Conjugated Linoleic Acid Induces Human Adipocyte Delipidation

AUTOCRINE/PARACRINE REGULATION OF MEK/ERK SIGNALING BY ADIPOCYTOKINES*

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Dietary conjugated linoleic acid (CLA) reduces body fat in animals and some humans. Here we show that trans-10, cis-12 CLA, but not cis-9, trans-11 CLA, when added to cultures of stromal vascular cells containing newly differentiated human adipocytes, caused a time-dependent decrease in triglyceride content, insulin-stimulated glucose and fatty acid uptake, incorporation into lipid, and oxidation compared with controls. In parallel, gene expression of peroxisome proliferator-activated receptor-γ and many of its downstream targets were diminished by trans-10, cis-12 CLA, whereas leptin gene expression was increased. Prior to changes in gene expression and metabolism, trans-10, cis-12 CLA caused a robust and sustained activation of mitogen-activated protein kinase kinase/extracellular signal-related kinase (MEK/ERK) signaling. Furthermore, the trans-10, cis-12 CLA-mediated activation of MEK/ERK could be attenuated by pretreatment with U0126 and pertussis toxin. In parallel, pretreatment with U0126 blocked the ability of trans-10, cis-12 CLA to alter gene expression and attenuate glucose and fatty acid uptake of the cultures. Intriguingly, the induction by CLA of MEK/ERK signaling was linked to hypersecretion of adipocytokines interleukin-6 and interleukin-8. Collectively, these data demonstrate for the first time that trans-10, cis-12 CLA decreases the triglyceride content of newly differentiated human adipocytes by inducing MEK/ERK signaling through the autocrine/paracrine actions of interleukins-6 and 8.

Conjugated linoleic acid (CLA) refers to a naturally occurring group of dienoic derivatives of linoleic acid. The two pre-

* This work was supported by NIDDK/Office of Dietary Supplements, National Institutes of Health, Grant R01DK-83070 and North Carolina Agriculture Research Service Grant 06520 (to M. K. M.) and by the Danish Daisy Research Foundation and the Innovation Act under the Danish Government (to S. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: CLA, conjugated linoleic acid; aP2, adipocyte-specific fatty acid-binding protein; BSA, bovine serum albumin; CAP, ca3/β3/α3; C/EBP-α, C/EBP/α; E2F-1, E2F-1; EB, EB; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-related kinase; FA, fatty acid; GLUT4, insulin-dependent glucose transporter 4; GPCR, G protein–coupled receptor; GPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hanks’ balanced salt solution; IL, interleukin; JNK, c-Jun-NH2-terminal kinases; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; PBS, phosphate-buffered saline; PPAR-γ, peroxisome proliferator-activated receptor-γ; PTX, pertussis toxin; SV, stromal vascular; TBS, Tris-buffered saline; TG, triglyceride; TNF-α, tumor necrosis factor-α.

This paper is available online at http://www.jbc.org 26735
Unlike TNF-α, which activates all three MAPKs (24). The importance of CLA-induced ERK activation was demonstrated by the fact that pretreatment with the MEK inhibitor U0126 blocked the ability of CLA to alter gene expression and attenuate glucose and fatty acid (FA) uptake of the cultures. The novel delayed, yet sustained kinetics of ERK activation by CLA, and the involvement of a pertussis toxin (PTX)-sensitive Gi protein in the activation, suggested that a second G protein-coupled receptor (GPCR) signaling network must exist for CLA to activate ERK. Finally, using protein array technology, we have identified IL-6 and IL-8 as critical autocrine/paracrine regulators of CLA-induced ERK activation. Collectively, these studies demonstrate for the first time that trans-10, cis-12 CLA induces the expression and secretion of the proinflammatory adipocytokines IL-6 and IL-8, and, through autocrine and paracrine networks, these mediators reduce human adipocyte TG content by activating MEK/ERK signaling.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**All cell cultureware and scintillation mixture (Scintisafe) were purchased from Fisher Scientific. d-[^14]C-Glucose was purchased from ICN Biochemicals, Inc. (Irvine, CA). d-[^1-14]C-Oleic acid and Western Lightning Plus chemiluminescence Substrate were purchased from PerkinElmer Life Sciences. Bicinchoninic acid protein assay and Restore™ Western blot stripping buffer were purchased from Pierce, NUPAGE precast gels and buffers for SDS-PAGE were purchased from Invitrogen. Gene-specific primers for real time PCR were purchased from DNA Technology A/S (Aarhus, Denmark), and the real time PCR kit was purchased from Applied Biosystems (Copenhagen, Denmark). Fetal bovine serum was purchased from Cambrex/BioWhittaker (Walkersville, MD). Linoleic acid (99% pure) was purchased from Nu-Chek-Prep (Elysian, MN), and isomers of CLA (≥ 98% pure) were purchased from Matreya (Pleasant Gap, PA). Rubber stoppers and an inverter cell well hanging bucket for oxidation assays were purchased from Knotes Glass Company (Vineland, NJ). Recombinant human TNF-α was purchased from Upstate Biotechnology (Lake Placid, NY). Recombinant IL-6 and IL-8 proteins were purchased from Research Diagnostics Inc. (Boston, MA). PTT, P2P, calpastatin C, rapamycin, C-89, brefeldin A, and bisindolylmaleimide-1 were purchased from Calbiochem. Antibodies for total and phospho-specific MAPKs and U0126 were purchased from Cell Signaling Technologies (Beverly, MA). Phospho-p38 (pT180/pY182) monoclonal antibody was purchased from BD Transduction Laboratories (Franklin Lakes, NJ). Mouse monoclonal PPAR-γ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rhodamine red and fluorescein isothiocyanate-isocyanate-conjugated IgG were purchased from Jackson Immunoresearch (West Grove, PA). Cytochrome arrays were purchased from Raybiotech, Inc. (Norcross, GA). Monoclonal neutralization antibodies targeted against TNF-α, IL-6, and IL-8 were purchased from R & D Systems, Inc. (Minneapolis). All other chemicals and reagents were purchased from Sigma Chemical, unless otherwise stated.

**Culturing of Stromal Vascular (SV) Cells Isolated from Human Adipose Tissue—**Abdominal adipose tissue was obtained from females with a body mass index <30.0 during liposuction or elective surgery with consent from the Institutional Review Board at University of North Carolina-Greensboro, or purchased from Zen Bio, Inc. (Research Triangle Park, NC) as described previously (13, 14). SV cells isolated from single subjects were used in some experiments, and another set of pooled SV cells obtained from Zen Bio, Inc. and from six independent human donors was used in parallel. Data obtained from single subject isolations and from pooled lots responded similarly, thus the data were merged. Cells were isolated and cultured as defined previously (11, 12). Under these isolation and culturing conditions, ~50–80% of the cells had visible lipid droplets when the treatments were initiated. Differentiation was initiated from the day 0 cultured cells. Newly differentiated adipocytes began on day 12–15 of differentiation.

**Fatty Acid Preparation—**Both isoforms of CLA and linoleic acid were complexed to FA-free (<98%) bovine serum albumin (BSA) at a 4:1 molar ratio using 1 mm BSA stocks.

**Lipid Staining and Triacylglycerol Content Determination—**The presence of intracellular lipid was visualized by staining the cultures with oil red O, and TG content was determined using a colorimetric assay (GPO Trinder, Sigma) as described previously (11, 12).

**[2-14]H]Deoxyglucose Uptake—**Cultures were seeded at 4 × 10^5 cells/cm^2 in 35-mm culture plates and allowed to differentiate for 12 days as described in the cell culture protocol. For chronic 72-h treatment (see Fig. 2), vehicle or FA treatments were added in 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 μM human insulin in the presence of vehicle or FA treatments, to give a total of 72-h exposure to treatments. For U0126 and PTX rescue experiments (see Fig. 8), 12-day-old cultures were treated for additional 24 h in DMEM/Ham’s F-12 medium, pretreated with inhibitors for 1 h, and then the medium was spiked with treatments for an additional 24 h. Immediately after experimental incubations, 2-[14]Hideoxyglucose uptake was measured as described previously (12).

**[^14]CO2 Production and de Novo Lipid Synthesis from[^14]C]Glucose—**Cultures were seeded at 4 × 10^5 cells/cm^2 in 35-mm culture plates and allowed to differentiate for 12 days as described in the cell culture protocol. For chronic 72-h treatment (see Fig. 2), vehicle or FA treatments were added in 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 μM human insulin in the presence of vehicle or FA treatments, to give a total of 72-h exposure to treatments. After experimental incubation, culture medium was removed and replaced with 1 ml of Hanks’ balanced salt solution (HBSS) buffer containing 100 ng/ml human insulin for 10 min. After insulin preincubation, 20 μl of HBSS containing 2.2 nmol of d-[^14]C-Glucose (specific activity = 231 mCi/mmol) was added to each plate, and the plate was placed quietly in an air-tight CO2 collector for 4 h (Fig. 3). The CO2 collector was incubated at 37 °C for 3 h (a time course study indicated a linear increase in radiolabeled CO2 production and incorporation into lipid from [^14]C-glucose over a 4-h period; data not shown). After the 3-h incubation, the center well collection bucket was cut out of the collection chamber and delivered to a liquid scintillation counting vial, the cells were extracted and partitioned, and fractions were subjected to liquid scintillation counting as described previously (11, 12).

**[^14]C]Oleic Acid Uptake and Metabolism—**Cultures were seeded at 4 × 10^5 cells/cm^2 in 35-mm culture plates and allowed to differentiate for 12 days as described in the cell culture protocol. For chronic 72-h treatment (see Fig. 3), vehicle or FA treatments were added in 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 μM human insulin in the presence of vehicle or FA treatments, to give a total of 72-h exposure to treatments. For U0126 and PTX rescue experiments (see Fig. 8), 12-day-old cultures were serum starved for 24 h in DMEM/Ham’s F-12 medium, pretreated with inhibitors for 1 h, and then the medium was spiked with treatments for an additional 24 h. After experimental incubation, culture medium was removed and replaced with 1 ml of HBSS buffer containing 100 ng/ml human insulin. Prior to incubation, [^14]C-oleic acid was complexed to FA-free (<98%) BSA at a 4:1 molar ratio using 1 mm BSA stocks. Then, 12.5 nmol of [^14]C-oleic acid (specific activity = 40–60 mCi/mmol) complexed to BSA was added to each plate, and the plate was placed quietly in an air-tight CO2 collector for 4 h. The CO2 collector was incubated at 37 °C for 3 h. After 4 h of incubation, the center well collection bucket was cut out of the collection chamber and delivered to a liquid scintillation counting vial, the cells were extracted and partitioned, and fractions were subjected to liquid scintillation counting as described previously (12). Total cellular uptake was derived by combining the dpm collected in both the lipid-soluble and the water-soluble cellular fractions, excluding those captured in CO2.

**RNA Isolation and Quantitative Real Time PCR—**Total RNA was isolated using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer’s protocol. First strand cDNA synthesis and real time quantitative PCR were carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems), as described previously (12). Primer sets for acyl-CoA-binding protein (ACBP) and fatty acid amide hydrolase (FAAH) were designed with SV adipocyte mRNA sequences (C/EBP-α), glycerol-3-phosphate dehydrogenase (GPDH), leptin, perilipin, PPAR-γ, PPAR-δ, and TATA-binding protein have been described previously (12). The following primer sets used were also used in this work: adiponectin (accession no. NM_004797) sense (5'-GAAGCTTTGGCAAGGATGTCCCTATG-3'), antisense (5'-CAATCCACCACATGAGTCGTC-3'), C/EBP-α (accession no. NM_021671) sense (5'-GGCCCCATGCGCCGAC-3'), antisense (5'-AGACCGTGCGCAGAAG-3'), insulin-dependent glucose transporter 4 (GLUT4) (accession no. NM_010042) sense (5'-GTCTACGATGCCCCTCCAGAA-3'), antisense (5'-CACGGCCTCCTTACG-3'). Lipoprotein lipase...
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RESULTS

Trans-10, Cis-12 CLA Decreases TG Content and Alters Cell Morphology—To determine the isomer-specific influence of CLA on TG content and lipid droplet morphology, cultures of SV cells containing newly differentiated adipocytes were treated with 30 μM trans-10, cis-12 CLA, cis-9, trans-11 CLA, linoleic acid (FA control), or vehicle (BSA) for either 1, 2, or 3 weeks. As shown in Fig. 1A, the TG content of cultures treated with trans-10, cis-12 CLA decreased over time, whereas the TG content increased over time in all other cultures. Changes in lipid droplet morphology in the trans-10, cis-12 CLA-treated cultures were apparent after 1 week of treatment (data not shown). After 3 weeks of treatment, cultures treated with trans-10, cis-12 CLA had fewer adipocytes with unilocular lipid droplets and more adipocytes with multilocular lipid droplets compared with cis-9, trans-11 CLA, linoleic acid, or control cultures (Fig. 1B).

Trans-10, cis-12 CLA Decreases Glucose Uptake, Incorporation into Lipid, and Oxidation—To determine the extent to which trans-10, cis-12 CLA decreased the TG content by decreasing glucose uptake or utilization, cultures of SV cells containing newly differentiated adipocytes were treated with...
30 μM trans-10, cis-12 CLA, cis-9, trans-11 CLA, or vehicle (BSA) for 72 h and then radiolabeled glucose uptake, conversion to lipid, and oxidation were measured. As seen in Fig. 2A, 2-[3H]deoxyglucose uptake was lower in insulin-stimulated, but not basal (e.g. −insulin), cultures treated with trans-10, cis-12 CLA compared with all other treatments. De novo lipogenesis, as measured by [14C]glucose incorporation into [14C]lipid, was lower in trans-10, cis-12 CLA-treated cultures under basal and insulin-stimulated conditions compared with their respective controls (Fig. 2B). Glucose oxidation, as determined by [14C]CO₂ production from [14C]glucose, was lower in insulin-stimulated cultures treated with trans-10, cis-12 CLA compared with all other treatments (Fig. 2C). In contrast, in the absence of insulin, glucose oxidation was not significantly affected by CLA. These data show that trans-10, cis-12 CLA decreases adipocyte TG content, in part, by reducing insulin-stimulated glucose uptake and de novo lipogenesis of the cultures.
differentiated adipocytes were treated with 30 μM cis-9, trans-11 CLA (9,11) or trans-10, cis-12 CLA (10,12). A, [14C]oleic acid (12.5 nmol) uptake was measured after a 2-h incubation; the control rate of uptake was ~15 nmol/(h mg of protein). B, [14C]oleic acid (12.5 nmol) incorporation into [14C]lipid was measured after a 2-h incubation; the control rate was ~12 nmol/(h mg of protein). C, [14C]CO₂ production from [14C]oleic acid was measured after a 2-h incubation; the control rate was ~100 pmol/(h mg of protein). Data are expressed as a percentage of vehicle control (BSA) rate. Means (± S.E.; n = 6) not sharing a common superscript differ, p < 0.05.

Fig. 3. Isomer-specific regulation of FA uptake and metabolism by CLA. Cultures of SV cells containing newly differentiated human adipocytes were treated (T) continuously for 72 h with either a BSA vehicle, 30 μM cis-9, trans-11 CLA (9,11) or trans-10, cis-12 CLA (10,12). A, [14C]oleic acid (12.5 nmol) uptake was measured after a 2-h incubation; the control rate of uptake was ~15 nmol/(h mg of protein). B, [14C]oleic acid (12.5 nmol) incorporation into [14C]lipid was measured after a 2-h incubation; the control rate was ~12 nmol/(h mg of protein). C, [14C]CO₂ production from [14C]oleic acid was measured after a 2-h incubation; the control rate was ~100 pmol/(h mg of protein). Data are expressed as a percentage of vehicle control (BSA) rate. Means (± S.E.; n = 6) not sharing a common superscript differ, p < 0.05.

Fig. 4. Chronic trans-10, cis-12 CLA treatment alters gene expression. Cultures of SV cells containing newly differentiated human adipocytes were treated continuously for 8, 24, 72, or 216 h with either a BSA (●) vehicle, or 30 μM cis-9, trans-11 CLA (●), or trans-10, cis-12 CLA (▲). After treatment, total RNA was harvested and used for first strand cDNA synthesis. Real time quantitative PCR analyses were performed to examine the expression of acyl-CoA-binding protein (ACBP), adiponectin, ap2, CAP, C/EBPα, GLUT4, GPDH, leptin, lipoprotein lipase (LPL), perilipin, and PPARγ-1 and PPARγ-2. Results shown are representative of three separate experiments from independent human subjects.

Chronic Trans-10, Cis-12 CLA Treatment Decreases the Expression of Markers of Adipocyte Differentiation—To determine the extent to which the trans-10, cis-12 CLA-mediated reductions in glucose and lipid uptake and utilization were caused by suppression of genes that regulate these processes, we examined the time-dependent effects of CLA on the expression of PPAR-γ, the master regulator of adipocyte differentiation, and several of its downstream targets. As seen in Fig. 4, there was a time-dependent decrease in the mRNA levels of all genes except leptin in cultures treated with trans-10, cis-12 CLA. Messenger RNA levels of PPAR-γ1 and PPAR-γ2 were markedly lower in cultures treated for 9 days with trans-10, cis-12 CLA compared with BSA or cis-9, trans-11 CLA-treated cultures. Similarly, mRNA levels of PPAR-γ target genes such as C/EBPα, ap2, lipoprotein lipase, acyl-CoA-binding protein, CAP, perilipin, adiponectin, and GLUT4 were lower in trans-
10, cis-12 CLA-treated cultures on day 9 compared with the other treatments. Messenger RNA levels of GPDH, a mid-late marker of adipocyte differentiation, were barely detectable on day 9 in CLA-treated cultures. In contrast, the expression of leptin, an adipocyte-specific secreted protein that has been shown to be repressed by ligand-bound PPAR-γ (52), was higher in cultures treated with trans-10, cis-12 CLA on day 9 compared with all other treatments. Within this targeted panel of genes which are known to be up-regulated during adipocyte differentiation, GPDH, perilipin, adiponectin, and GLUT4 seemed to be the earliest genes affected (i.e. reduced levels seen within 8–24 h), and these genes were essentially nondetectable after 9 days of trans-10, cis-12 CLA treatment. Collectively, these data suggest that the isomer-specific effects of CLA on TG content and gene expression are greatest during the first 9 days of treatment. Indeed, a 30-min treatment of cultures of SV cells containing newly differentiated human adipocytes with TNF-α exhibited the transient activation seen when we used dimethyl sulfoxide as a vehicle to deliver CLA acutely (5 min–24 h), we did not see ERK1/2 activation by CLA other than the transient activation caused by the vehicle alone (data not shown).

To determine whether this novel robust and sustained activation of MEK/ERK was isomer-specific, cultures were treated with 30 μM cis-9, trans-11 CLA or trans-10, cis-12 CLA for 24–72 h. As shown in Fig. 5C, MEK/ERK activation was specific for the trans-10, cis-12 isomer of CLA. These data show that the isomer-specific effects of CLA on TG content and gene expression of the cultures correlate with MEK/ERK activation.

To determine critical signaling steps involved in trans-10, cis-12 CLA-induced MEK/ERK signaling, we used pharmacological inhibitors to block upstream regulators of ERK1/2. First, the effects of the MEK inhibitor U0126 were tested in the presence and absence of trans-10, cis-12 CLA. U0126 binds to MEK, thereby inhibiting its catalytic activity and phosphorylation of ERK1/2. As shown in Fig. 6A, pretreatment with U0126 blocked the trans-10, cis-12 CLA activation of ERK1/2 after 24 h of treatment. However, U0126 pretreatment paradoxically increased MEK hyperphosphorylation. Similarly, PTX, an inhibitor of GPCR-Gi/o coupling, blocked trans-10, cis-12 CLA activation of MEK and ERK1/2, indicating that a PTX-sensitive GPCR is critical for CLA-induced MEK/ERK activation. In contrast, neither a c-SRC kinase inhibitor (PP2; Fig. 6A), two protein kinase C inhibitors (calphostin C; Fig. 6A or bisindolylmaleimide-1; data not shown), a protein kinase A inhibitor (H-89; data not shown), a FRAP/mTOR inhibitor (rapamycin; data not shown), nor a phosphatidylinositol 3-kinase inhibitor (LY294002; data not shown) blocked the CLA activation of MEK/ERK signaling. Consistent with these data, trans-10, cis-12 CLA treatment conferred the ability of ERK1/2 to phosphorylate its nuclear substrate Elk-1 in vitro (Fig. 6B).
parallel with the inhibition of ERK1/2 phosphorylation, pretreatment with either U0126 or PTX blocked the CLA-mediated ability of ERK1/2 to phosphorylate ELK-1 in vitro. Enzyme activation of ERK1/2 (as evidenced by in vitro phosphorylation of ELK-1) by TNF-α and trans-10, cis-12 CLA could be attenuated by pretreatment with the PPAR-γ agonist BRL49653. Lastly, we demonstrated in Fig. 6C that both TNF-α and trans-10, cis-12 CLA treatment for 30 min and 24 h, respectively, increased the abundance of P-ERK1/2 in the nucleus of both nonadipocyte SV cells and newly differentiated adipocytes using immunofluorescence microscopy. This is important because nuclear ERK1/2 has been shown to phosphorylate a variety of transcription factors including PPAR-γ (33–36), which could potentially be implicated in CLA transcriptional attenuation of adipogenesis. However, trans-10, cis-12 CLA-mediated activation of MEK/ERK signaling did not result in the hyperphosphorylation of PPAR-γ in total cell extracts (data not shown), whereas phorbol ester (12-O-tetradecanoylphorbol-13-acetate) treatment caused PPAR-γ1 and PPAR-γ2 hyperphosphorylation as demonstrated previously (33).

Taken together, these data demonstrate that: 1) CLA-mediated hyperphosphorylation and activation of ERK enzyme activity are isomer-specific and can be blocked by pretreatment with the MEK inhibitor U0126, the GPCR-Gi/o coupling inhibitor PTX, and the PPAR-γ agonist BRL49653; and 2) neither c-SRC kinase, protein kinase C, protein kinase A, FRAP/mTOR, nor phosphatidylinositol 3-kinase is required for CLA sustained activation of MEK/ERK signaling. These data suggest CLA activation of MEK/ERK signaling is dependent on an upstream GPCR-Gi/o protein-coupled receptor. Additionally, because of the time lag prior to MEK/ERK activation (e.g. 12 h after CLA treatment), the impact of CLA on the MEK/ERK signaling pathway is most likely an indirect, rather than a direct effect.

**Fig. 6. Effect of pharmacological inhibitors on trans-10, cis-12 CLA-mediated activation and nuclear accumulation of P-ERK1/2.** Cultures of SV cells containing newly differentiated human adipocytes were serum starved for 24 h and then treated as follows. A, cultures were pretreated with the MEK inhibitor U0126 (10 μM), the GPCR-Gi/o coupling inhibitor PTX (100 ng/ml), the c-SRC kinase inhibitor PP2 (1 μM), or the protein kinase C inhibitor calphostin C (Cal. C, 200 nM) for 1 h and subsequently treated with either a BSA vehicle (B) or 30 μM trans-10, cis-12 CLA complexed to BSA (C) for an additional 24 h. Cell extracts were immunoblotted for the active phosphorylated forms of MEK (P-MEK) and ERK (P-ERK1/2) and subsequently were stripped and reprobed with antibodies recognizing total MEK and total ERK (ERK1/2). Data shown are representative of two to three independent experiments for each panel. B, cultures were pretreated with a vehicle (dimethyl sulfoxide) for 1 h, 10 μM U0126 for 1 h, 100 ng/ml PTX for 1 h, or 1 μM BRL49653 for 24 h, and subsequently treated with a BSA vehicle (B), 30 μM cis-9, trans-11 CLA complexed to BSA (9), or 30 μM trans-10, cis-12 CLA complexed to BSA (10) for an additional 24 h. Active ERK was then immunoprecipitated from total cell extracts and used in an in vitro kinase reaction with its known substrate, recombinant ELK-1. The resulting kinase reaction was subjected to SDS-PAGE and probed for phosphorylated ELK-1 using a phosphospecific antibody. A 30-min TNF-α treatment (100 ng/ml) of human adipocytes and active ERK-2 (ERK) was used as positive control for enzyme activation. C, cultures were pretreated with either a BSA vehicle control, 30 μM cis-9, trans-11 CLA (9,11), or 30 μM trans-10, cis-12 CLA (10,12) for 24 h. A 30-min treatment with TNF-α was used as a positive control for MAPK activation. Active ERK1/2 was then detected using immunofluorescence microscopy. Data shown are representative of two to three independent experiments for each panel.

whether other adipocytokines known to oppose adipocyte differentiation and promote insulin resistance were involved. To this end, we treated cultures for 24 h with either vehicle (BSA) or 30 μM trans-10, cis-12 CLA with or without U0126 pretreatment and then measured the levels of 42 cytokines or chemokines in conditioned media using a membrane-based human
cytokine antibody array and the mRNA levels of selected adipocytokines in the cells using real time qPCR. We used TNF-α treatment as a positive control for induction of cytokine synthesis and secretion. As seen in Fig. 9A, the levels of IL-6 and IL-8 were increased markedly in conditioned media of cultures treated with CLA compared with controls, which were reversed by U0126. As expected, TNF-α treatment also increased IL-6 and IL-8 levels (data not shown). Similarly, the mRNA levels of IL-6 and IL-8 were increased 7- and 40-fold, respectively, in cultures treated with CLA compared with controls, and this induction was attenuated by U0126 pretreatment (Fig. 9B). These data demonstrate for the first time in cultures of SV cells containing newly differentiated human adipocytes that trans-10, cis-12 CLA increases mRNA levels and protein secretion of IL-6 and IL-8, which are dependent, at least in part, on MEK/ERK signaling.

Trans-10, Cis-12 CLA Increases IL-6 and IL-8 Production Predominately from SV Cells—Using immunolocalization mi-
CLA-induced Adipocyte Delipidation: IL-6 Regulation of MEK/ERK

**DISCUSSION**

Several studies (13, 54–56) have demonstrated that although trans-10, cis-12 CLA decreased adipose tissue mass in vivo, it promoted insulin resistance and/or lipodystrophy. However, the underlying mechanism causing these effects is unclear. In this article, we demonstrate for the first time that trans-10, cis-12 CLA decreases the TG content of cultures of SV cells containing newly differentiated human adipocytes through MEK/ERK-dependent transcriptional control of genes involved in glucose and FA uptake and metabolism. Furthermore, our data demonstrate that CLA-induced MEK/ERK activation depends on a proinflammatory signaling network involving hypersecretion of IL-6 and IL-8 and activation of their respective receptor systems in the cultures. Taken together, we propose in our working model (Fig. 12) that trans-10, cis-12 CLA 1) increases the production of proinflammatory cytokines and chemokines, particularly IL-6 and IL-8, which in turn 2) activate their respective cell surface receptors IL-6R and CXCR1 3) through unidentified signaling transducers, 4) activate MEK/ERK phosphorylation, which 5) promotes P-ERK1/2 translocation to the nucleus and subsequent phosphorylation of transcription factors, including ELK-1, and potentially others that then 6) down-regulate PPAR-γ and downstream adipogenic gene expression, thereby attenuating insulin-sensitive glucose and FA uptake and 7) lead to insulin resistance, impaired adipocyte FA clearance, and reduced cellular TG content. Importantly, trans-10, cis-12 CLA is the first noncytokine (nutrient) effector of MEK/ERK signaling described in human adipocytes. These data are also the first to demonstrate a specific mechanism by which trans-10, cis-12 CLA reduces the TG content of cultures of SV cells containing newly differentiated human adipocytes. They also support the notion raised by several human studies that trans-10, cis-12 CLA supplementation causes insulin resistance (54, 55), which could potentially be linked IL-6 and IL-8 hypersecretion from adipose tissue. However, further studies are needed to test this hypothesis.

In what has become an exciting new area of adipocyte biology, several groups have demonstrated that cytokines and chemokines are either expressed at the mRNA level or secreted from adipose tissue. The ever-growing list (e.g., TNF-α, IL-6, IL-8, MCP-1, MIP-1α, adiponectin, plasminogen activator inhibitor-1, resistin, leptin) of secreted proteins that arise from adipose tissue have been collectively coined adipocytokines, yet this name may be misleading in light of several recent reports. Recent reports from Xu et al. (57) and Weisberg et al. (58) suggest that the actual amount of cytokines and chemokines secreted by adipocytes is minimal compared with that of neighboring reticuloendothelial cells, adipocyte precursors, or macrophages that all reside in adipose tissue. For example, expression analyses of macrophage and nonmacrophage cells from adipose tissue demonstrate that almost all of the TNF-α expression and significant amounts of IL-6 are derived from
FIG. 9. Trans-10, cis-12 CLA induces MEK/ERK-dependent cytokine secretion and mRNA expression. Cultures of SV cells containing newly differentiated human adipocytes were serum starved for 24 h and pretreated with or without 10 μM U0126 for 1 h. Subsequently, cultures were treated with either a BSA vehicle or 30 μM trans-10, cis-12 CLA for an additional 24 h. A, conditioned medium was collected and utilized to detect the secretion of multiple cytokines using protein array technology. Positive control spots (used to normalize between membranes) are located in the upper left corner (n = 4) and the lower right corner (n = 2), and CLA-induced cytokines spotted in duplicate are indicated as IL-6 and IL-8. B, total RNA was harvested and used for first strand cDNA synthesis. Real time quantitative RT-PCR analyses were performed to examine the expression of IL-6 and IL-8. Results shown are representative of two (A) or four (B) separate experiments from independent human subjects.

FIG. 10. CLA-induced IL-6 and IL-8 production predominates from nonadipocyte SV cells. Cultures of SV cells containing newly differentiated human adipocytes were serum starved for 24 h and pretreated with 1 μg/ml brefeldin A for 1 h to prevent cytokine or chemokine secretion. Subsequently, cultures were treated with either a BSA vehicle or 30 μM trans-10, cis-12 CLA for an additional 12 h. Cultures were double stained for aP2 (rhodamine-conjugated anti-rabbit IgG = red) and subsequently for individual cytokines IL-6 or IL-8 (fluorescein isothiocyanate-conjugated anti-mouse IgG = green). Hoechst staining was conducted to identify nuclei positively. Fluorescent images (magnification, ×20) were captured as described under “Experimental Procedures.” Results shown are representative of two separate experiments from independent human subjects.
adipose tissue macrophages (58). Similarly, inflammatory- and macrophage-specific genes were up-regulated in white adipose tissue in a mouse model of obesity, and rosiglitazone reversed these changes in gene expression (57). Adipose tissue of obese mice were infiltrated with macrophages, but not neutrophils or lymphocytes, suggesting that macrophage-related inflammatory activities correlate with insulin resistance associated with obesity. Therefore, based on these studies and our data, we propose (Fig. 12) that CLA induces IL-6 and IL-8 synthesis and secretion primarily in the supporting SV cells, which through paracrine signaling, leads to decreased adipogenesis and insulin resistance in adipocytes. However, future studies are needed to test this hypothesis. To this end, we are currently examining the impact of CLA on cytokine production and gene expression in cultures of freshly isolated mature adipocytes and in undifferentiated cultures of SV cells isolated from human adipose tissue.

TNF-α, by far, has been the most comprehensively studied of all the adipocytokines, yet the importance of adipocyte-derived IL-6 has come to light more recently. Unlike TNF-α, which is secreted in low abundance and is thought to act primarily through autocrine/paracrine actions locally in adipose tissue, IL-6 has been reported to be secreted abundantly from adipose tissue, contributing ~30% of total systemic IL-6 (59). Furthermore, adipose tissue-derived IL-6 is thought to act through autocrine, paracrine, and endocrine networks because its receptor system is present in many cell types including human adipocytes (41, 59). The IL-6 receptor system utilizes classical JAK/STAT signal transduction, but ERK has been shown to be a critical downstream intermediate of IL-6 signaling in multiple cell types, including adipocytes (60, Fig. 11A). Importantly, IL-6 treatment of murine and human adipocytes has pleiotropic effects on differentiation, metabolism, and gene expression similar to those seen with trans-10, cis-12 CLA treatment. In support of this concept, IL-6 treatment of murine and human adipocytes results in diminished TG content, insulin-stimulated glucose uptake, de novo lipogenesis, and increased lipolysis (41, 42, 59). Furthermore, IL-6 treatment down-regulates adipogenic markers FA synthase, GPDH, aP2, PPAR-γ, and C/EBP-α in murine adipocytes (42). In addition, IL-6 treatment of 3T3-L1 cells reduces the expression and secretion of adiponectin, an effect that was blocked by pharmacological inhibition of ERK (60). These data demonstrate that IL-6 reduces the TG content, insulin sensitivity, and adipocyte-specific gene expression in a manner strikingly similar to what we see after treatment of cultures of SV cells containing newly differentiated human adipocytes with trans-10, cis-12 CLA. In further support of this concept, dietary supplementation with trans-10, cis-12 CLA in humans results in increased circulating levels of C-reactive protein (55), an acute phase protein that is well known to be transcriptionally induced by IL-6 in the liver (61).

In contrast to IL-6, the role of IL-8 in adipocytes is less clear, and only recently has IL-8 been implicated as a true adipose tissue-derived cytokine (43, 62). Intriguingly, elevated IL-8 mRNA expression in subcutaneous adipose tissue has been correlated positively with insulin resistance (41) and lipodystrophy (63) in humans, two metabolic phenomena also seen with in vivo administration of trans-10, cis-12 CLA (13, 54–56). Gerhardt et al. (43) was the first to demonstrate that primary cultures of SV cells containing newly differentiated human adipocytes produce significant amounts of IL-8. In addition, they demonstrated that mature human adipocytes, but not preadipocytes, express the receptor systems for IL-8 (i.e. CXCR1 and CXCR2), which are known to be classic PTX-sensitive heptahelical GPCRs (46). Importantly, using immuno-localization techniques Gerhardt et al. (43) also found that CXCR1 and CXCR2 receptors were only present on the surface of adipocytes in culture and not present in the supporting SV cells (43). Collectively, the selective expression of CXCR1 and CXCR2 in adipocytes and the fact that they are induced with differentiation point toward a functional role for IL-8 in mature human adipocytes and not in the supporting stroma. Furthermore, CXCR1 and CXCR2 can functionally couple, through PTX-sensitive G proteins, to ERK activation (46, 47, Fig. 11A). This could potentially explain the PTX-sensitive portion of CLA-induced ERK activation (Fig. 6, A and B) because IL-8 is hypersecreted in response to trans-10, cis-12 CLA (Fig. 9A).

The fact that adipocytokines (e.g. TNF-α, IL-6, IL-8, MCP-1, MIP-1α) that oppose adipocyte differentiation all activate ERK downstream of their respective receptors (45–50) indicates that ERK activation may be a critical TG-lowering signal in adipocytes. Although the role of MEK/ERK signaling in adipocytes has been studied extensively, conclusions from these studies are conflicting. In support of this concept, although MEK/ERK activation during the early stages of adipocyte differentiation promotes adipogenesis (34), MEK/ERK activation in mature adipocytes promotes delipidation and insulin resistance (24, 26, 38, 53, 64, 65). Importantly, trans-10, cis-12 CLA-induced...
MEK/ERK activation is sustained for several days (Fig. 5B) and has even been detected after weeks of treatment (data not shown). To our knowledge, this is the longest activation of ERK ever documented in cultures of adipocytes (e.g. cell lines and primary cultures) by any known effector.

In summary, our in vitro data identify molecular mechanisms that may explain the observations from several in vivo studies that trans-10, cis-12 CLA causes insulin resistance and lipodystrophy. We propose that primarily IL-6, and potentially IL-8, are autocrine and paracrine activators of MEK/ERK signaling, leading to impaired glucose and FA uptake and TG synthesis. Based on new emerging data on the source of antiaipocytokine-initiated, MEK/ERK signaling in nonadipocyte SV cells, which in turn, through paracrine actions impacts MEK/ERK signaling in newly differentiated adipocytes, leading to insulin resistance and delipidation. Studies are under way in our laboratory to determine how trans-10, cis-12 CLA increases IL-6 and IL-8 gene expression and secretion, the specific type of cells secreting these adipocytokines (i.e. adipocytes versus undifferentiated SV cells), and the direct versus indirect effects of these proinflammatory agents on MEK/ERK signaling.

Acknowledgments—We are very grateful to GlaxoSmithKline for supplying the Rosiglitazone. We also thank Dr. D. Bernlohr for a polyclonal rabbit anti-aP2 antibody and Dr. Robin Hopkins for excellent technical assistance.

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