Original Article

Nutritional Supplementation With *trans*-10, *cis*-12–Conjugated Linoleic Acid Induces Inflammation of White Adipose Tissue

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Conjugated linoleic acids (CLAs) are conjugated dienoic isomers of linoleic acid. Many people supplement their diets with CLAs to attempt weight loss, and the *trans*-10,*cis*-12 isomer (*t10*,*c12*-CLA) of CLA reduces adiposity in animal models and humans. However, CLA treatment in mice causes insulin resistance that has been attributed to the lipoatrophic state, which is associated with hyperinsulinemia and hepatic steatosis. Here, we investigated the effect of *t10*,*c12*-CLA on adipose tissue inflammation, another factor promoting insulin resistance. We confirmed that *t10*,*c12*-CLA daily gavage performed in mice reduces white adipose tissue (WAT) mass and adiponectin and leptin serum levels and provokes hyperinsulinemia. In parallel, we demonstrated that this CLA isomer led to a rapid induction of inflammatory factors such as tumor necrosis factor-α and interleukin-6 gene expression in WAT without affecting their serum levels. In vitro, *t10*,*c12*-CLA directly induced IL-6 secretion in 3T3-L1 adipocytes by an nuclear factor-κB–dependent mechanism. In vivo, however, the lipoatrophic adipose tissue of CLA-treated mice was notable for a dramatic increase in macrophage infiltration and gene expression. Thus, CLA supplementation directly induces inflammatory gene expression in adipocytes and also promotes macrophage infiltration into adipose tissue to a local inflammatory state that contributes to insulin resistance. *Diabetes* 55:1634–1641, 2006

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Received for publication 7 January 2006 and accepted in revised form 6 March 2006.

c9,*t11*-CLA, *cis*-9,*trans*-11 isomer of conjugated linoleic acid; CLA, conjugated linoleic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IRβ, insulin receptor β; IRS-1, insulin receptor substrate 1; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; PAI-1, plasminogen activator inhibitor-1; PPAR, peroxisome proliferator–activated receptor; SOCS3, suppressors of cytokine signaling 3; *t10*,*c12*-CLA, *trans*-10,*cis*-12 isomer of conjugated linoleic acid; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.

DOI: 10.2337/db06-0036

Excess body weight is the sixth most important risk factor contributing to the overall burden of disease worldwide. Over 1 billion adults and 10% of children are now classified as overweight or obese. Average life expectancy is already diminished, and the main adverse consequences are cardiovascular disease, type 2 diabetes, and several cancers (1). Therefore, there is a growing interest in finding molecules capable of fighting obesity. Conjugated linoleic acid (CLA) is a class of geometric and positional conjugated dienoic isomers produced during biological or industrial hydrogenation of linoleic acid (C18:2, n-6, Δc9, c12). The major dietary sources of CLA are ruminant meat, dairy products, and partially hydrogenated vegetable oils. The two predominant isomers found in foods and commercial preparations are *cis*-9,*trans*-11 isomer of CLA (*c9*,*t11*-CLA) and *trans*-10,*cis*-12 isomer of CLA (*t10*,*c12*-CLA). In the rumens of ovines and bovines, microbial bioconversion mainly produces the *c9*,*t11*-CLA isomer, whereas commercial preparations, sold as dietary supplements, contain *c9*,*t11*-CLA and *t10*,*c12*-CLA isomers in approximately equal amounts (2).

The growing interest in CLAs is due to their putative beneficial health effects found in various experimental models. Besides anticarcinogenic and antiatherogenic effects, CLA-enriched diets lead to a rapid and marked decrease in fat stores in several species, including pig, rat, hamster, chicken, and mouse (3–5), suggesting that CLAs might be useful as weight-loss agents. However, adverse side effects have been recently reported in mice fed a commercial CLA mixture. In this highly CLA-sensitive model, the fat loss triggered by CLA is associated with insulin resistance, robust hyperinsulinemia, and massive liver steatosis (5–8). This CLA-mediated lipoatrophic syndrome is strictly dependent on the *t10*,*c12*-CLA isomer (8). *t10*,*c12*-CLA may impart its delipidation effects by increasing energy expenditure, fecal loss, apoptosis, fatty acid oxidation, and lipolysis as well as decreasing preadipocyte differentiation and lipogenesis (3). In humans, CLA effects observed on body fat mass and insulin sensitivity are still controversial. However, three randomized double-blinded placebo-controlled trials (9–11) presently published showed a weak but significant reduction in fat stores in CLA-treated subjects, and three others (12–14) described an impairment of insulin sensitivity.

It is now recognized that white adipose tissue (WAT), besides its role in energy storage, is also an active endocrine organ. WAT secretes a variety of proinflammatory and anti-inflammatory factors, including the adipokines

leptin, adiponectin, and resistin in addition to cytokines and chemocytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), or plasminogen activator inhibitor-1 (PAI-1). Proinflammatory molecules produced by WAT have been implicated as active participants in the development of insulin resistance in obesity (15). The derivation and role of these cytokines have recently become complicated by the discovery of increased macrophage content of adipose tissue in the setting of obesity (16,17).

Although excess adiposity is commonly associated with insulin resistance, insulin resistance is also observed in rodents and people with too little adipose tissue (18). Lipoatrophy-associated insulin resistance can be ameliorated by leptin in rodents (19,20) as well as humans (21,22). Along with reduced adipose mass, CLA supplementation reduces plasma levels of leptin (8), and leptin infusion reversed hyperinsulinemia in CLA-fed mice (7). An induction of TNF-α expression has been reported in the WAT of CLA-fed mice (7,23), but the role and derivation of adipokines and cytokines that promote insulin resistance in the lipoatrophic syndrome triggered by CLA remain to be determined. The present study was designed to determine whether adipose tissue inflammation plays a role in the insulin resistance syndrome triggered by *t10,c12*-CLA. We found that proinflammatory cytokine levels are increased in the WAT of CLA-treated mice. This is due to a direct inflammatory action of CLA on adipocytes as well as recruitment of macrophages to lipoatrophic adipose tissue of CLA-supplemented mice.

RESEARCH DESIGN AND METHODS

Antibodies. The rabbit polyclonal antibody to insulin receptor β (IRβ) subunit was purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal anti-insulin receptor substrate 1 (IRS-1) and mouse monoclonal anti-phosphotyrosine (PY20) were obtained from Upstate Biotechnology (Lake Placid, NY). The anti-phosphoserine (307) specific for IRS-1 and the polyclonal anti–nuclear factor-κB (anti–NF-κB) p65 were purchased from Cell Signaling (Danvers, MA). The F4/80 (MCA 497R) was purchased from Serotec (Raleigh, NC) and peroxisome proliferator–activated receptor (PPAR)γ from ref. 24.

Animals. Animal care and procedures were in accordance with the guidelines and regulations of the institutional animal care and use committee of the University of Pennsylvania. Wild-type C57Bl/6J females from The Jackson Laboratories (Bar Harbor, MA) at 2.5 months of age were housed ($n = 5$ per cage) under 12-h light/dark cycles (lights on at 7:00 A.M.) with ambient temperature at 23°C and allowed unrestricted access to semisynthetic laboratory chow and water. After 5 days of acclimatization, daily gavage was performed with 0.1 g sunflower oil or 0.1 g of a mixture with 0.08 g sunflower oil and 0.02 g triglycerides containing 79% of *t10,c12*-CLA (total CLA isomers 90.4% [79% *t10,c12*; 7% *c9,t11*; and 3.1% t/t], oleic acid 2.5%, linoleic acid 0.2%, and saturated fatty acids 6.6% [Loders Croklaan, Wormerveer, Holland]). Gavage and body mass gain were monitored every day. The morning after the first, third, and seventh gavages, mice were killed by CO_2 asphyxiation, and the blood was rapidly removed by aortic puncture. After killing, liver and periuteral adipose tissues were collected, weighed, and snap frozen in liquid nitrogen before being stored at −80°C until RNA determinations. A piece of freshly collected periuteral adipose tissue was also fixed in paraformaldehyde (4%, overnight at 4°C), dehydrated, cleared, and then embedded in paraffin for immunohistochemistry analysis.

Cell culture and treatment. 3T3-L1 preadipocytes from the American *Type Culture* Collection (Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37°C in 5% CO_2. Passage numbers 7–15 were used in all studies. 3T3-L1 fibroblasts were seeded on six-well plates, and differentiation was initiated 2 days after confluence in DMEM containing 10% FBS, 10 μg/ml insulin, 1 μmol/l dexamethasone, and 0.5 mmol/l 3-isobutyl-1-methylxanthine for 2 days. They were then grown in DMEM containing 10% FCS and 10 μg/ml insulin for an additional 48 h. After 4 days of differentiation, adipocytes were incubated in the presence of BSA (Fraction V) vehicle or 50 μmol/l linoleic acid (Sigma, St. Louis, MO), *c9,t10*-CLA, or *t10,c12*-CLA (Matreya, Pleasant Gap, PA) in DMEM and 10% FBS. The medium containing fresh BSA or complex BSA–linoleic acid was changed every 2 days. Aspirin was purchased from Sigma, and lactacystin and helenalin were purchased from Biomol (Plymouth Meeting, PA).

Glucose uptake assay. Glucose transport was assayed by measuring [^{3}H]2-deoxyglucose uptake on cells treated with complex BSA–linoleic acid 7 days after differentiation. Twelve hours after serum starvation, cells were treated with insulin (100 nmol/l) for 5 min. Differentiated 3T3-L1 cells in six-well plates were rendered serum free for up to 12 h. They were washed twice and placed into Leibovitz L-15 medium containing 0.2% BSA for an additional 2 h. Krebs-Ringer phosphate buffer with or without insulin (100 nmol/l) was added for 15 min, and then cells were incubated for 4 min with radiolabeled glucose (final concentration 0.1 mmol/l [^{3}H] 2-deoxyglucose; 0.5 μCi/ml) (PerkinElmer LifeSciences, Boston, MA). Cells were washed five times with ice-cold PBS and lysed with 0.05% SDS, and then radioactivity was counted.

Insulin signaling analysis. After 3 days of treatment, cells were incubated for 5 min in the presence of insulin (100 nmol/l), then quickly washed in ice-cold PBS and lysed in buffer (50 mmol/l Tris, 150 mmol/l NaCl, 10 mmol/l EDTA, 1% Triton X-100, 10% glycerol, 200 mmol/l NaF, and 4 mmol/l sodium orthovanadate–containing protease inhibitors, pH 7.5). IRβ and IRS-1 were immunoprecipitated from cell lysates (100 μg) with 2 μg antibody at 4°C overnight. Protein A-agarose was added to collect the immune complexes, and the antibody conjugates were washed four times with lysis buffer. All immune complexes were solubilized in Laemmli buffer and resolved by SDS-PAGE.

Immunohistochemistry. Sections of 5 μm were mounted on charged glass sides, deparaffinized in xylene, stained with hematoxylin, and processed for immunohistochemical detection of F4/80 according to standard immunoperoxidase procedure. The detection was performed using the avidin-biotin peroxidase method.

Nuclear and cytoplasmic extracts. Nuclear extracts were prepared from 3T3-L1 cells using the NE-PER kit from Pierce Biotechnology (Rockford, IL).

RNA isolation and quantitative RT-PCR analysis. For quantitative RT-PCR analysis, total RNA was isolated with RNeasy kits (Qiagen, Valencia, CA). cDNA was synthesized from total RNA with Superscript RT II kit (Gibco BRL, Gaithersburg, MD) after DNase treatment. This cDNA then served as a template for the real-time (Taqman) quantitative PCR performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). 36B4 was used as the housekeeping gene. The following primers and fluorigenic probes were used: 36B4 (forward primer, 5′-GCGGGCACCTTTCT TATCC-3′; reverse primer, 5′-CAAATTAAGCAGGCTGACTTGGT-3′; and probe, 5′-6-FAM-CGGTCTCTTCGACTAATCCCGCCAAA-TAMRA), suppressors of cytokine signaling 3 (SOCS3) (forward primer, 5′-GCGGGCACCTT TCTTATCC-3′; reverse primer, 5′-TCCCCGACTGGGTCTTGAC-3′; and probe, 5′-6-FAM-CTCGGACCAGCGCCACTTCTTCA-TAMRA), and resistin (forward primer, 5′-AGCCATCAATGATAGGATCCA-3′; reverse primer, 5′-TCCAGGC CAATGCTGCTTAT-3′; and probe, 5′-6-FAM-AGGTCGCCGGCTCCCTAATATT TAGGG-TAMRA). Primers and probes for all other genes were obtained from Applied Biosystems. The cycle number at which the transcripts of the gene of interest were detectable (CT) was normalized to the cycle number of 36B4 detection, referred to as ΔCT. The fold change in expression of the gene of interest in the compound-treated group relative to that in the vehicle-treated group was expressed as 2-ΔΔCT, in which ΔΔCT equals the ΔCT of the compound-treated group minus the ΔCT of the chosen control group, which was normalized to 1.

Enzyme-linked immunosorbent assays. Serum insulin, leptin, resistin, MCP-1, and PAI-1 were measured with a commercially available LINCOplex Mouse Adipokine Immunoassay kit from Linco Research (St. Charles, MO) capable of simultaneously measuring several adipokines in mouse serum. Adiponectin was measured in that group by the mouse adiponectin ELISA kit from Linco. To measure IL-6 secretion from 3T3-L1 cells, the medium was collected at 0, 8, 16, 24, and 48 h and between 120 and 168 h, centrifuged at 12,000*g* for 10 min to remove cell debris, and quantified using a commercial ELISA (R&D Systems, Minneapolis, MN).

Statistical analysis. The results are expressed as means ± SE. The significance of differences between groups was determined by the Student's t test. A P value of <0.05 was considered statistically significant.

RESULTS

Short-term oral supplementation with *t10,c12*-CLA leads to reduced adipose tissue mass, hyperinsulinemia, and increased proinflammatory cytokine gene expression. In a previous mouse study, we found that the *t10,c12*-CLA isomer is specifically responsible for the reduction of fat mass, severe hyperinsulinemia, and massive liver steatosis (8). Therefore, we investigated the effect of this isomer on adipose tissue inflammation, a key factor promoting insulin resistance. Daily oral administration of *t10,c12*-CLA to

TABLE 1
Effects of *t10,c12*-CLA supplementation on body composition and circulating factors in mice

	Gavages					
	One		Three		Seven	
	Control	*t10,c12*-CLA	Control	*t10,c12*-CLA	Control	*t10,c12*-CLA
Body mass (g)	20.1 ± 0.3	20.1 ± 0.3	20.4 ± 0.3	19.5 ± 0.4	20.1 ± 0.3	19.1 ± 0.4*
Periuteral adipose tissue (% body mass)	1.23 ± 0.08	1.31 ± 0.08	1.23 ± 0.17	1.42 ± 0.19	1.29 ± 0.05	1.01 ± 0.11*
Liver (% body mass)	4.69 ± 0.12	4.88 ± 0.18	4.67 ± 0.16	4.82 ± 0.05	4.83 ± 0.10	4.72 ± 0.10
Glucose (g/l)	1.54 ± 0.06	1.36 ± 0.05	1.63 ± 0.10	1.50 ± 0.11	1.43 ± 0.16	1.56 ± 0.04
Insulin (pg/ml)	494 ± 243	773 ± 161	470 ± 183	643 ± 101	459 ± 87	1136 ± 130*
Adipokines						
Adiponectin (μg/ml)	30.6 ± 8.9	21.2 ± 3.8	24.5 ± 4.9	22.8 ± 4.3	24.1 ± 4.3	10.3 ± 1.1†
Leptin (ng/ml)	1.2 ± 1.6	1.1 ± 0.6	1.2 ± 0.3	1.6 ± 1.3	1.0 ± 0.2	0.6 ± 0.1†
Resistin (ng/ml)	6.3 ± 0.8	5.1 ± 0.7	5.2 ± 1.0	7.0 ± 0.4‡	6.2 ± 0.5	7.4 ± 1.0*
IL-6 (pg/ml)	7.9 ± 0.8	23.4 ± 5.6	8.6 ± 1.6	8.1 ± 1.7	9.7 ± 2.5	6.4 ± 0.9
TNF-α (pg/ml)	2.7 ± 0.1	3.2 ± 0.4	2.7 ± 0.2	3.1 ± 0.2	2.8 ± 0.2	3.2 ± 0.3
MCP-1 (pg/ml)	63.2 ± 8.2	95.7 ± 49.0	44.5 ± 12	72.3 ± 9.6	44.1 ± 6.4	26.7 ± 5.4
PAI-1 (ng/ml)	1.7 ± 0.1	2.2 ± 0.8	1.4 ± 0.4	2.2 ± 0.4	1.6 ± 0.2	2.2 ± 0.2

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

female C57Bl/6J mice led to a significant decrease in body weight and adipose tissue mass (Table 1). This decrease was accompanied by an insulin-resistant state characterized by a hyperinsulinemia triggered after seven gavages. During this short-term exposure, serum glycemia and liver mass were unaffected.

To explore whether *t10,c12*-CLA triggered an inflammatory state, we analyzed the serum profiles of several adipokines and cytokines and their gene expression in WAT after one, three, and seven gavages (Table 1; Fig. 1). Both leptin and adiponectin serum levels were decreased in mice supplemented seven times with *t10,c12*-CLA, although their mRNA levels decreased at different rates (Table 1; Fig. 1*A* and *B*). By contrast, serum levels of the mouse adipocyte-specific insulin resistance factor resistin increased significantly with CLA supplementation, al-

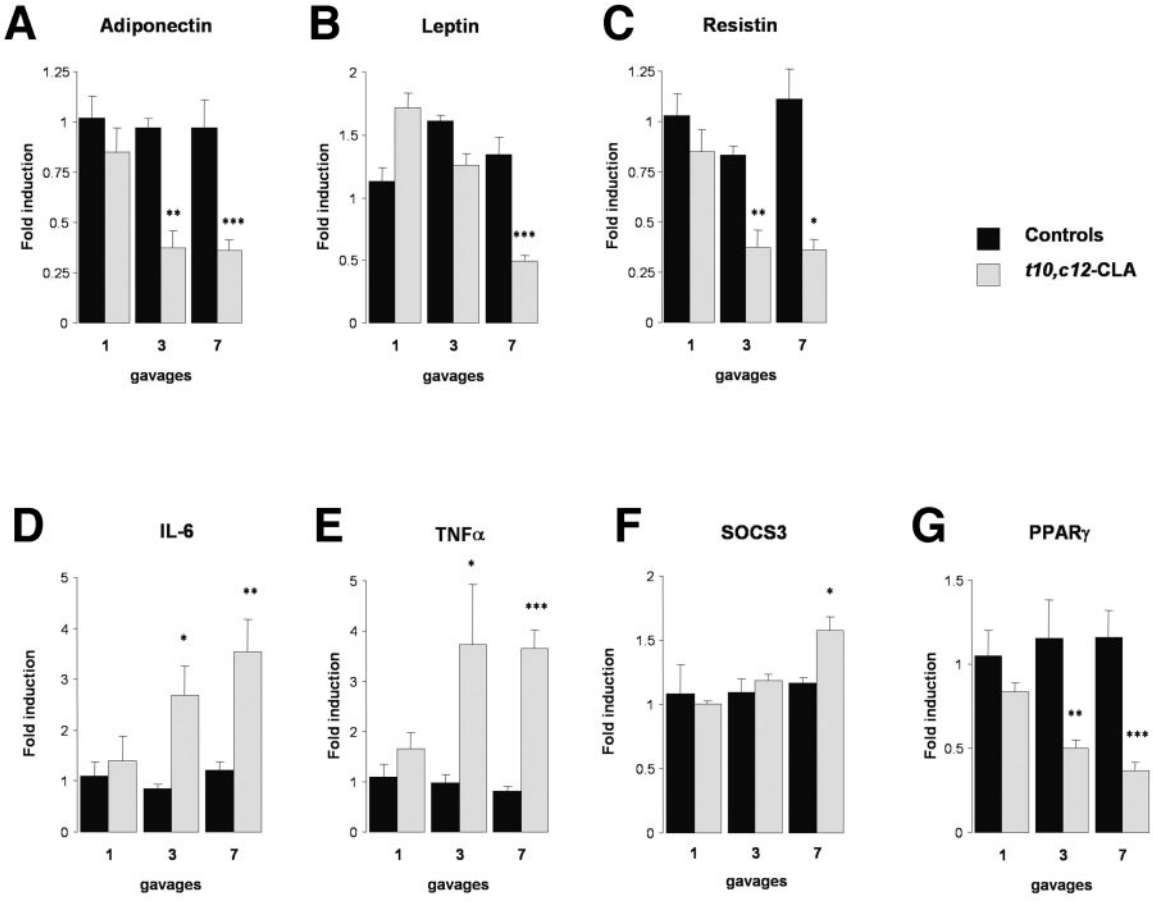


FIG. 1. Effect of *t10,c12*-CLA supplementation on adipokines (*A–C*), cytokines (*D* and *E*), SOCS3 (*F*), and PPARγ (*G*) gene expression in WAT. C57Bl/6J mice were supplemented with 0.1 g sunflower oil or 0.08 g sunflower oil and 0.02 g *t10,c12*-CLA (purity 79%) and killed the next morning after one, three, or seven daily gavages. Means ± SE, $n = 5$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

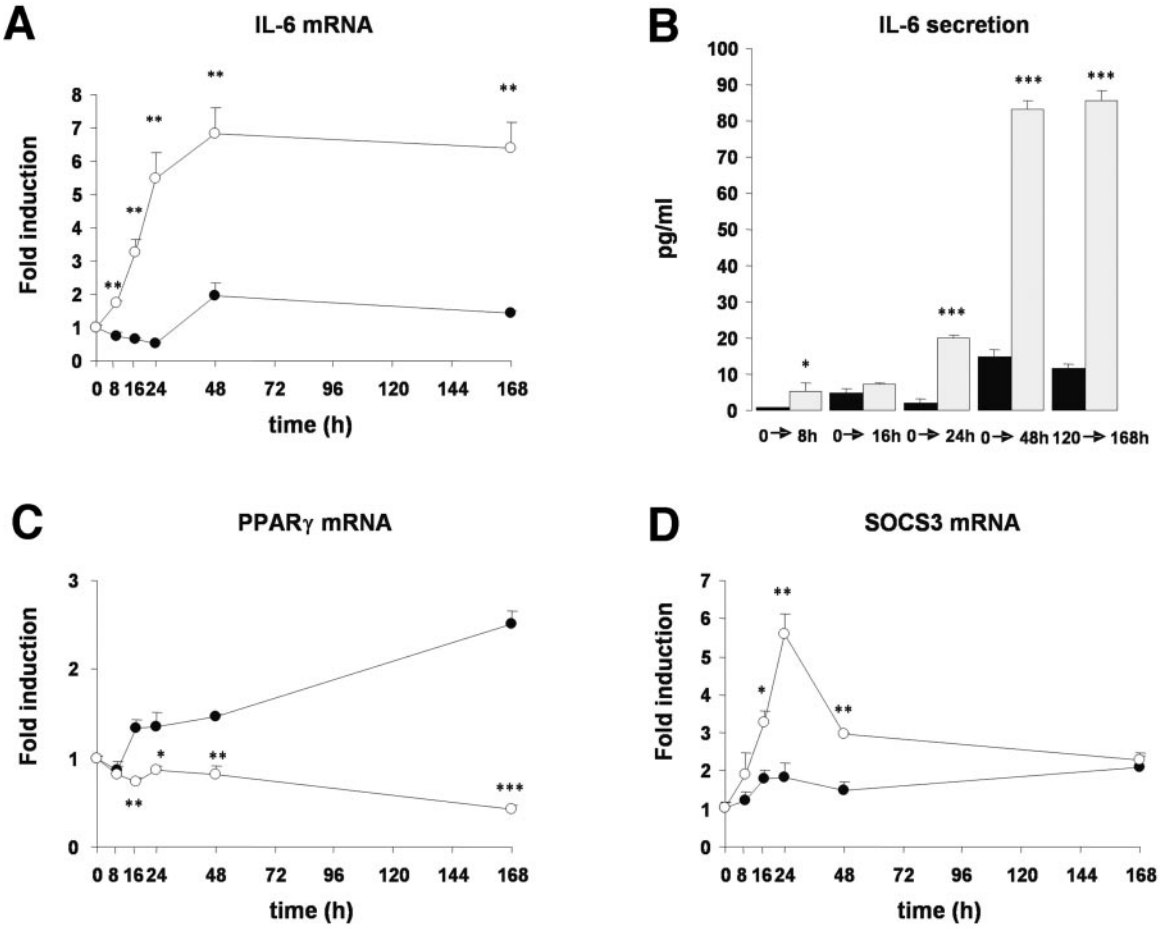


FIG. 2. *t10,c12*-CLA induces IL-6 gene expression and secretion in 3T3-L1 cells. 3T3-L1 cells were differentiated 2 days after confluence in the presence of a cocktail containing dexamethasone (1 μmol/l), isobutylmethylxanthine (0.5 mmol/l), and insulin (10 μmol/l) and then 2 days with insulin (10 μmol/l). After differentiation, cells were incubated in the presence of *t10,c12*-CLA (50 μmol/l) complexed with 12.5 μmol/l BSA (Fraction V) for 0, 8, 16, 24, 48, and 168 h. IL-6 (*A*), PPARγ (*C*), and SOCS3 (*D*) gene expression were analyzed by real-time quantitative RT-PCR, and IL-6 secretion was quantified in medium by enzyme-linked immunosorbent assay (*B*). Black and open circles or bars represent control and *t10,c12*-CLA treatments, respectively. Means ± SE, $n = 5$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

though resistin mRNA actually decreased (Table 1; Fig. 1*C*). A similar dissociation between levels of adipocyte resistin mRNA and circulating protein has been noted in obesity (25). In contrast to resistin, gene expression of IL-6 and TNF-α, two insulin resistance–promoting cytokines, increased in WAT, whereas their serum levels were not significantly altered by CLA supplementation (Fig. 1*D* and *E*). CLA supplementation also induced SOCS3, which is known to be induced by IL-6 and a mediator of insulin resistance (Fig. 1*F*) (21). The lipoatrophic state of the adipose tissue was mirrored by a marked reduction in PPARγ gene expression (Fig. 1*G*).

CLA supplementation induces IL-6 gene expression and secretion along with an insulin-resistant state in 3T3-L1 cells. To explore the mechanism by which CLAs induced IL-6 and TNF-α in mouse WAT, we studied the effect of *t10,c12*-CLA in differentiated 3T3-L1 mouse adipocytes. *t10,c12*-CLA (50 μmol/l) markedly induced IL-6 gene expression and secretion of the protein (Fig. 2*A* and *B*). Thus *t10,c12*-CLA has a direct and specific action on IL-6 expression in adipocytes. Unlike IL-6, in our hands, TNF-α expression was barely detectable in 3T3-L1 adipocytes, and no induction was observed in the presence of *t10,c12*-CLA (data not shown). In parallel and as observed in vivo, gene expression of PPARγ was diminished by *t10,c12*-CLA treatment, whereas SOCS3 mRNA levels were increased (Fig. 2*C* and *D*). Induction of SOCS3 impairs insulin action (26), and insulin-stimulated [^{3}H]2-deoxyglucose uptake was markedly attenuated in adipocytes treated with *t10,c12*-CLA (Fig. 3*A*). This was mediated at least in part by a decrease in IRβ and IRS-1 expression and tyrosine phosphorylation, although phosphorylation of IRS-1 at serine 307, which is known to inhibit the insulin signaling cascade (27), was unaffected (Fig. 3*B*).

IL-6 induction by *t10,c12*-CLA is NF-κB dependent. To explore whether IL-6 induction is dependent on NF-κB activation, the effect of three specific chemical inhibitors of this pathway was investigated in the 3T3-L1 adipocytes treated with *t10,c12*-CLA. These inhibitors were aspirin (0.5, 5, or 10 μmol/l), which has anti-inflammatory properties; lactacystin (1 or 2.5 μmol/l), which blocks proteasomal degradation of inhibitor of κBα; and helenalin (1 μmol/l), which inhibits NF-κB–DNA binding activity by selectively alkylating the p65 subunit of NF-κB. All three inhibitors caused a dose-dependent inhibition of CLA induction of IL-6 gene expression (Fig. 4*A*). Consistent with this, nuclear localization of the p65 subunit of NF-κB was markedly increased in the CLA-treated cells (Fig. 4*B*). By contrast, PPARγ protein, which is present almost exclusively in the nucleus, was decreased in the presence of *t10,c12*-CLA (Fig. 4*B*).

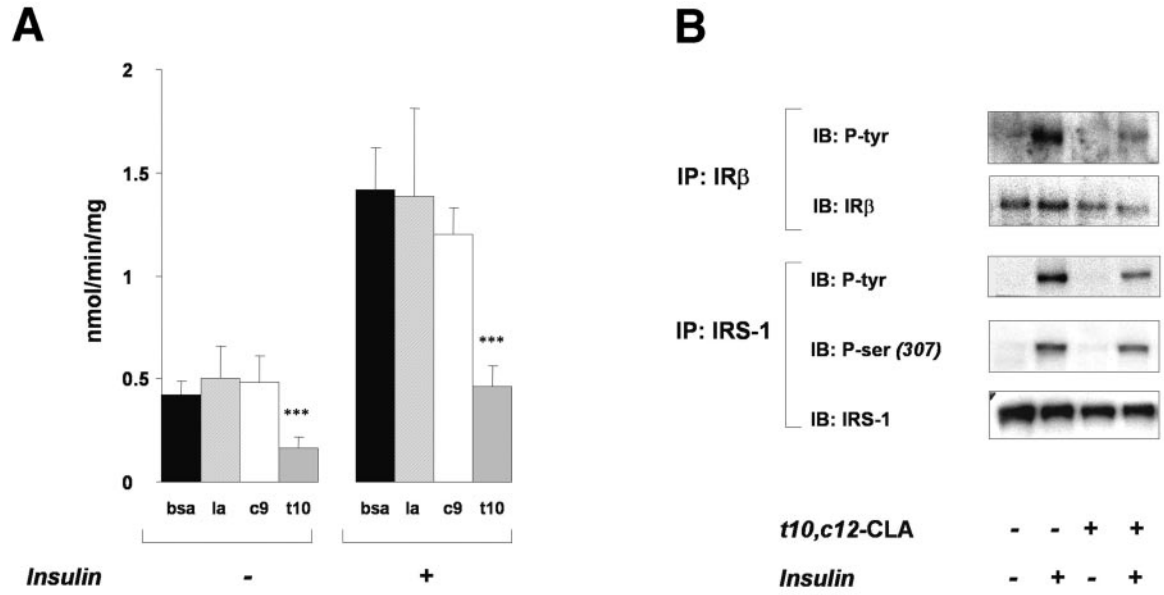
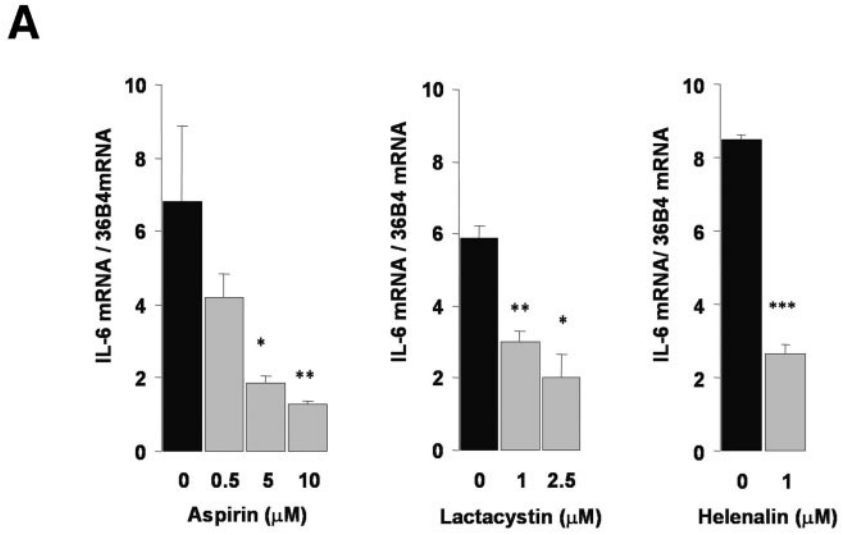


FIG. 3. *t10,c12*-CLA reduces insulin-stimulated glucose transport in 3T3-L1 cells and alters the insulin signaling cascade. Differentiated 3T3-L1 cells were incubated with *t10,c12*-CLA (50 μmol/l) complexed with 12.5 μmol/l BSA (Fraction V) or BSA alone for 72 h. *A*: Basal and insulin-stimulated uptake of 0.1 mmol/l [^{3}H]2-deoxyglucose was measured for 4 min in the absence (−) or presence (+) of insulin (100 nmol/l). *B*: Insulin signaling cascade was studied for 5 min in the absence (−) or presence (+) of insulin (100 nmol/l), and total cell extracts were immunoprecipitated with IRβ and IRS-1 antibodies then immunoblotted with (*p*-tyr)IRβ, IRβ, (*p*-tyr)IRS-1, IRS-1, and (ser307)IRS-1 antibodies. Means ± SE, $n = 3$. ***$P < 0.001$.



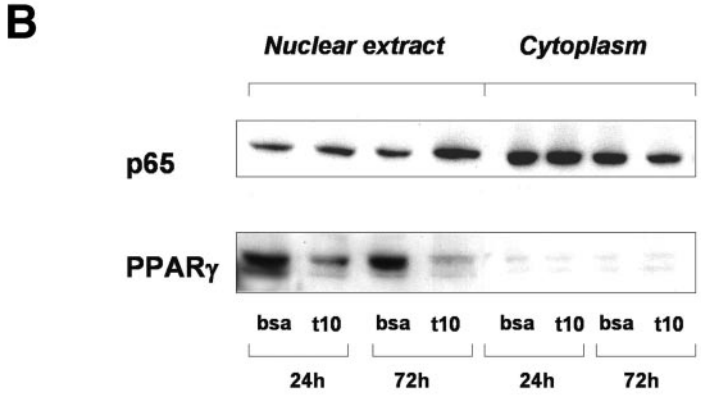


FIG. 4. IL-6 induction due to *t10,c12*-CLA is NF-κB dependent. *A*: Differentiated 3T3-L1 cells were incubated in the presence of *t10,c12*-CLA (50 μmol/l) complexed with 12.5 μmol/l BSA (Fraction V) for 72 h. NF-κB pathway was inhibited with aspirin (0, 0.5, 5, and 10 μmol/l), lactacystin (1 and 2.5 μmol/l), helenalin (1 μmol/l), or vehicle (DMSO), and IL-6 mRNA level was determined by real-time quantitative RT-PCR. *B*: NF-κB active form (p65) and PPARγ proteins were visualized by immuoblotting the nuclear extract and cytoplasm of 3T3-L1 cells treated for 1 or 3 days with *t10,c12*-CLA (50 μmol/l) complexed with BSA (12.5 μmol/l) or BSA alone. Means ± SE, $n = 3$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

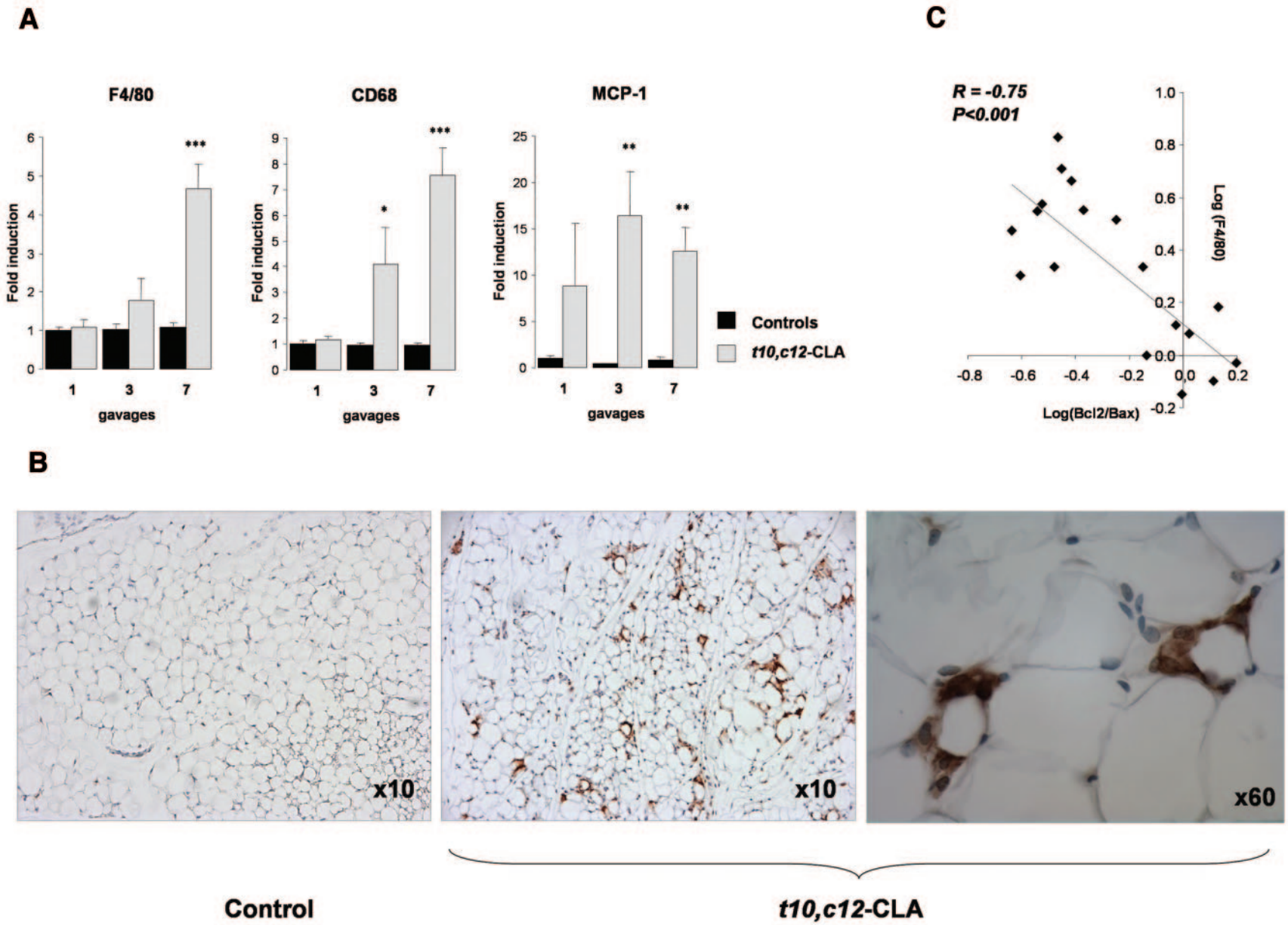


FIG. 5. ***t10,c12*-CLA supplementation causes macrophage infiltration in WAT. C57BL/6J mice were force fed with 0.1 g sunflower oil or 0.08 g sunflower oil and 0.02 g *t10,c12*-CLA (purity 79%) and then killed the next morning after one, three, or seven daily gavages. *A*: Macrophage markers (CD68 and F4/80) and MCP-1 gene expression were analyzed by quantitative RT-PCR. *B*: F4/80 protein was visualized on WAT by immunostaining. Representative sections are shown from control and CLA-gavage fed mice ($n = 5$). *C*: Correlation between F4/80 and Bcl2-to-Bax ratio gene expressions. Means ± SE, $n = 5$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.**

***t10,c12*-CLA triggered a macrophage infiltration in WAT.** The observation that CLA induced IL-6 and SOCS3 gene expression in isolated adipocytes suggested a direct effect on these cells that could explain the induction of these inflammatory markers in WAT from CLA-supplemented mice. However, TNF-α gene expression was also markedly induced in WAT yet was not increased in adipocytes treated with CLAs in vitro. TNF-α is a major inflammatory product of macrophages, and macrophage infiltration of WAT has been recently described in obesity. Therefore, we hypothesized that CLA supplementation might be a novel cause of macrophage infiltration, which could explain the increased TNF-α expression in the WAT from CLA-treated mice. Immunohistochemistry using the macrophage marker F4/80 revealed marked macrophage infiltration into adipose tissue, consistent with a dramatic induction in the gene expression of macrophage-specific markers CD68 and F4/80 after three and seven gavages with CLAs (Fig. 5*A*). Interestingly, gene expression of MCP-1 involved in the chemotaxis and maintenance of circulating monocytes in tissue developing inflammation was dramatically induced in the WAT from CLA-supplemented mice (Fig. 5*A*). This could contribute to the increased macrophage homing to WAT. Also, the ratio between the gene expression of apoptosis-inducing Bax and the repressor of cell death Bcl2 decreased in WAT from *t10,c12*-CLA supplemented mice, suggesting an increase in apoptosis (Fig. 5*C*). This is consistent with several previous studies that showed increased apoptosis in adipose tissue in CLA-treated mice (7,28,29) and was highly correlated with macrophage presence because a high correlation was observed between Bcl2-to-Bax ratio and macrophage markers (Fig. 5*C*).

DISCUSSION

In this study, we demonstrated for the first time that *t10,c12*-CLA leads to a local inflammation of WAT characterized by macrophage infiltration and induction of TNF-α, IL-6, and MCP-1 gene expression without alteration of their serum levels. In 3T3-L1 adipocytes, *t10,c12*-CLA induces IL-6 expression and inhibition of insulin-stimulated glucose uptake. However, the induction of TNF-α observed in vivo is likely due to the increased macrophage infiltration of the WAT from CLA-supplemented mice.

TNF-α and IL-6 gene expression in WAT are positively correlated with insulin resistance (30), and these genes were induced within a week of CLA supplementation, which also caused insulin resistance. However, circulating levels of these cytokines were not elevated at those times. By contrast, we did observe changes in circulating adipokines, with decreases in insulin-sensitizing adiponectin and leptin, and an increase in serum resistin levels. Changes in adipokine secretion may be due to local production of TNF-α (31,32) and IL-6 (33). Therefore, these data support the existence of a chronology of events beginning with an induction of TNF-α and IL-6 gene expression in WAT followed by a reduction of adiponectin and leptin gene expression before a significant drop in fat stores and hyperinsulinemia. Although not observed in the present, acute experiments, hepatic steatosis *t10,c12*-CLA–induced lipoatrophy would be expected to occur with more chronic treatment, as shown previously (7,34).

Studies in murine 3T3-L1 adipocytes suggest that *t10,c12*-CLA induction of IL-6 in WAT is due to a direct effect on adipocytes. This effect appeared to be related to activation of NF-κB, because three chemically and mechanistically distinct inhibitors of this pathway abrogated the IL-6 induction. This finding is consistent with a recent report of NF-κB activation by *t10,c12*-CLA in mature human adipocytes (35). In human adipocytes, IL-6 induction is reported to perturb insulin action via the mitogen-activated extracellular signal protein kinase/extracellular signal–related kinase pathway (36). However, our study performed in 3T3-L1 adipocytes showed that insulin resistance is partially due to an alteration of the insulin signaling cascade via a decrease in IRβ and IRS-1 expression and tyrosine phosphorylation that is potentially due to an induction of SOCS3. IL-6 induces SOCS3 mRNA levels, and SOCS3 inhibits IRS-1 phosphorylation and induces ubiquitination and proteasomal degradation of IRS-1 and IRS-2 (37). Therefore, we proposed that CLA treatment of 3T3-L1 adipocytes induces local insulin resistance by activation of NF-κB, which induces IL-6 expression and secretion. IL-6 induces SOCS3 and thereby attentuates insulin signaling leading to a decrease in insulin-stimulated glucose uptake.

Although the induction of IL-6 and SOCS3 in WAT from CLA-supplemented mice can be attributed to direct adipocyte effects of *t10,c12*-CLA, this is not the case for the induction in TNF-α gene expression that is observed in vivo but not in vitro. We found that CLA supplementation led to a large increase in macrophage infiltration into WAT, detected both by analysis of macrophage-specific gene expression as well as by immunohistochemistry. This finding is consistent with the finding of Weisberg et al. (17) that in WAT, macrophages produce the vast majority of TNF-α, whereas mature adipocytes secrete the majority of adiponectin, leptin, resistin, and IL-6.

The macrophage infiltration into WAT may be attributed to MCP-1, which is involved in macrophage recruitment (38) and is induced 16-fold after only three gavages with CLAs. Although macrophage infiltration into WAT has been most well documented in obesity (15), this is also seen in lipodystrophic syndromes, including HIV-1–infected lipodystrophic patients (39). Intriguingly, changes in WAT gene expression and circulating adipokines that we have observed in CLA-induced lipoatrophy are similar to those described in HIV lipoatrophy syndrome (40). Scherer and colleagues (41) recently demonstrated that macrophage accumulation in WAT is also observed in a mouse model of inducible lipoatrophy through targeted activation of caspase 8 leading to apoptosis. *t10,c12*-CLA causes apoptosis in vivo as observed here and previously (7,28,29) but also in vitro (42). This suggests that apoptosis would precede macrophage infiltration into WAT.

Because CLA impact appears to be highly species specific, with mice generally more sensitive than other species, it is too early to extrapolate our results to other species. However, several studies (12,14,43) have shown that *t10,c12*-CLA supplementation induces an alteration of insulin sensitivity in humans. The inflamed lipoatrophic adipose tissue that we have observed in CLA-treated mice provides a potential mechanism of insulin resistance that adds to concerns about the safety of dietary supplements containing *t10,c12*-CLA that are widely promoted as nonprescription antiobesity agents.

ACKNOWLEDGMENTS

M.A.L. has received National Institutes of Health Grant PO1-DK-49210.

The Penn Diabetes and Endocrinology Research Center (supported by National Institutes of Health Grant P30-DK-

19525) assisted with cytokine and hormone assays (H. Collins, RIA Core), and the Morphology Core (supported by National Institutes of Health Grant DK-49210) assisted with immunohistochemistry (G. Swain). We thank Dr. M. Lehrke for helpful discussions and Loders Crocklaan for supplying CLA preparations.

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