cultures on day 12 of differentiation. Clearly, this is not the situation *in vivo*, where immune cells may be recruited to WAT (Weisburg et al. 2003; Xu et al. 2003) and non-adipocytes in tissue matrix associated with adipose tissue robustly secrete cytokines (Fain et al. 2004). This discrepancy is most likely due to the loss of macrophages during growth and differentiation of the cultures. Therefore, because our model lacks of macrophages as occurs *in vivo* in adipose tissue, it provides a model for examining the role that preadipocytes play in inflammation and insulin resistance.

To access the role of non-adipocytes in inflammation and insulin resistance, cultures of newly differentiated human adipocytes (AD50) were initially fractionated after LPS stimulation and the expression of markers of preadipocytes (e.g., AEBP-1) and adipocytes (e.g., aP2, APM-1, PPARγ) and inflammatory cytokines (e.g., IL-6, IL-8, TNF-α, IL-1B) and markers (e.g., COX-2) were measured (Fig 4.3). These data are consistent with work by Harkins et al. (2004) showing that preadipocytes produce cytokines in response to LPS to a much greater extent than adipocytes using the 3T3-L1 cell line. These data demonstrate that preadipocytes have a greater inflammatory response to LPS than adipocytes in our cultures, and are associated with attenuated expression of adiponectin and PPARγ, two markers of insulin sensitivity.

Using a second approach, we manipulated the degree of differentiation of the cultures (e.g., AD0, AD50, or AD90) before LPS treatment, and then measured cytokine expression (Fig 4.4), glucose uptake (Fig 4.5), PPARγ activity and phosphorylation (Fig 4.6), and NFκB (Fig 4.7) and MAPK activation (Fig 4.7) and signaling (Fig 4.8). Both of these approaches gave consistent results demonstrating that the presence of preadipocytes in the cultures was positively associated with the degree of inflammatory gene expression, implicating preadipocytes as dominant source of cytokine production that adversely affect PPARγ activity and insulin sensitivity involving NFκB and MAPK activation and signaling.

Role of PPARγ in Inflammation and Insulin Resistance

Despite increasing evidence of the casual link between inflammation and insulin resistance, elucidating the precise mechanism by which cytokines impair glucose uptake has proved difficult (reviewd in Rajala and Scherer 2003; Wellen and Hotamisiligil 2005). Our data highlight the importance of preadipocytes in mediating insulin resistance. One possible explanation for this observation is the suppression of adiponectin gene expression by LPS, which is exclusively excreted from adipocytes and positively associated with insulin sensitivity (Ajuwon and Spurlock 2005 a,b). In addition to its role in the modulation of glucose and lipid metabolism, adiponectin has been reported to have potent anti-suppressive properties due to its ability to induce the production of anti-inflammatory cytokines (i.e., IL-10), and to inhibit proinflammatory cytokine production (Reviewed in Gil-Campos et al. 2004). Thus, it seems reasonable to presume that LPS

attenuation of insulin sensitivity in AD50 model is due, at least in part, by the suppression of adiponectin expression. Consistent with this notion, LPS administration to cultures containing almost exclusively adipocytes (AD90) did not adversely affect insulin-stimulated glucose uptake (Fig 4.5) or adiponectin gene expression (data not shown). Ajuwon and Spurlock (2005a) reported direct induction of PPARγ by adiponectin, coupled with suppression of NFκB activation, suggesting mutual transcriptional activation of PPARγ and adiponectin may determine adipocyte susceptibility to inflammatory stimuli.

The PPAR subfamily of nuclear receptors controls many different target genes involved in both lipid metabolism and glucose homeostasis. Loss of function, PPARγ mutations in humans cause insulin resistance (Zhang et al. 2004; Freedman et al 2005; reviewed in Hegele 2005), and activation of PPARγ by thiazolidinediones act as insulin sensitizers (reviewed in Yki-Jarvinen 2004). However, detailed mechanisms describing how inflammation suppresses PPARγ activity in human WAT are unclear. In our study, LPS suppressed ligand-induced, but not basal, PPARγ activity. Similarly, the PPARγ antagonist GW9662 decreased ligand-dependent, but not basal, PPARγ activity (our unpublished data), implicating ligand-inducible PPARγ activity is critical in regulating insulin sensitivity.

One of the putative mechanisms modulating PPARγ activity is phosphorylation. It has been suggested that phosphorylation of PPARγ 1) impairs PPARγ affinity for its ligand (Shao et al. 1998), 2) controls interactions between PPARs and corepressors and/or coactivators of transcription (Guan et al. 2005), or 3) alters PPARγ binding to the

PPRE (reviewed in Diradourian et al. 2005). ERK1/2 and JNK are two candidate transcription factors reported to phosphorylate PPARγ (Hu et al. 1996; Adams et al. 1997). Thus, LPS-mediated impairment of insulin sensitivity (Fig 4.5) and PPARγ activity (Fig 4.6B) may be due to changes in PPARγ affinity for its ligand via phosphorylation, as suggested by the data in Fig 4.6C. We propose that post-transcriptional modification of PPARγ activity through phosphorylation may be one of the mechanisms by which cytokines affect the transcription of genes involved in glucose and lipid metabolism. However, other potential mechanisms (*i.e.*, recruitment/dismissal of corepressors/activators or binding affinity to the PPRE) remain to be examined.

Role of NFκB and MAPK in Inflammation and Insulin Resistance

In addition to controlling gene expression, PPARγ has been linked to NFκB regulation through physical interactions which blocks its transcriptional activity (Ruan et al. 2003). Conversely, cytokine-induced NFκB activation suppresses PPARγ DNA binding (Suzawa et al. 2003). Consistent with these data, activation of NFκB (Ruan et al. 2002; Ruan et al. 2003; Bailey and Ghosh 2005) and MAPK (Camp and Tafuri 1997; de Mora et al. 1997; Adams et al. 1997) hinders PPARγ DNA binding affinity or transcriptional activation, providing a mechanism by which LPS-induced cytokine production suppresses PPARγ activity. The anti-inflammatory role of PPARγ is also demonstrated in this work showing that the more adipocytes in the culture, and consequently more PPARγ activity, the less robust NFκB signaling observed in Fig. 7. These data support recent findings showing that PPARγ mediates transcriptional

repression of NFκB target gene expression (Pascual et al. 2005). Also consistent with data from Berg et al. (2004) comparing 3T3-L1 preadipocytes to mature adipocytes, LPS activation of NFκB was substantially attenuated in human adipocytes compared to preadipocytes in our study. However, constitutive NFκB activation found in 3T3-L1 adipocytes (Berg et al. 2004) was absent in our human adipocyte cultures.

The mechanism by which LPS signals to its downstream targets in adipocytes has yet to be clearly established. In macrophages, LPS signals through TRL4. TRL4 activates at least two downstream pathways PI3K/AKT and IRAK1 (interleukin-1 receptor associated kinase 1) /TRAF6 (TNF receptor-associated factor 6)/NIK/IKK pathway, which depend on adaptor protein MyD88 (Lee et al. 2003). Additionally, Covert et al. (2005) suggested that MyD88-independent, but interferon-regulatory factor 3 (IRF3) dependent, pathways are involved in LPS activation of NFκB. Based on their work in macrophages, we used specific inhibitors to block potential pathways involved LPS signaling. Consistent with these data, activation of the MAPK pathway, including ERK and JNK, and NFκB were critical for LPS-induced cytokine expression in our cultures. PI3K/AKT pathway appeared to play a minor role judged by TNF-α gene expression, implicating IRAK1/TRAF6/NIK pathway may be the major signaling pathway by which LPS induces cytokine synthesis in human (pre)adipocytes. However, the role of MyD88-indepenent pathways in this study was not examined.

In summary, we have shown in cultures of newly differentiated human adipocytes that LPS-induced proinflammatory cytokine production is primarily from preadipocytes.

This proinflammatory response is mediated by NFκB and MAPK signaling and is associated with decreased PPARγ activity and insulin sensitivity in adipocytes.

EPILOGUE

The "Obesity epidemic' is not a foreign concept anymore in the United States or in most developing countries. Americans are consuming dietary supplements to lose weight or prevent weight gain, including supplements containing equal mixtures of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. Although conjugated linoleic acid (CLA) has been studied extensively for its potential use as a pharmacological strategy to prevent or cure human obesity, its efficacy and potential side effects are unclear.

Research from our group has been focused on identifying the mechanism by which CLA decreases human adiposity using an in vitro system. Our studies revealed that CLA inhibits preadipocyte differentiation (Brown et al. 2003), and promotes adipocyte delipidation (Brown et al. 2004). Based on these initial studies, investigations were first designed to determine CLA's impact on lipolysis and the changes in lipid-storage capacity of adipocytes following CLA treatment. The acute increase in lipolysis following CLA treatment was described in Chapter II. To address the second question, I developed immunocytochemistry techniques to examine cell-specific changes in the expression and localization of lipid droplet coating proteins. The expression of ADRP protein in mature human adipocytes had not been reported in the literature prior to my research. I discovered that atypical ADRP protein expression by CLA appeared to be regulated at the translational, rather than transcriptional, level. This was a novel finding

(Chung et al. 2005). Unfortunately, I was not able to obtain reliable data supporting a band shift of perilipin using a western blotting, which would demonstrate that CLA caused the phosphorylation of perilipin. Ideally, the best approach to determine the extent to which CLA increased perilipin phosphorylation would have been to immunoprecipitate perilipin and then probe with phospho-serine antibody to detect serine phosphorylation of perilipin. However, I was unable to perform immunoprecipitation due to the lack of resoures at that period.

During the first and second years of research, I was unable to correlate attenuation of TG deposition by CLA with insulin sensitivity. However, recent research has suggested that activation of mTOR pathway (translational activation) is positively associated with insulin resistance, showing that the insulin sensitivity is restored by rapamycin treatment, a specific inhibitor of mTOR pathway (Khamzina et al. 2005). Therefore, it would be interesting in future studies to investigate the impact of CLA-induced activation of mTOR pathway and ADRP protein expression on insulin sensitivity in human adipocytes in the presence or absence of rapamycin treatment.

Unexpectedly, our research with CLA revealed that it caused potentially undesirable metabolic consequences including insulin resistance and inflammation (Brown et al. 2005; Chung et al. 2005). Even though our in vitro results can not be directly applied to in vivo studies, our data are supported by several human clinical trials (Riserus et al. 2004a, b), suggesting that *trans*-10, *cis*-12 CLA may cause hyperglycemia, hyperlipidemia and inflammation (summarized in Chapter I).

Apart from our initial goal for this research (*i.e.*, development of new dietary treatment for obesity using CLA), we have started to examine mechanisms by which dietary fatty acids (*i.e. trans*-10, *cis*-12 CLA, saturated fatty acid) initiate insulin resistance through NFκB- and MAP Kinase-dependent inflammation. The investigations in Chapter III involved examining CLA's impact on IL-6 and IL-8 secretion and NFκB activation using chemical inhibitors and specific knockdown of NFκB by siRNA transfection. These studies revealed a mechanism by which CLA promotes insulin resistance; *e.g.*, through NFκB-dependent cytokine production. In the RNA interference study, I was able to transfect human adipocytes using NFκB siRNA, which had previously not been reported in the literature. If I had the time and resources, I would like to able to transfect the cells with a NFκB-luc reporter construct. Transfection with a NFκB reporter would allow a unique opportunity to investigate NFκB transcription activity in the presence and absence of CLA.

One of the interesting discoveries from our previous work (Brown et al. 2004) and research in Chapter III were the different capacities of preadipocytes and adipocytes for adipokines production in response to CLA. Theses observations led me to hypothesize that cytokine production from preadipocytes plays an important role in regulating adipocyte metabolism through paracrine interactions. Our story has been confirmed by simulating acute inflammation by LPS. In Chapter IV, the susceptibility of preadipocytes to inflammatory stimuli (*e.g.*, LPS) and their paracrine effects on adipocytes have been demonstrated by using a fractionation method to separate preadipocytes from adipocytes, or by modulating the degree of differentiation of the cultures. However, because our

differentiated cultures lack the macrophages, which are expected to reside in adipose tissue in vivo, it is difficult to extrapolate our in vitro results to in vivo physiological conditions. To tease out this problem, I propose future experiments. Using a co-culture system with human adipocytes and macrophages (U937 cells) in either compartment, I would like to investigate localization and quantification of cytokine production in each cell type in response to LPS or CLA, and signal cross-talk between each cell type. Such an experiment would allow us to determine the role that macrophages play in mediating inflammation and insulin resistance in adipose tissue.

In summary, I was able to demonstrate that CLA induces inflammatory gene expression and protein secretion, which mediates insulin resistance and delipidation in primary cultures of human adipocytes. It was consistent with the evolving concept that adipose tissue is a source of inflammatory products that play a central role in systemic insulin resistance. However, there are still large gaps in our understanding of the relationship between CLA induced inflammation and insulin resistance that beg the following questions. First, are there any specific receptors for CLA directly or indirectly mediate its induction of inflammation? Second, is CLA itself or its metabolites that trigger NFκB activation? Third, what is the role of chronic MEK/ERK activation in low grade chronic inflammation? Fourth, can CLA's side effect *(i.e,* insulin resistance) be prevented or attenuated with fish oil intervention, without altering CLA's delipidation action, given recent reports that fish oil blocks insulin resistance in CLA-fed mice (Yanagita et al. 2005)?

Concerning membrane specific receptors for CLA, no studies have been reported. We assumed that CLA uptake may be regulated by fatty acid transporters (*e.g.,* CD36) or by possibly fatty acid flip-flap. One hypothesis that I would like to test is whether CLA activates toll-like-receptors (TLRs), which are abundantly expressed both in preadipocytes and adipocytes (Lin et al. 2000), and mediate innate immunity. This hypothesis is supported by work by Lee et al. (2001) demonstrating that saturated fatty acids increases TLR4-mediated cyclooxygenase-2 (COX2) production, and unsaturated fatty acids inhibit TLR4 activation. Given the general concept that *trans*-fatty acids behave similar to saturated fat, it would be a great interest to investigate this possibility.

Several labs including ours have reported that *trans*-10, *cis*-12 CLA itself are found in phospholipid and neutral lipid fractions of the cultures, suggesting CLA is incorporated into cell membrane phospholipids or lipid droplets directly. Unfortunately, we lack the facilities to conduct the studies with radio-labeled CLA to identify its metabolites. Alternatively, by blocking fatty acid metabolism we might gain insights about the involvement of CLA metabolites in inflammation. It is expected that pretreatment of Triacsin C, a potent inhibitor of fatty acylCoA synthase, would provide a piece of information about the importance of CLA or its metabolites in inducing inflammation.

One of the distinguished features of CLA treatment in our study was the chronic activation of MEK/ERK. Acute ERK phosphorylation seems to be necessary for NFκB activation. However, the physiological importance of chronic ERK activation is not yet understood, especially with respect to its role in low degree inflammation. To uncover the

role of chronic ERK activation by CLA, the silencing of ERK using RNA interference followed by the examination of signaling cascade would be good strategy to take.

Polyunsaturated fatty acids are known to play an anti-inflammatory role. Parallel to this concept, it is suggested recently that docosahexaenoic acid (DHA) supplementation to the diet can attenuate CLA-induced fatty liver through the reduction of hepatic fatty acid synthesis without affecting adipokine production in C57BL/6N mice (Yanagita et al. 2005). In chapter III, I have proposed that inflammation contributes to adipocytes delipidation by CLA, but there is a possibility the two pathways (*i.e.*, inflammation and delipidation) are regulated in a distinct manner. Taking advantage of anti-inflammatory properties of fish oil, attenuation of adiposity by CLA without triggering inflammation might be achievable by co-supplementation with DHA.

My research questions focused on inflammation, and its impact on insulin resistance and type II diabetes. In the future, I would like to investigate potential factors secreted from visceral fat comparing to subcutaneous fat that promote insulin resistance in response to inflammatory stimuli. Recently, visfatin has been identified as an adipokine, which is predominantly secreted from visceral adipose tissue both in humans and mice. Visceral and subcutaneous adipose tissue display important metabolic differences that underlie the association of visceral obesity with obesity-related cardiovascular and metabolic alterations. Therefore, research revealing the association of visfatin to insulin resistance, and its mechanism of action offer promising data that could potentially provide insights about why visceral adipose tissue is a more important predictor of insulin resistance compare to subcutaneous adipose tissue. Another future

research topic I would like to examine is systemic regulation of inflammation. I have performed exciting experiments using human adipocyte cultures to explore the dynamics of CLA-induced signaling pathway mediating delipidation. However, cellular level research is limited its scope and dose not allow for understanding systemic responses to inflammation. Thus, studies using knockout animal would provide more global and physiological insights about critical clues that we would enhance our comprehension about the interrelation between obesity-induced adipocyte inflammation and diabetes, cardiovascular disease, and liver and islet dysfunction.

Insights obtained from the research reported in this dissertation and the proposed future research would have a potential to lead to novel investigations and therapeutic approaches that may improve our understating of Metabolic Syndrome.

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149

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