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Conjugated Linoleic Acid Promotes Human Adipocyte Insulin Resistance through NFκB-dependent Cytokine Production*

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We previously demonstrated that *trans*-10, *cis*-12 conjugated linoleic acid (CLA) reduced the triglyceride content of human adipocytes by activating mitogen-activated protein kinase kinase/extracellular signal-related kinase (MEK/ERK) signaling via interleukins (IL) 6 and 8. However, the upstream mechanism is unknown. Here we show that CLA increased (≥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 isomer-specific induction of IL-6 and tumor necrosis factor-α was associated with the activation of nuclear factor κB (NFκB) as evidenced by 1) phosphorylation of IκBα, IκBα kinase, 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 suppression of insulin-stimulated glucose uptake at 24 h was associated with decreased total and plasma membrane glucose transporter 4 proteins. Inhibition of NFκB activation or depletion of NFκB by RNA interference using small interfering NFκB p65 attenuated CLA suppression of glucose transporter 4 and peroxisome proliferator-activated receptor γ 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 peroxisome proliferator-activated receptor γ target gene expression and insulin sensitivity in mature human adipocytes.

Conjugated linoleic acid (CLA)[2] 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 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 (for review, see Ref. 1) and some humans (for review, see Ref. 2). We have reported that *trans*-10, *cis*-12 CLA, but not *cis*-9, *trans*-11 CLA, inhibited human preadipocyte differentiation (3) and caused delipidation of newly differentiated human adipocytes (4). CLA 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 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 (5–7), and atherosclerosis (for review, see Ref. 8). 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 (nuclear factor for IL-6 expression, a.k.a. CCAAT enhancer-binding protein β (C/EBPβ)), activator protein-1, and cAMP response element (CRE)-binding protein (CREB) depending on cell type and stimulus (9, 10). Activation of NFκB is critical for fatty acid-induced IL-6 secretion and insulin resistance in myotubes *in vitro* (11, 12) and muscle *in vivo* (13). Interestingly, several studies have shown that chronic exposure to IL-6 reduces adipogenic gene expression (14, 15) and insulin signaling and glucose uptake in adipocytes (16).

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 (for review, see Refs. 17 and 18). After phosphorylation by mitogen-activated protein kinases such as ERK1/2 and p38 or mitogen and stress-activated kinase 1 (MSK1) (for review, see Refs. 19 and 20), phospho-p50/p65 binds to the NFκB response elements of target genes, thereby inducing their transcriptional activation (for review, see Refs. 17 and 21).

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[2] The abbreviations used are: CLA, conjugated linoleic acid; aP2, adipocyte-specific fatty acid-binding protein; ERK, extracellular signal-related kinase; P-ERK, phosphorylated ERK; FITC, fluorescein; Glut4, insulin-dependent glucose transporter 4; GPCR, G protein-coupled receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; IL-8, interleukin-8; IRS-1, insulin receptor substrate-1; IκBα, inhibitory κB protein α; IKK, IκBα kinase; NEMO BP, NEMO (IKKγ) binding domain-binding peptide; MEK, mitogen-activated protein kinase kinase; NFκB, nuclear factor κB; PM, plasma membrane; PPARγ, peroxisome proliferator-activated receptor γ; RISC, RNA interference-induced silencing complex; siRNA, double-stranded small interfering RNA; STAT3, signal transducer and activator of transcription 3; SV, stromal vascular; TG, triglyceride; TNF-α, tumor necrosis factor α; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription; BSA, bovine serum albumin; siP65, p65 siRNA.

CLA TG-lowering actions in human adipocytes are consistent with NFκB activation based on our data (3, 4) 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 before inducing IL-6 expression and insulin resistance in cultures of 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 targeting NFκB p65. Last, our data suggest that non-adipocytes or stromal vascular (SV) cells secrete a significant amount of cytokines in response to treatment with *trans*-10, *cis*-12 CLA.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—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). [2-^{3}H]deoxyglucose and Western Lightning Plus Chemiluminescence substrate were purchased from PerkinElmer Life Sciences. 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. One-step RT-PCR kits were purchased from Qiagen Inc. (Valencia, CA), and ribosomal 18 S 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 (NIDDK, National Institutes of Health, Bethesda, MD). aP2 antibody was a generous gift from Dr. D. Bernlohr (University 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 (Ser-32) 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 (Ser-176/-180, Ser-177/-181)-IKK, and anti-IκBβ antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Polyclonal anti-phospho (Tyr-891)-IRS-1 was purchased from Oncogene Science (Cambridge, MA). Monoclonal anti-nucleoporin was obtained from BD Transduction Laboratories. 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, Bay11-7082, pertussis toxin, kamebakaurin, 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), nonspecific control pool (mock), and siGLO-RISC- free were purchased from Dharmacon (Lafayette, CO). All the other chemicals and reagents were purchased from Sigma unless otherwise stated.

Cell Culture of Primary Human SV Cells—Primary human SV cells were obtained from subcutaneous adipose tissues of non-obese (body mass index <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 (4) 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 fatty acid-free (>98%) bovine serum albumin (BSA) at a 4:1 molar ratio using 1 mM BSA stocks.

[2-^{3}H]Deoxyglucose 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 Dulbecco's modified Eagle's medium (1000 mg/liter D-(+)-glucose) in the presence or absence of 20 pM insulin. For the 72 h of treatment, BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA were added to adipocyte media for 48 h on day 12. Then, for an additional 24 h, cultures were incubated in 1 ml of serum-free basal Dulbecco's modified Eagle's medium containing 1000 mg/liter D-(+)-glucose with or without 20 pM 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. After the experimental treatments, [2-^{3}H]deoxyglucose uptake was measured as described previously (4).

Plasma Membrane (PM) Isolation and Glut4 Levels—For the detection of Glut4 translocation, PM was fractionated according to Carvalho *et al.* (23). Briefly, cultures were washed once with TES buffer (20 mM Tris-HCl, 1 mM EDTA, 225 mM sucrose, 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 the 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 Co.).

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 media directly. Conditioned media 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 Hanks' balanced salt solution, and cells were harvested and dedicated for protein determination. IL-6 and IL-8 secretion to the media was quantified using a commercial ELISA (R&D Systems) 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.* (24). Each minced explant was then preincubated in Dulbecco's modified Eagle's medium-Ham's F-12 (1:1) medium containing 100 units/ml penicillin, 100 units/ml streptomycin, 50 μg/ml gentamicin, and 0.25 mg/ml amphotericin-B at 37 °C overnight. Then explants were transferred to 10 ml of fresh media containing either BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA in culture tubes. Subsequently, conditioned media 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 (semiquantitative) RT-PCR was carried out using One-step RT-PCR kit (Qiagen Inc) as we described previously (3). Primer sets for adipose fatty acid-binding protein (aP2) were previously described (3). Primer sequences for IL-6 were (accession number NM_000600) sense (5′-CCAGCTATGAACTCCTTCTC) and 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 (4, 25). For phospho-IKK immunostaining, cells were permeabilized 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. 4*C*). For NFκB p65 and aP2 double staining (Fig. 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-labeled siGLO fluorescent, and phase contrast images were captured without fixation under the Olympus IX60 microscope equipped with a SPOT digital camera (Fig. 8*B*). For PPARγ immunostaining (Fig. 8*E*), transfected cells grown on coverslips were permeabilized 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 μg/ml Hoechst dye was used for nuclei staining (Fig. 8*E*).

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 1× 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™ 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 media (AM-1, Zen Bio Inc., Research Triangle Park, NC) were supplemented with either 25 nM NFκB p65 siRNA or non-targeting siRNA complexed with *Trans*IT-TKO (6 μl/ml), a non-lipid-based transfection reagent from Mirus Corp. Transfection reagent and undelivered siRNA were removed 24 h post-transfection by removing the media and washing the cells twice with Hanks' balanced salt solution. 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 the 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 (4). 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 [2-^{3}H]deoxyglucose uptake, phosphorylation of downstream targets of insulin, and Glut4 abundance in cultures of differentiated human adipocytes. As shown in Fig. 1*A*, insulin-stimulated [2-^{3}H]deoxyglucose uptake was significantly lower in cultures treated with 30 μM *trans*-10, *cis*-12 CLA for either 24 or 72 h compared with 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. 1*B*). 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 (16). The basal levels of Glut4 were markedly lower in all three cellular fractions from cultures treated with *trans*-10, *cis*-12 CLA (Fig. 1*C*) compared with BSA controls, consistent with CLA-induced reductions in Glut4 gene expression (4). 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 [2-^{3}H]deoxyglucose uptake after 24 h of treatment (~30%, Fig. 1*A*) was relatively similar to the degree to which it attenuated Glut4 abundance in both membrane fractions (~35%, Fig. 1*C*). 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*.

Trans-10, Cis-12 CLA Induces Cytokine Secretion and/or Expression in SV Cells, Adipocytes, and Tissue Explants—Recent reports, including our own data using human primary cultures (4), suggest that adipocytes are molecular targets for IL-6, resulting in insulin resistance (16). 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 (4), we did not know how early this occurred, which cells in the cultures were secreting these cytokines, or if CLA 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. 2*A*), whereas IL-6 and IL-8 secretion in control cultures increased only marginally with time (Fig. 2*A*). Accumulations of IL-6 and IL-8 in the media of cultures treated with *trans*-10, *cis*-12 CLA were apparent after 6 h of treatment, reaching a plateau after 24 h (Fig. 2*A*). Interestingly, *trans*-10, *cis*-12 CLA induced IL-6 and IL-8 secretion in non-differentiated cultures of SV cells (Fig. 2*B*) and adipose tissue explants (Fig. 2*C*) 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. 2*B*) compared

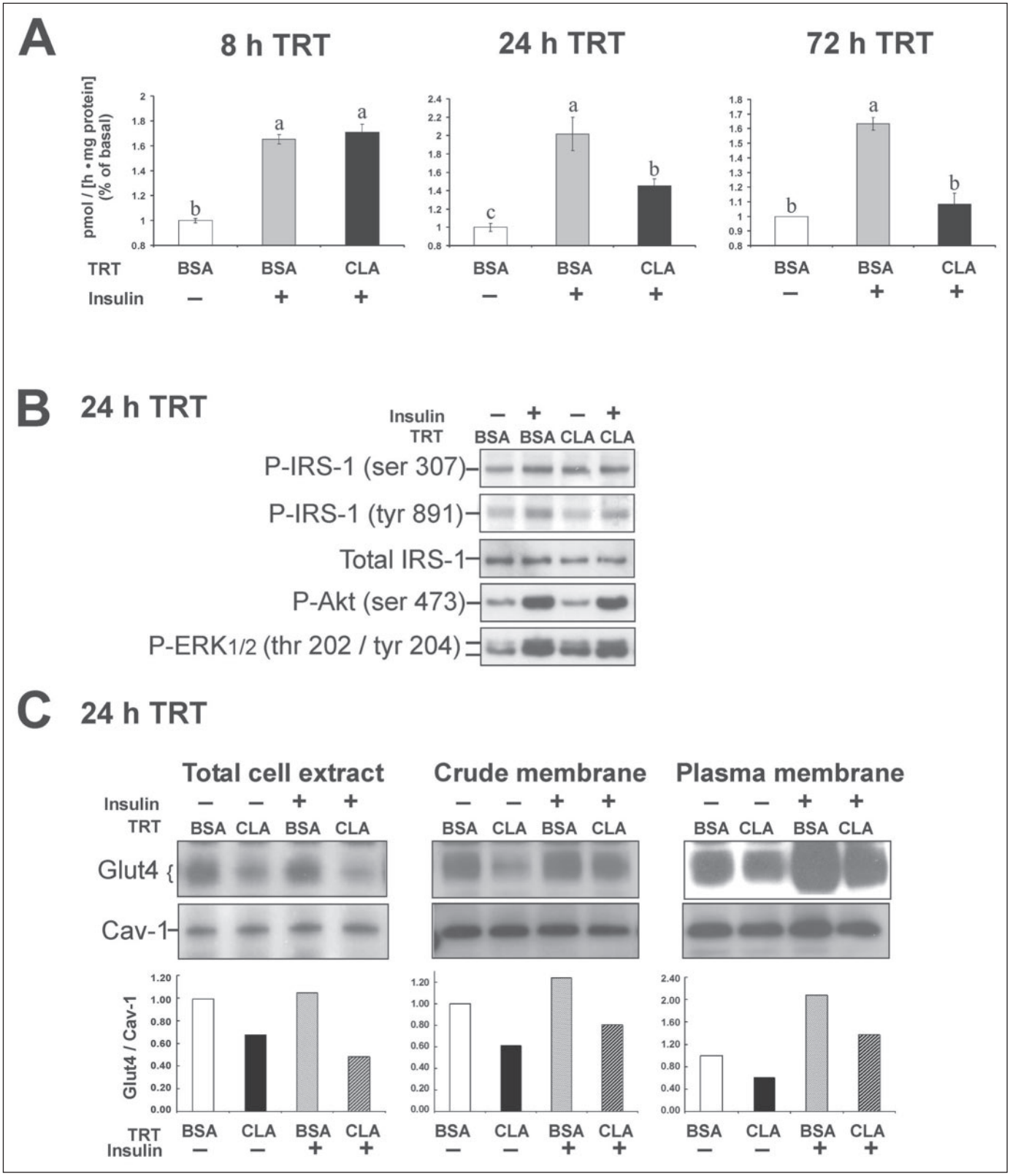

FIGURE 1. ***Trans*-10, *cis*-12 reduces insulin-stimulated glucose uptake and Glut4 protein expression.** Differentiated cultures of human adipocytes were treated for 8, 24, or 72 h with either a BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA. *A*, basal and insulin-stimulated uptake of 4 nmol of [2-^{3}H]deoxyglucose were measured for 90 min after treatment (*TRT*) 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 (BSA, −*insulin*) rate. Means (±S.E.; $n = 6$) not sharing a common *lowercase letter* differ, $p < 0.05$. *B*, after 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 (Ser-307), p-IRS-1 (Tyr-891), p-Akt (Ser-473), p-ERK1/2 (Thr-202,204), and total IRS-1. *C*, cultures were treated with either BSA vehicle or *trans*-10, *cis*-12 CLA for 24 h before 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 the amount of Glut4 relative to caveolin-1 was expressed as a *bar graph under* each *blot*.

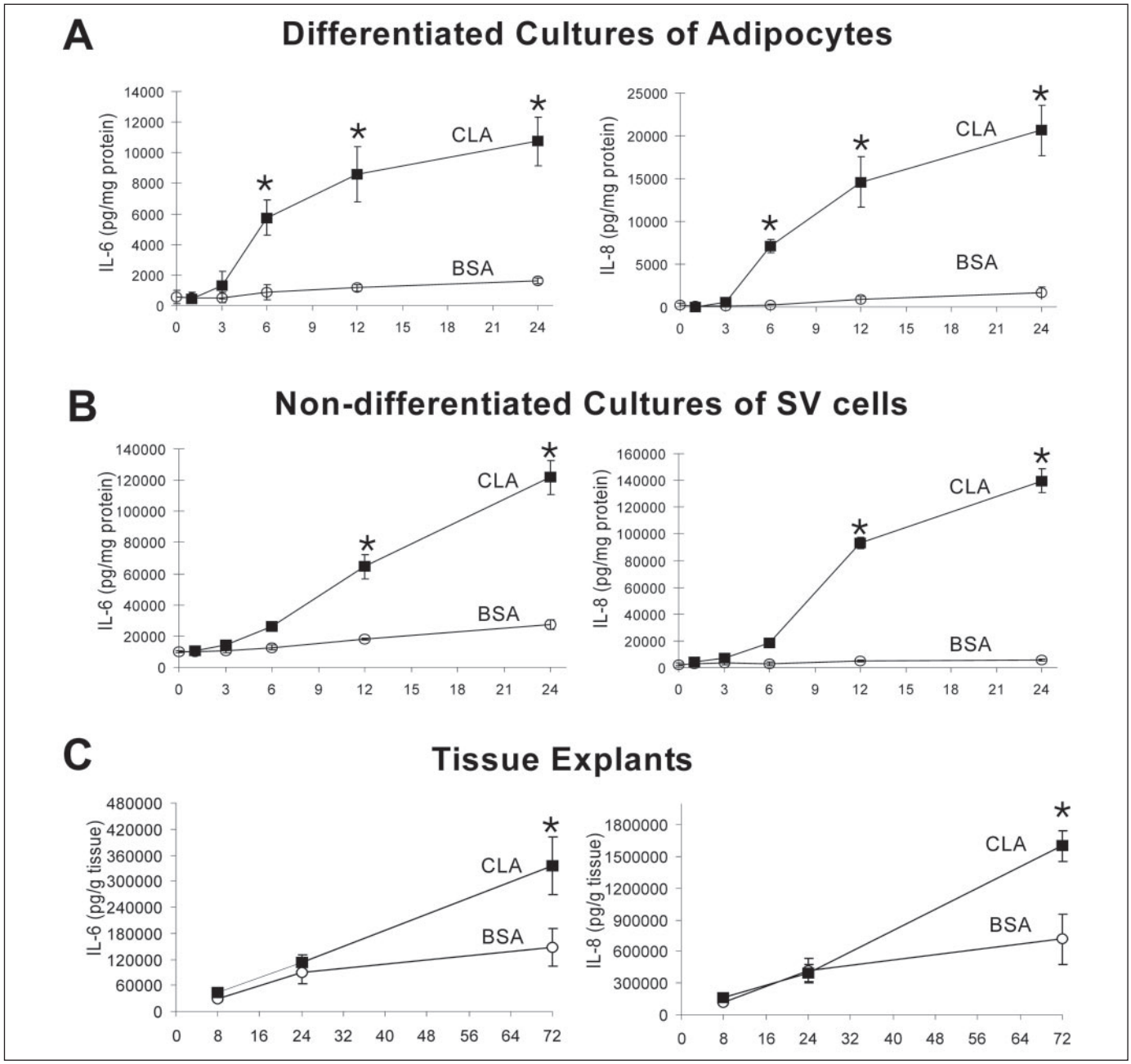

FIGURE 2. ***Trans*-10, *cis*-12 CLA increases IL-6 and IL-8 secretion.** Cultures were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for the indicated times (0–72 h), and IL-6 and IL-8 in the media were determined at each time point by ELISA from cultures of human differentiated adipocytes (*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* and *B*, $n = 9$) or from adipose tissue from three different subjects (*C*, $n = 8$). Data are expressed as pg of cytokine/mg of protein (*A* and *B*) for the cell culture studies and as pg of cytokine/g of tissue for the tissue explant studies. Means with *asterisks* differ significantly ($p < 0.05$) from the BSA controls at each time point.

with differentiated cultures of adipocytes treated with CLA (Fig. 2*A*). 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 (4).

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*A*, *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. 2*A*. 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 with those receiving the BSA vehicle (data not shown).

To determine the extent to which CLA 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 human recombinant TNF-α for 30 min. As seen in Fig. 3*B*, *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*B*) as well as in cultures of non-differentiated SV cells (data not

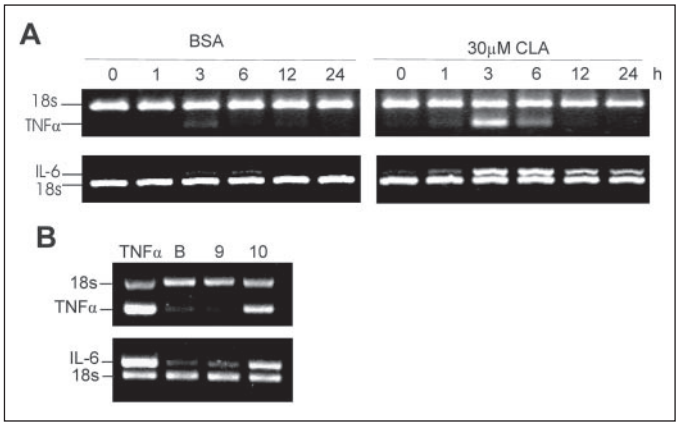

FIGURE 3. ***Trans*-10, *cis*-12 CLA increases TNF-α and IL-6 gene expression.** *A*, differentiated cultures of human adipocytes were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for the indicated times (0–24 h). mRNA levels of TNF-α and IL-6 were analyzed using semiquantitative RT-PCR. *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. 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 semiquantitative RT-PCR. Results shown are representative of three (*A*) or four (*B*) separate experiments from independent human subjects.

shown). Collectively, these data demonstrate for the first time that cells isolated from human adipose tissue secrete proinflammatory cytokines *in vitro* 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 (21); 2) transient TNF-α gene expression is positively linked to NFκB activation (26, 27); 3) ERK1/2 phosphorylation of nuclear p50/p65 is important for its activation (20); 4) NFκB-mediated IL-6 and TNF-α production are associated with insulin resistance in myotubes (11, 12, 28); 5) human adipose tissue is a target for IL-6-mediated insulin resistance (16). To test this hypothesis, cultures were treated with either BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA, and IκBα protein levels were 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. 4*A*), implying that 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 with cultures treated with *cis*-9, *trans*-11 CLA or BSA (Fig. 4*B*). 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 (Ser-176/180, Ser-177/181). *Trans*-10, *cis*-12 CLA treatment for 2 h increased the phosphorylation of IKKα/β compared with the vehicle controls (Fig. 4*C*), suggesting that *trans*-10, *cis*-12 CLA activates the IKK-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) (for review, see Ref. 17), 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′-GGGACTTTCC-3′) 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. 4*D*, 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. 4*E*, 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 with 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. 4*E* 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 the cytosol (Fig. 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. 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 induction of TNF-α (Fig. 6). Similarly, the GPCR-$G_{i/o}$ inhibitor pertussis toxin attenuated CLA-induced IL-6 gene expression without affecting CLA-induced TNF-α gene expression. Chemical inhibition of NFκB activation was performed using 1) proteasome inhibitor I, which blocks proteasomal degradation of IκBα, 2) Bay11-7082, which inhibits IκBα phosphorylation (29, 30), and 3) kamebakaurin, which prevents NFκB p50 DNA binding (Fig. 6). Chemical inhibition of NFκB activation abolished CLA-inducible IL-6 and TNF-α gene expression. However, pretreatment with antioxidant *N*-acetylcysteine did not block CLA induction of either TNF-α or IL-6 (data not shown), suggesting CLA does not activate NFκB or induce cytokines by producing prooxidants. 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 Suppression of Glut4 and PPARγ Protein Levels—Given the reported antagonistic interaction between PPARγ and NFκB (31), we investigated the role of NFκB in mediating *trans*-10, *cis*-12 CLA 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 syn-

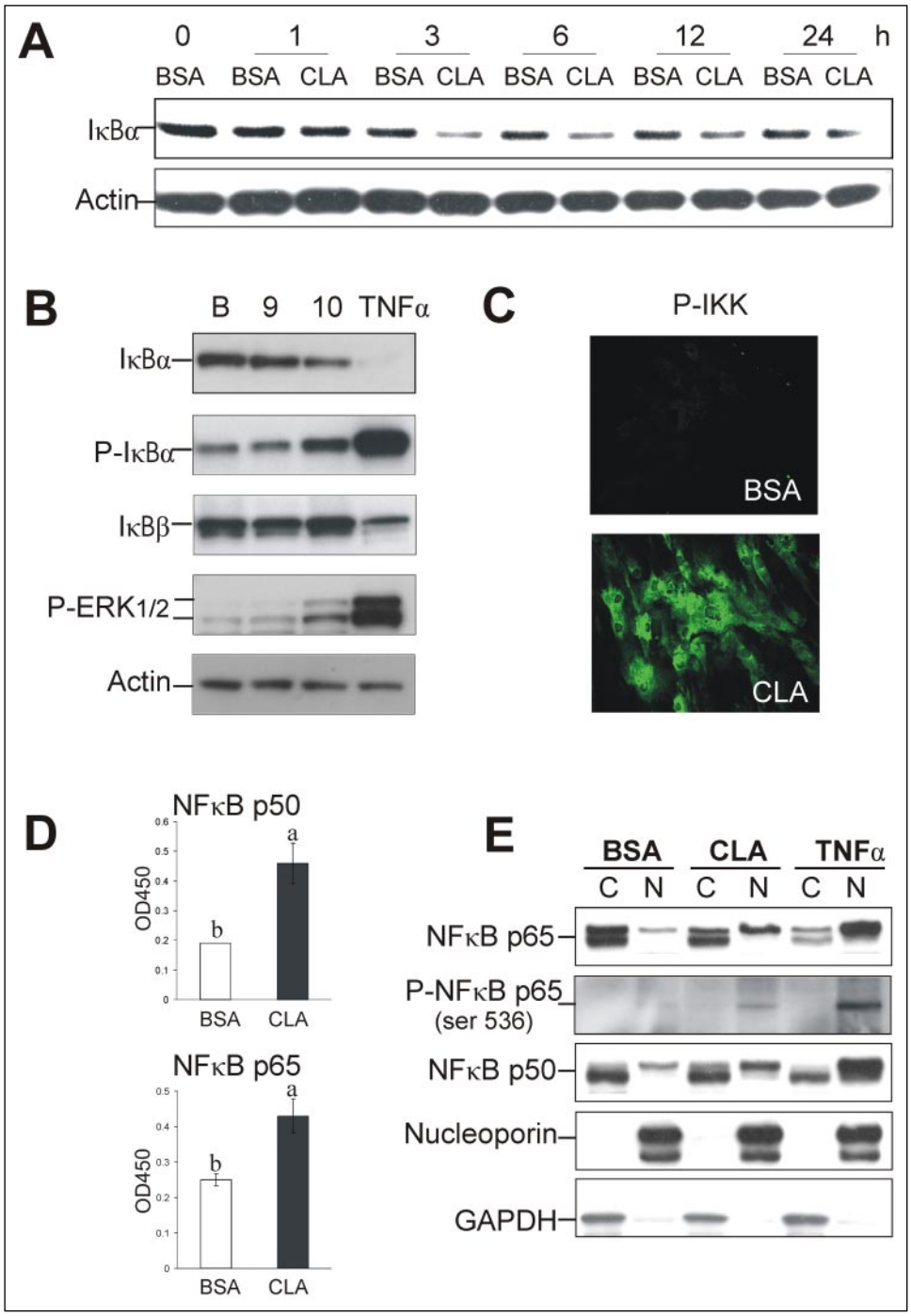

FIGURE 4. ***Trans*-10, *cis*-12 CLA activates NFκB.** *A*, differentiated cultures of human adipocytes were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for 0, 1, 3, 6, 12, or 24 h, and the abundance of IκB was determined using immunoblotting. *B*, cultures were treated with BSA, 30 μM *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 for 2 h and immunostained with p-IKK (Ser-177/180, Ser-177/181). 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 (±S.E.; $n = 2$) not sharing a common *lowercase letter* differ ($p < 0.05$). *E*, nuclear (*N*) and cytosol (*C*) extracts were immunoblotted with antibodies targeting NFκB p65, p-NFκB p65 (Ser-536), and NFκB p50. Fractionation was validated by immunoblotting nucleoporin and GAPDH.

thetic peptide that blocks the regulatory site of the active IKK complex, thereby inhibiting IκBα degradation (32, 33). As shown in Fig. 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 RNA Interference Attenuates CLA 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 suppression of adipogenic protein expression (Fig. 7). Using a second approach, selective depletion of NFκB p65 using siRNA was performed before treatment with *trans*-10, *cis*-12 CLA. As shown in Fig. 8*A*, 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. 8*A*). 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 (≥90%) were positive to Cy3 fluorescence of siGLO 24 h post-transfection (Fig. 8*B*), indicating that both adipocytes and non-adipocytes were efficiently transfected (Fig. 8*B*). Specific depletion of NFκB p65 was examined using immunoblotting as shown in Fig. 8*C*. We consistently obtained ≥50% NFκB depletion. NFκB p65 protein

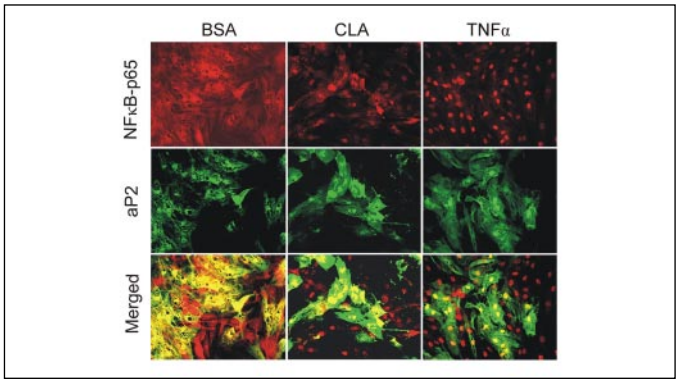

FIGURE 5. ***Trans*-10, *cis*-12 CLA promotes NFκB localization to the nucleus in adipocytes and non-adipocytes.** Differentiated cultures of human adipocytes were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for 24 h, and then cells were double-stained for NFκB p65 (*red*, cy3 conjugated anti-mouse IgG) and aP2 (*green*, FITC-conjugated, anti-rabbit IgG). 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.

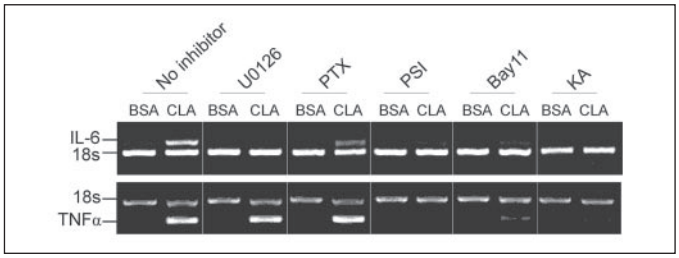

FIGURE 6. ***Trans*-10, *cis*-12 CLA induction of IL-6 and TNF-α gene expression is blocked by NFκB inhibitors.** Differentiated cultures of human adipocytes were serum-starved for 20 h and then pretreated for 1 h with either with the MEK/ERK inhibitor U0126 (10 μM), the GCPR-$G_{i/o}$ inhibitor pertussis toxin (*PTX*; 100 ng/ml), the NFκB inhibitors proteasome inhibitor I (*PSI*; 50 μM), Bay11-7082 (Bay11; 2.5 μM), or kamebakaurin (*KA*; 26 μM) and subsequently treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for an additional 3 h. Total RNA was harvested, and semiquantitative 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.

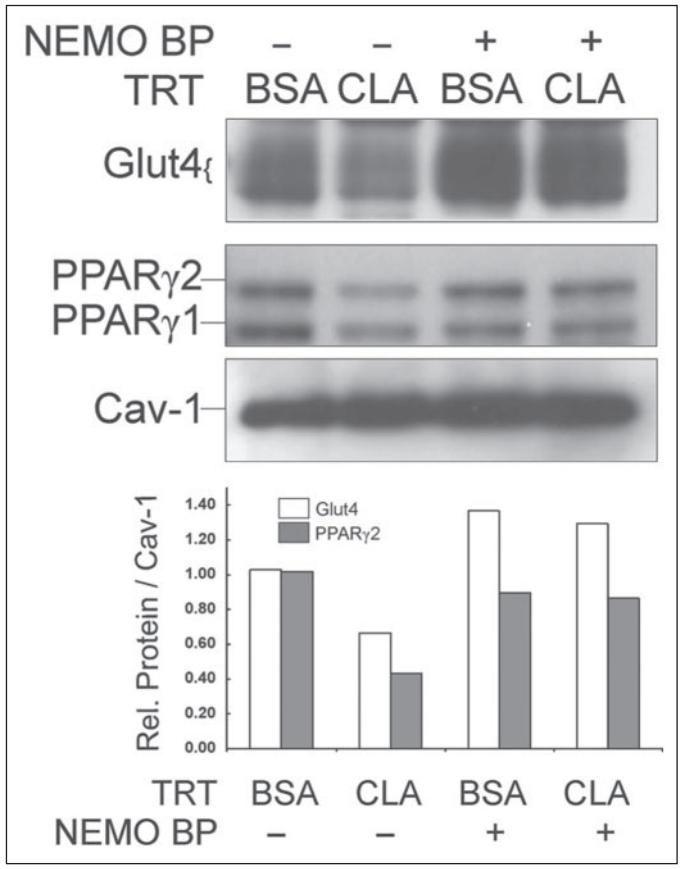

FIGURE 7. **Inhibiting IKK complex formation by NEMO BP blocks CLA suppression of Glut4 and PPARγ protein levels.** Differentiated cultures of human adipocytes were serum-starved for 20 h and pretreated with 100 μg/ml NEMO BP for 1 h. Subsequently, cultures were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for 24 h. Cellular fractions containing nuclei and membranes were collected and immunoblotted for the Glut4, 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 (*Rel. Protein*) to caveolin-1 (*Cav-1*) were expressed as *bar graphs under* the *blot*. *TRT*, treatment.

expression was severely blunted in the NFκB p65 siRNA-transfected cultures compared with untreated or non-targeting control or lamin-siRNA-transfected cultures (Fig. 8*C*). 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 knock-down was validated using lamin siRNA as a positive control (Fig. 8*C*).

To investigate the impact of CLA regulation on adipocytes under conditions of NFκB depletion, we examined PPARγ expression and the activation of ERK1/2 and STAT3. As shown in Fig. 8*D*, depletion of NFκB p65 modestly attenuated CLA suppression of PPARγ2 (also confirmed by immunostaining in Fig. 8*E*) 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 suppression of PPARγ was attenuated by knocking down NFκB activity using siRNA, we hypothesized that silencing NFκB would block or attenuate CLA suppression of insulin-stimulated glucose uptake demonstrated in Fig. 1. To test this hypothesis, we measured insulin-stimulated [2-^{3}H]deoxyglucose uptake in NFκB-depleted cultures of adipocytes treated for 24 h with BSA vehicle or *trans*-10, *cis*-12 CLA. CLA suppression of glucose uptake was attenuated in siP65-transfected cultures compared with CLA-treated cultures not receiving siP65 (Fig. 9*A*). 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 knock-down achieved for NFκB-p65 in this experiment (~54%; densitometry not shown) (Fig. 9*B*). 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 (11, 28) and adipocytes (16).

DISCUSSION

We demonstrate in this article 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 (3, 4, 25), we propose in our working model (Fig. 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

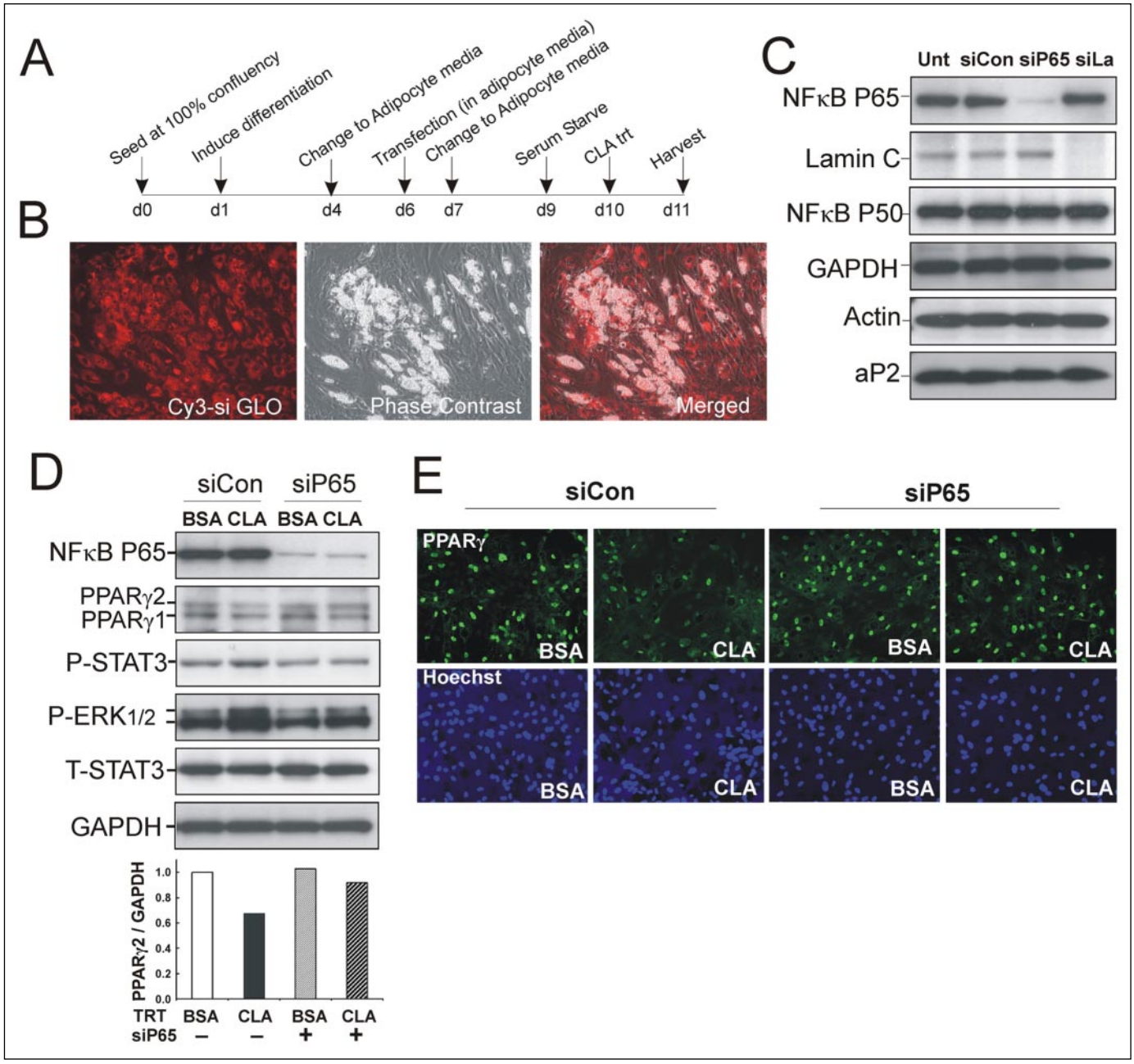

FIGURE 8. **Specific depletion of NFκB p65 attenuates CLA suppression of PPARγ and activation of MEK/ERK signaling in cultures of primary human adipocytes.** *A*, experimental design of transfection protocol with siRNA NFκB p65 in primary cultures of human adipocytes. *trt*, treatment. *B*, transfection efficiency was evaluated by transfection with siGLO, fluorescence (Cy3)-tagged non-targeting siRNA. *C*, knock-down 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 non-targeting control siRNA or siP65, cultures were treated with BSA vehicle or a 30 μM *trans*-10, *cis*-12 CLA for an 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 and 72 h later treated with either BSA or 30 μM *trans*-10, *cis*-12 CLA for 24 h and then immunostained for PPARγ. Hoechst staining was conducted to identify nuclei.

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, lipoprotein lipase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase. 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. 10) and data showing that NFκB is activated by CLA in both SV cells and adipocytes (Fig. 5), we propose a differential role of NFκB

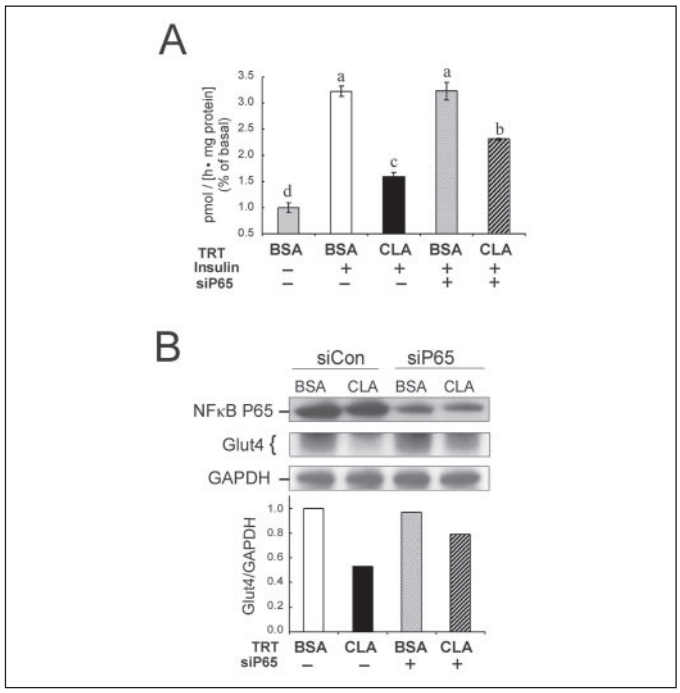

FIGURE 9. **Depletion of NFκB p65 attenuates CLA suppression of Glut4 levels and insulin-stimulated glucose uptake.** *A*, cultures of differentiated human adipocytes were transfected with either control siRNA (*siCon*) or NFκB p65 siRNA (*siP65*) as described in Fig. 8 and 72 h later were treated with BSA vehicle or 30 μM *trans*-10, *cis*-12 CLA for 24 h. Basal and insulin-stimulated uptake of 4 nmol of [2-^{3}H]deoxyglucose 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 (BSA, −*insulin*) rate. Means (±S.E.; $n = 4$) not sharing a common *lowercase letter* differ, $p < 0.05$. *B*, immunoblotting for NFκB p65, Glut4, and GAPDH were carried out as described in Fig. 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*.

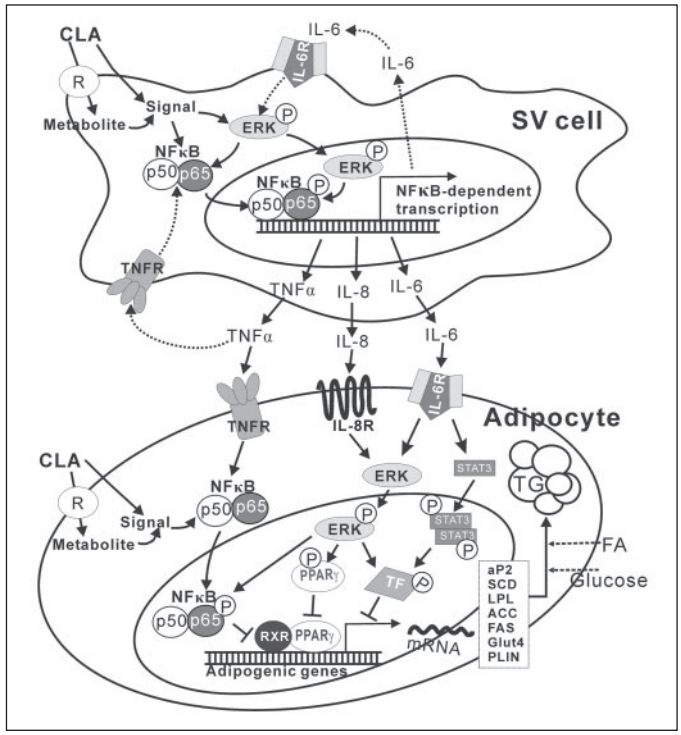

FIGURE 10. **Working model; *trans*-10, *cis*-12 CLA reduces glucose and fatty acid uptake and TG synthesis via activation of NFκB and ERK1/2 signaling and cytokine production.** Upon entry into SV cells, *trans*-10, *cis*-12 CLA generates an unidentified signal, thereby activating NFκB and ERK1/2. NFκB 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 (*FA*) synthase (*FAS*), 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.

activation in SV cells *versus* 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. 2*B*) and in differentiated cultures of adipocytes (Fig. 2*A*). Quantitatively, the non-differentiated SV cells (Fig. 2*B*) produced 10- and 7-fold more IL-6 and IL-8, respectively, in response to 24 h of treatment with *trans*-10, *cis*-12 CLA than did the differentiated cultures of adipocytes (Fig. 2*A*). In agreement with these data, Harkins *et al.* (34) showed that 3T3-L1 preadipocytes stimulated with lipopolysaccharide express more IL-6 than adipocytes. Consistent with these data, IL-6, IL-8, and TNF-α possess a nuclear factor κB response element (21). Furthermore, 1) cytokine secretion was preceded by IκBα degradation (Figs. 4, *A* and *B*), IKK phosphorylation (Fig. 4*C*), increased p50 and p65 nuclear translocation (Figs. 4*E* and 5) and binding to a consensus NFκB oligomer (Fig. 4*D*) compared with controls, and 2) selective chemical inhibitors of NFκB acutely blocked CLA-mediated increases in IL-6 and TNF-α gene expression (Fig. 6).

Once these cytokines are secreted into the media, 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 (4). Consistent with these data, cytokines secreted from adipose tissue regulate glucose and lipid metabolism locally and peripherally (14, 16, 36, 37). 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-Janus kinase (JAK), IL-8 via GPCR-$G_{i/o}$).

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. 7) or depletion of NFκB (Fig. 8, 9) attenuated CLA 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 (16), myotubes (12, 28), and hepatocytes (39). We demonstrated that *trans*-10, *cis*-12 CLA activates the IKK-IκB-NFκB axis (Figs. 4 and 5), and NFκB inhibitors block or attenuate CLA-induced IL-6 gene expression (Fig. 6), Glut4 protein levels (Figs. 7 and 9), and suppression of glucose uptake (Fig. 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 with controls.[3] Furthermore, we previously demonstrated that neutralization of IL-6 blocked CLA activation of ERK1/2 (4), supporting the important role of IL-6 in mediating CLA anti-adipogenic actions.

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

[3] S. Chung, J. M. Brown, J. N. Provo, Robin Hopkins, and M. K. McIntosh, unpublished data.

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.* (40) 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 with the classic downstream signaling of TNF-α receptor, we cannot rule out the possibility that TNF-α plays an important role in mediating CLA induction of insulin resistance and delipidation (4) in adipocytes.

The term adipokines has been used to identify cytokines or chemokines originated from adipocytes (*e.g.* IL-6, IL-8, TNF-α, leptin, resistin, adiponectin). However, this term is misleading based on data suggesting that nonfat cells from adipose tissue are the major site for cytokine production rather than adipocytes (37, 41, 42). 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 with 3T3-L1 adipocytes, which are almost exclusively adipocytes. Studies are under way in our laboratory 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 (3) and newly differentiated adipocytes (4). Herein, we demonstrated that this CLA-mediated suppression of insulin-stimulated glucose uptake (Fig. 1*A*) was due primarily to decreased levels of Glut4 (Fig. 1*C*), 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. 1*B*), 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 that depletion of NFκB attenuates CLA suppression of glucose uptake (Fig. 9). To our knowledge this is the first published report documenting depletion of NFκB p65 using an RNA interference technique in primary human adipocytes. In addition, there is evidence that phosphorylation of IKK is a potential inhibitor of insulin-stimulated glucose uptake (43, 44). Based on these reports, our data showing that *trans*-10, *cis*-12 CLA activates IKK (Fig. 4*C*) reinforces our working model, demonstrating that CLA promotes the IKK-IκB-NFκB cascade, which is critical for suppression of PPARγ and Glut4 proteins (Fig. 7).

Collectively, these data and our previously published data (3, 4, 25) 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 (45, 46) and animal (47) 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 (46) and the insulin resistance (48, 49) and/or lipodystrophy (50) 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 mitogen-activated protein kinase is not yet fully understood. P-ERK1/2 is an important regulator of PPARγ as demonstrated by its inhibition of adipogenesis (51). Furthermore, cytokine expression suppressed PPARγ activity in mesenchymal stem cells, showing direct interaction between PPARγ and NFκB (52). Similar reports demonstrating that activation of NFκB (27, 31, 53) and mitogen-activated protein kinase (22, 38, 54) 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 before that of PPARγ itself, leading to insulin resistance and delipidation (4). 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 suppression of PPARγ2 (Figs. 7 and 8, *D* and *E*) and Glut4 (Figs. 7 and 9*B*) but also prevented CLA-mediated ERK1/2 phosphorylation (Fig. 8*D*). 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 under way in our laboratory to test this hypothesis.

CLA suppression of insulin sensitivity and TG accumulation (3, 4, 25) 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 the following evidence. 1) NFκB activation antagonizes apoptosis and promotes cells survival (19); 2) caspase 3 is not activated by 30 μM CLA for up to 72 h (unpublished data[3]); 3) Hoechst and 4,6-diamidino-2-phenylindole staining indicate similar numbers of nuclei and no differences in nuclear condensation or fragmentation in CLA-treated cultures compared with controls (Fig. 8*E* and Ref. 4); 4) protein and RNA levels are not reduced after CLA treatment; 5) CLA reduces the TG content of adipocytes without reducing the number of adipocytes (4, 25). Similarly, CLA does not appear to cause de-differentiation *per se* because of the following evidence. 1) Adipocytes still contain small lipid droplets after 3 weeks of treatment even though CLA suppresses adipogenic gene expression and protein levels and impairs glucose uptake within 24 h of treatment (4); 2) CLA increases adipose differentiation-related protein and leptin expression (4, 25), suggesting that CLA-treated cultures still contain adipocytes; 3) Pref-1 gene expression, which is abundant in our non-differentiated SV cells, is low in CLA-treated cultures containing adipocytes,[3] 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 most likely does not impair the differentiation of new adipocytes. Instead, we postulate that CLA is causing delipidation, primarily by blocking *de novo* TG synthesis (3, 4) and to a lesser extent by increasing lipolysis (25).

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 under way 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.

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