Original Article

Antidiabetic Effects of *cis*-9, *trans*-11–Conjugated Linoleic Acid May Be Mediated via Anti-Inflammatory Effects in White Adipose Tissue

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Adipose tissue may be the source of insulin desensitizing proinflammatory molecules that predispose to insulin resistance. This study investigated whether dietary fatty acids could attenuate the proinflammatory insulin-resistant state in obese adipose tissue. The potential antidiabetic effect of *cis*-9, *trans*-11–conjugated linoleic acid (c9,t11-CLA) was determined, focusing on the molecular markers of insulin sensitivity and inflammation in adipose tissue of *ob/ob* C57BL-6 mice. Feeding a c9,t11-CLA–enriched diet reduced fasting glucose ($P < 0.05$), insulin ($P < 0.05$), and triacylglycerol concentrations ($P < 0.01$) and increased adipose tissue plasma membrane GLUT4 ($P < 0.05$) and insulin receptor ($P < 0.05$) expression compared with the control linoleic acid–enriched diet. Interestingly, after the c9,t11-CLA diet, adipose tissue macrophage infiltration was less, with marked downregulation of several inflammatory markers in adipose tissue, including reduced tumor necrosis factor-α and CD68 mRNA ($P < 0.05$), nuclear factor-κB (NF-κB) p65 expression ($P < 0.01$), NF-κB DNA binding ($P < 0.01$), and NF-κB p65, p50, c-Rel, p52, and RelB transcriptional activity ($P < 0.01$). To define whether these observations were direct effects of the nutrient intervention, complimentary cell culture studies showed that c9,t11-CLA inhibited tumor necrosis factor-α–induced downregulation of insulin receptor substrate 1 and GLUT4 mRNA expression and promoted insulin-stimulated glucose transport in 3T3-L1 adipocytes compared with linoleic acid. This study suggests that altering fatty acid composition may attenuate the proinflammatory state in adipose tissue that predisposes to obesity-induced insulin resistance. *Diabetes* 56:574–582, 2007

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c9,t11-CLA, *cis*-9, *trans*-11–conjugated linoleic acid; t10,c12-CLA, *trans*-10, *cis*-12–conjugated linoleic acid; CLA, conjugated linoleic acid; HOMA-IR, homeostasis model assessment for insulin resistance; IRS, insulin receptor substrate; LXR, liver X receptor; NEFA, nonesterified fatty acid; NF, nuclear factor; QUICKI, quantitative insulin sensitivity check index; SREBP, sterol regulatory element binding protein; TNF, tumor necrosis factor; TZD, thiazolidinedione.

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The metabolic syndrome is characterized by obesity, insulin resistance, and dyslipidemia (1). It is associated with a high risk of type 2 diabetes and cardiovascular disease (2). The incidence of the metabolic syndrome is increasing exponentially as a consequence of the escalating rise in obesity, the key etiological factor in the development and severity of the metabolic syndrome (3). To date, public health strategies have been largely unsuccessful at reducing the prevalence of obesity. Therefore, dietary interventions that attenuate the severity of the metabolic syndrome, within the context of obesity, are required. Dietary fatty acids play an inherent role in the development of obesity, insulin resistance, and the metabolic syndrome. First, dietary fats are an enriched source of dietary energy, and excessive consumption can induce obesity with subsequent insulin resistance. Second, nutrient-derived metabolic stressors including a high-fat diet and elevated plasma nonesterified fatty acid (NEFA) concentrations induce insulin resistance by inhibiting insulin signaling (4,5). It has been proposed that the combination of excessive nutrient-derived metabolic stressors coupled with proinflammatory stressors further augment the risk of insulin resistance (6).

Adipose tissue may be the source of insulin desensitizing proinflammatory molecules that predispose to insulin resistance (7,8). Adipokines, including tumor necrosis factor (TNF)-α, interleukin-6, resistin, and adiponectin (acrp30), have all been shown to influence insulin sensitivity (9–11). The insulin desensitizing effects of TNF-α are probably best characterized. TNF-α inhibits autophosphorylation of tyrosine residues of the insulin receptor, promotes serine phosphorylation of insulin receptor substrate (IRS)-1, and reduces transcription of key targets in the insulin-signaling cascade, all of which impede transduction of the insulin signal (12). Knocking out the TNF-α and TNF-α receptor genes improves insulin resistance in animal models of obesity-induced insulin resistance (13,14). Other groups have proposed that IκB kinase-β is central to the interplay between dietary fatty acids and insulin resistance. In vivo salicylate treatment, a known inhibitor of IκB kinase-β, prevented fat-induced insulin resistance (15). The c-Jun NH_2-terminal kinases also play a role in obesity-induced insulin resistance. Mice with a targeted mutation in *Jnk1* are leaner, are more insulin sensitive, and show enhanced insulin receptor signaling capacity in genetic and dietary-induced models of obesity (16). These studies suggest that downregulation of several

components of the inflammatory response affords protection from obesity-induced insulin resistance.

Altering dietary fatty acid composition may play a role in insulin resistance. Diets rich in saturated fatty acids desensitize peripheral tissue responsiveness to insulin (5), whereas some polyunsaturated fatty acids counteract this effect. Our work suggests that some polyunsaturated fatty acids, in particular, a specific isomer of conjugated linoleic acid (CLA), may improve insulin sensitivity. CLA refers to a group of positional and geometric conjugated dienoic isomers of linoleic acid (C18:2 n-6). The metabolic health effects of CLA are isomer specific, a phenomenon that may explain the controversial effects of CLA on glucose and insulin metabolism. Diets enriched with c9,t11-CLA resolved the dyslipidemic component of the metabolic syndrome, through altered hepatic sterol regulatory element binding protein (SREBP)-1c– and liver X receptor (LXR)-α–mediated gene expression events (17). In contrast, feeding a diet containing *trans*-10, *cis*-12–conjugated linoleic acid (t10,c12-CLA) induced insulin resistance and hyperlipidemia in *ob/ob* and C57BL/6J mice (17,18). Interestingly, recent research has shown that feeding t10,c12-CLA promoted macrophage infiltration into adipose tissue, inducing adipose tissue inflammation and insulin resistance (19). The pro-diabetic effect of t10,c12-CLA was confirmed in obese men with signs of the metabolic syndrome with negative effects on insulin resistance, biomarkers of oxidative stress, and inflammation demonstrated (20,21). Nevertheless, detailed analysis of the potential insulin-sensitizing effects of *cis*-9, *trans*-11–conjugated linoleic acid (c9,t11-CLA), particularly focusing on the anti-inflammatory capabilities of this CLA isomer (22), had not been fully investigated.

This study addressed the hypothesis that some dietary fatty acids may attenuate the proinflammatory insulin-resistant state in obese adipose tissue. The potential antidiabetic effect of a c9,t11-CLA–enriched diet was determined, focusing on the molecular markers of insulin sensitivity and inflammation in adipose tissue of *ob/ob* C57BL-6 mice, a well-characterized model of obesity and insulin resistance. Feeding the c9,t11-CLA–enriched diet improved glucose and insulin metabolism compared with the control linoleic acid–rich diet. This was associated with less adipose tissue macrophage infiltration and down-regulation of inflammatory markers associated with insulin resistance in adipose tissue. Complimentary cell culture studies also showed that c9,t11-CLA attenuated the insulin desensitizing effect of TNF-α treatment in 3T3-L1 adipocytes compared with linoleic acid. This result supports the concept that altering fatty acid composition may provide a means to attenuate the proinflammatory state in adipose tissue that predisposes to insulin resistance.

RESEARCH DESIGN AND METHODS

Animal experiment and nutritional intervention. This study was conducted at the BioResources Unit at Trinity College, Dublin, Ireland, according to good animal welfare protocols that comply with European legislation governing the use of animals in research. Six-week-old male *ob/ob* mice were purchased from Harlan U.K. The mice were exposed to a 12-h light/12-h dark cycle and maintained at a constant temperature of 22°C. Sixteen were randomly assigned to a control diet or c9,t10-CLA–enriched diet for 6 weeks. The diets contained 131 g fat/kg (30% energy); provided equal amounts of saturated fatty acid, monounsaturated fatty acids, and polyunsaturated fatty acids; and were prepared by Unilever (Vlaardingen, the Netherlands), as previously described (17). The polyunsaturated fatty acid fraction of the control diet contained linoleic acid as free fatty acid, and the CLA-enriched diet contained an equivalent amount of the CLA isomers as free fatty acids (Table 1). CLA was supplied by Loders Croklaan (Wormerveer, the Netherlands). In addition, eight *ob/ob* mice received the control diet containing 0.2% rosiglitazone (a gift from SmithKline Beecham Pharmaceuticals, Worthing, West Sussex, U.K.) to act as a positive control thiazolidinedione (TZD) group. Feed consumption and mouse weight was measured weekly. In this study, the investigations were completed in the fasted state. Food was removed at 6:00 P.M., and the animals were killed the following morning between 8:00 and 10:00 A.M. The mice were killed and blood was collected by cardiac puncture. The blood was centrifuged, and the serum was harvested and stored (−20°C). White adipose tissue and liver samples were harvested. Tissue samples for RNA analysis were immersed in RNase Later (Ambion, AMS Technology, U.K.), and those for protein analysis were snap-frozen in liquid N_2. All tissue samples were stored at −70°C until analysis.

TABLE 1
Fatty acid composition of the control (linoleic acid) and c9,t11-CLA–enriched diets expressed as a percentage of the total diet (g/100 g feed)

	Linoleic acid	c9,t11-CLA
Saturated fatty acids	5.29	5.11
Monounsaturated fatty acids	4.27	4.47
Polyunsaturated fatty acids	3.50	3.48
Linoleic acid	0.87	0.04
c9,t11 CLA	—	0.58
t10,c12 CLA	—	0.11
Other CLA isomers	—	0.01

Cell culture experiments. 3T3-L1 cells were cultured and differentiated into fully mature adipocytes as previously described (23). 3T3-L1s were incubated with 50 μmol/l of c9,t11-CLA (Cayman Chemical, Ann Arbor, MI), linoleic acid (Sigma-Aldrich, Dorset, U.K.), or an equivalent volume of dimethyl sulfoxide (DMSO; Sigma-Aldrich), which served as the vehicle control. Fatty acids were added with the differentiation media and replaced with each media change (daily for a 7-day duration), to test the effect of chronic fatty acid supplementation as in the feeding study in vivo. Insulin resistance was induced in adipocytes by incubating mature adipocytes for 3 days in the presence of 3 ng/ml TNF-α (R&D Systems, Minneapolis, MN), with fresh media changes each day. This TNF-α treatment has previously been shown to significantly reduce the rate of insulin-stimulated glucose uptake by ~60% without inducing morphological changes in mature adipocytes (24). Adipocytes treated in parallel but without TNF-α were considered normal adipocytes. Oil-Red O staining was performed according to standard protocol to confirm that the fibroblast treatment with either DMSO or fatty acids differentiated into mature adipocytes. An MTS assay (Promega, Madison, WI) was performed according to the manufacturer's instructions to ensure that neither the fatty acids nor TNF-α treatment adversely affected cellular viability.

Biochemical analysis. Serum glucose (Glucose PAP; BioMerieux, Lyon, France), triacylglycerol (TAG PAP; BioMerieux), cholesterol (Cholesterol PAP; BioMerieux), and NEFA (NEFA; Randox Laboratories, Antrim, U.K.) concentrations were determined using enzymatic colorimetric assays on a Technicon RA-XT analyzer (Bayer, Dublin, Ireland) (25). Cell culture TNF-α levels were determined using a mouse TNF-α Quantikine enzyme-linked immunosorbent assay kit (R&D Systems). Serum insulin concentrations were measured using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem, Chicago, IL) (17). Fasting serum insulin and glucose concentrations were used to calculate insulin resistance using the homeostasis model assessment for insulin resistance (HOMA-IR) [$(\text{glucose}_{\text{fasting}} \times \text{insulin}_{\text{fasting}})/22.5$] (26), although it should be acknowledged that the HOMA-IR model has not been validated for use in animal models (27). Insulin sensitivity was calculated with use of the revised quantitative insulin sensitivity check index (QUICKI) [$1/(\log \text{insulin}_{\text{fasting}} + \log \text{glucose}_{\text{fasting}} + \log \text{NEFA}_{\text{fasting}})$] (28).

mRNA expression analysis. Total RNA was extracted from animal tissues and 3T3-L1 adipocytes using TRI Reagent (Molecular Research Centre, Cincinnati, OH). RNA purity and integrity was assessed, and RT was performed as previously described (29). RNA expression was quantified by real-time RT-PCR (ABI 7700 Sequence Detection System; Perkin-Elmer Applied Biosystems, Warrington, U.K.) using reaction conditions outlined previously (29). Predeveloped primer and probe kits (Perkin-Elmer Applied Biosystems) were used to detect most mRNA targets. Custom-made oligonucleotide primers and TaqMan probes were designed using Primer express (version 1.0; Perkin-Elmer Applied Biosystems) for isoform-specific components of peroxisome proliferator–activated receptor-γ and SREBP-1, as previously described (17,29). Target gene mRNA expression was normalized to the internal control GAPDH and expressed relative to the control group.

Protein extraction and Western blot analysis. Various protein fractions were isolated from the adipose tissue of *ob/ob* mice using specific lysis buffers.

The total protein fraction was isolated using ice-cold lysis buffer as previously described by Takemori et al. (30). The plasma membrane fraction was prepared from the pooled fat of two animals according to dietary intervention to yield sufficient tissue using lysis buffer and conditions previously outlined (31). The cytosolic and nuclear fractions of adipose tissue were isolated as described by Loscher et al. (22). Total protein and plasma membrane protein concentrations were determined using the modified Lowry assay, and nuclear and cytosolic extracts were quantified using the Bradford assay, using BSA as a standard. Equal quantities of protein were acetone precipitated overnight at −20°C. Proteins were subsequently separated using a 10% SDS-PAGE gel or a 6% SDS-PAGE gel in the case of phospho IRS-1 (Ser^{307}) and electrophoresed onto polyvinylidene fluoride membrane (Pall Corporation, Pensacola, FL) for immunoblotting with the appropriate antibody. Samples treated with TZD were included on some blots as a positive control. An appropriate molecular weight marker was run concurrently to aid protein identification. Immunoreactive bands were visualized using an enhanced chemiluminescence reaction (Supersignal West Pico Chemiluminescent Substrate, Pierce, IL). Antibodies used in Western blot analysis were IRS-1 (Ser^{307}) (Upstate, Charlottesville, VA), GLUT4 (Chemicon International, Tennecula, CA), nuclear factor (NF)-κB p65, and IκBα (both Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemistry. Paraffin-embedded sections (n = 10 per dietary treatment, two sections from five animals in each dietary group) were incubated in primary antibody against F4/80 (Abcam, Cambridge, U.K.). Slides were washed and incubated in secondary biotinylated antibody and the immunocomplex was visualized using the diaminobenzidine chromogen (Vectastain Elite Lit; Vector Laboratories, Burlingame, CA). Macrophage surface area was quantified using Improvision software (Improvision, Warwick, U.K.).

Electrophoretic mobility shift assay. Nuclear extracts were isolated as described by Loscher et al. (22). The 4 μg white adipose tissue nuclear extracts were incubated with 10,000 cpm of ^{32}P-labeled NF-κB oligonucleotide (5′-AGTTGAG*GGGACTTTCC*CAGGC-3′) for 30 min at room temperature, before separation of the DNA-protein complexes on a 4% polyacrylamide gel as previously described (22). Quantification of both electrophoretic mobility shift assay and Western blotting autoradiographic bands was performed using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation and Analysis System; Syngene). Results were normalized to the control group and expressed as fold change relative to control.

NF-κB transcription factor assay. NFκB activation was measured using the TransAm NFκB family kit (Active Motif Europe, Belgium). The kit contains a 96-well plate on which has been immobilized an oligonucleotide containing the NF-κB consensus site. The active form of NF-κB contained in nuclear extracts specifically binds to this oligonucleotide. The primary antibodies used to detect NF-κB recognize an epitope on p50, p52, p65, c-Rel, or RelB that is accessible only when NF-κB is activated and bound to its target DNA. A horseradish peroxidase–conjugated secondary antibody provides a colorimetric readout that can be measured by reading the absorbance at 450 nm.

Glucose uptake assay. Glucose uptake in 3T3-L1s was measured using Cytostar-T scintillating microplates (Amersham Biosciences, Buckinghamshire, U.K.) according to the manufacturer's instructions. Fatty acid and TNF-α treatments were performed as outlined above. Cells were grown overnight in serum-free Dulbecco's modified Eagle's medium containing 0.5% (wt/vol) BSA. This was removed, and cells were washed with 200 μl buffer (50 mmol/l HEPES, pH 7.4, 120 mmol/l NaCl, 1.85 mmol/l $CaCl_2$, 1.3 mmol/l $MgSO_4$, and 4.8 mmol/l KCL) and cultured for 5 h to ensure glucose depletion. Insulin (0.1, 1, and 10 nmol/l) was added to the appropriate wells, and the cells were cultured for a further 20 min. This was followed by the addition of 10 μl (0.1 μCi) 2-deoxyglucose (Amersham Biosciences) for 10 min. Glucose uptake was monitored, as an increase in scintillation counts, using a Wallac MicroBeta 1450 liquid scintillation counter (Perkin Elmer, Wellesley, MA).

Statistical analysis. Statistical analyses were performed with Data Desk 6.0 (Data Description, Ithaca, NY). The data were transformed as appropriate to yield a normal Gaussian distribution. One-way ANOVA, with dietary intervention as the independent variable, was used to investigate differences between treatments. Post hoc statistical analysis was performed using the Scheffe test, to identify the significance of the individual dietary treatments.

RESULTS

Antidiabetic effects of c9,t11-CLA in *ob/ob mice*. Table 2 shows that feeding a diet enriched with the c9,t11-CLA isomer improved several physiological indexes of the metabolic syndrome. The c9,t11-CLA diet significantly reduced fasting serum glucose ($P < 0.05$), insulin ($P < 0.05$), and triacylglycerol ($P < 0.01$) concentrations, compared with the control diet. Also, intervention with c9,t11-CLA significantly decreased the HOMA-IR and increased the index of insulin sensitivity (revised QUICKI) ($P < 0.05$). Reduced glucose and triacylglycerol concentrations were equivalent to that achieved in the positive TZD-treated group (10.05 ± 0.596 and 0.930 ± 0.092 mmol/l, respectively). The c9,t11-CLA diet had no significant effect on serum NEFA or cholesterol concentrations compared with the control linoleic acid diet. Neither body weight, epididymal adipose tissue mass, or feed intake were significantly different between the control and c9,t11-CLA–fed mice.

TABLE 2
Fasting serum glucose and insulin concentrations, HOMA-IR, revised QUICKI, lipid concentrations, and body weight in C57/BL-6 *ob/ob* mice fed the control (linoleic acid) and c9,t11-CLA–enriched diets for 4 weeks

	Control	c9,t11-CLA
n	6	5
Glucose (mmol/l)	12.06 ± 0.61	10.76 ± 0.71*
Insulin (pmol/l)	202.9 ± 56.5	79.5 ± 16.4*
HOMA-IR	96.38 ± 28.35	40.84 ± 4.86*
Revised QUICKI	0.32 ± 0.01	0.36 ± 0.01†
Triacylglycerol (mmol/l)	1.11 ± 0.07	0.95 ± 0.04†
NEFA (mmol/l)	0.74 ± 0.04	0.65 ± 0.12
Cholesterol (mmol/l)	6.46 ± 0.32	6.98 ± 0.17
Body weight (g)	47.59 ± 2.08	46.72 ± 1.34
Adipose tissue mass (g)	2.30 ± 0.25	2.25 ± 0.28

Data are group means ± SE. Significantly different to the control diet, *$P < 0.05$, †$P < 0.01$.

c9,t11-CLA altered the expression of molecular markers of insulin resistance in white adipose tissue. Adipose tissue is a key organ involved in systemic insulin sensitivity and insulin desensitizing proinflammatory molecules (7,8). Plasma membrane GLUT4 expression was significantly increased (17%) after the c9,t11-CLA diet ($P < 0.05$) in white adipose tissue (Fig. 1*A*). An equivalent increase in GLUT4 expression was demonstrated in TZD-treated *ob/ob* mice compared with the control diet group (15%; $P < 0.05$). The c9,t11-CLA diet also increased adipose tissue insulin receptor expression by 23% ($P < 0.05$), compared with the control group. Similarly, this increase was comparable to the TZD-treated group (29.5%; $P < 0.05$) (Fig. 1*B*). The c9,t11-CLA–enriched diet had no significant effect on the expression of phospho IRS-1 (Ser^{307}) in adipose tissue (data not shown).

The c9,t11-CLA diet increased GLUT4 and IRS-1 mRNA expression, but these changes were not statistically significant (Fig. 1*D*). The most remarkable effect of c9,t11-CLA on adipokine mRNA expression was evident for TNF-α, which was significantly reduced by almost 50% compared with the control linoleic acid–fed mice ($P < 0.05$) (Fig. 1*D*). Interestingly, the level of the macrophage marker CD68mRNA was also significantly reduced by 48% in adipose tissue of the c9,t11-CLA–fed mice compared with the control diet ($P < 0.05$). The effect of c9,t11-CLA on a range of transcription factors involved in insulin sensitivity was also determined. It has recently been shown that LXRα activation induces GLUT4 transcription in adipose tissue (31–33), while also inhibiting the inflammatory response in macrophages (34). Interestingly the c9,t11-CLA diet significantly increased LXRα (0.85-fold; $P < 0.05$) and SREBP-1c mRNA expression in white adipose tissue (0.4-fold; $P = 0.05$). Adipose tissue SREBP-1a or peroxi-

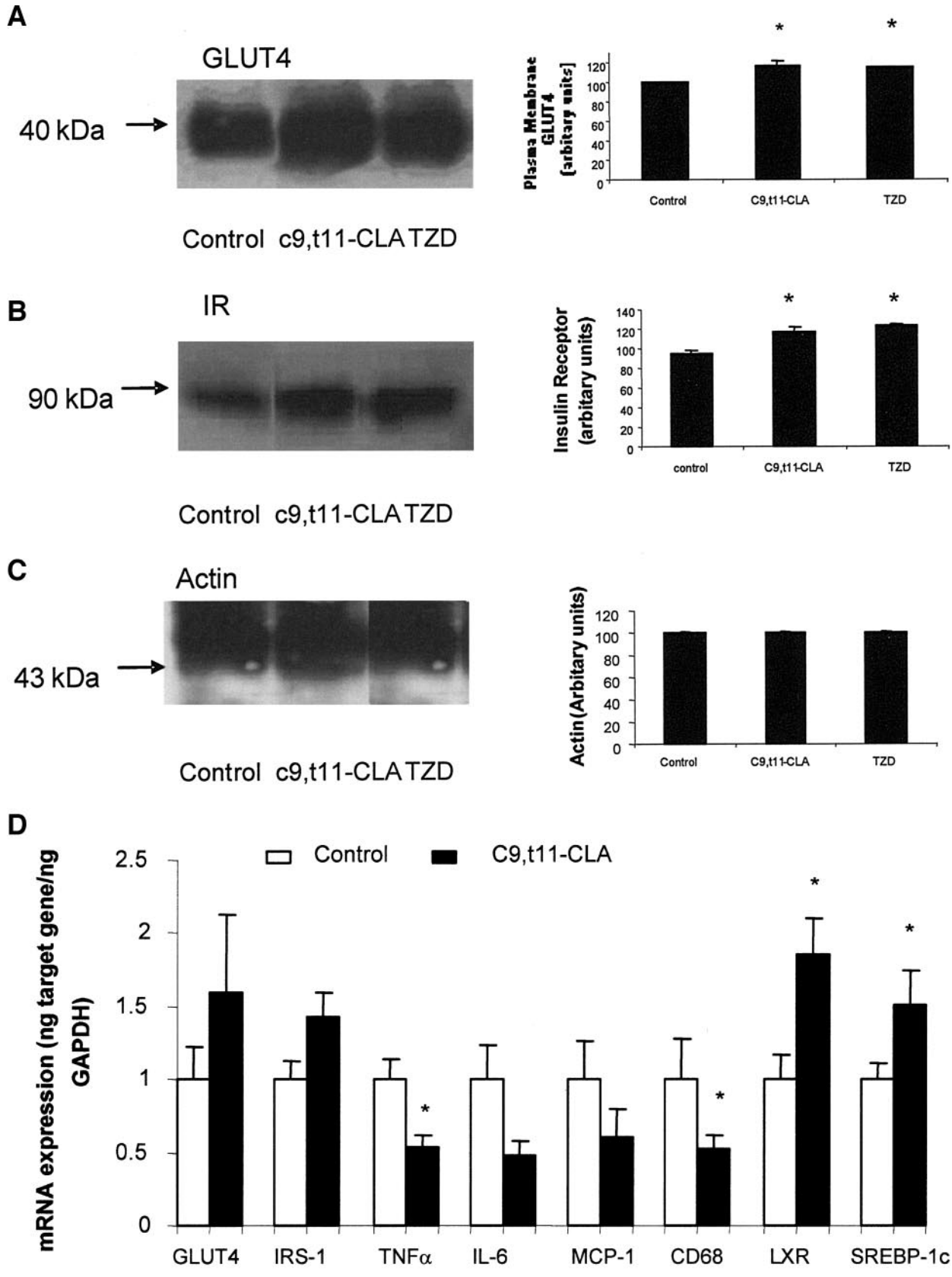

FIG. 1. Plasma membrane GLUT4 (*A*), insulin receptor (*B*), and actin (*C*) expression in adipose tissue extracts from control (linoleic acid), c9,t11-CLA (CLA) dietary treatments, and TZD-treated C57Bl-6 *ob/ob* mice fed specialized diets for 4 weeks ($n = 3$ per group). Representative blot is displayed. Histogram from densitometric analysis expressed as arbitrary units are means ± SE, normalized to the actin control. Significantly different from control (linoleic acid), *$P < 0.05$. *D*: The effect of feeding a control (linoleic acid) or c9,t11-CLA–enriched diet for 4 weeks ($n = 8$ per group) on mRNA expression of molecular markers of insulin sensitivity and adipocytokine expression in white adipose tissue of fasting male *ob/ob* mice. Target gene mRNA levels were normalized to GAPDH and are expressed relative to the control group. Values represent group means ± SE. Significantly different from the control diet, *$P = 0.05$.

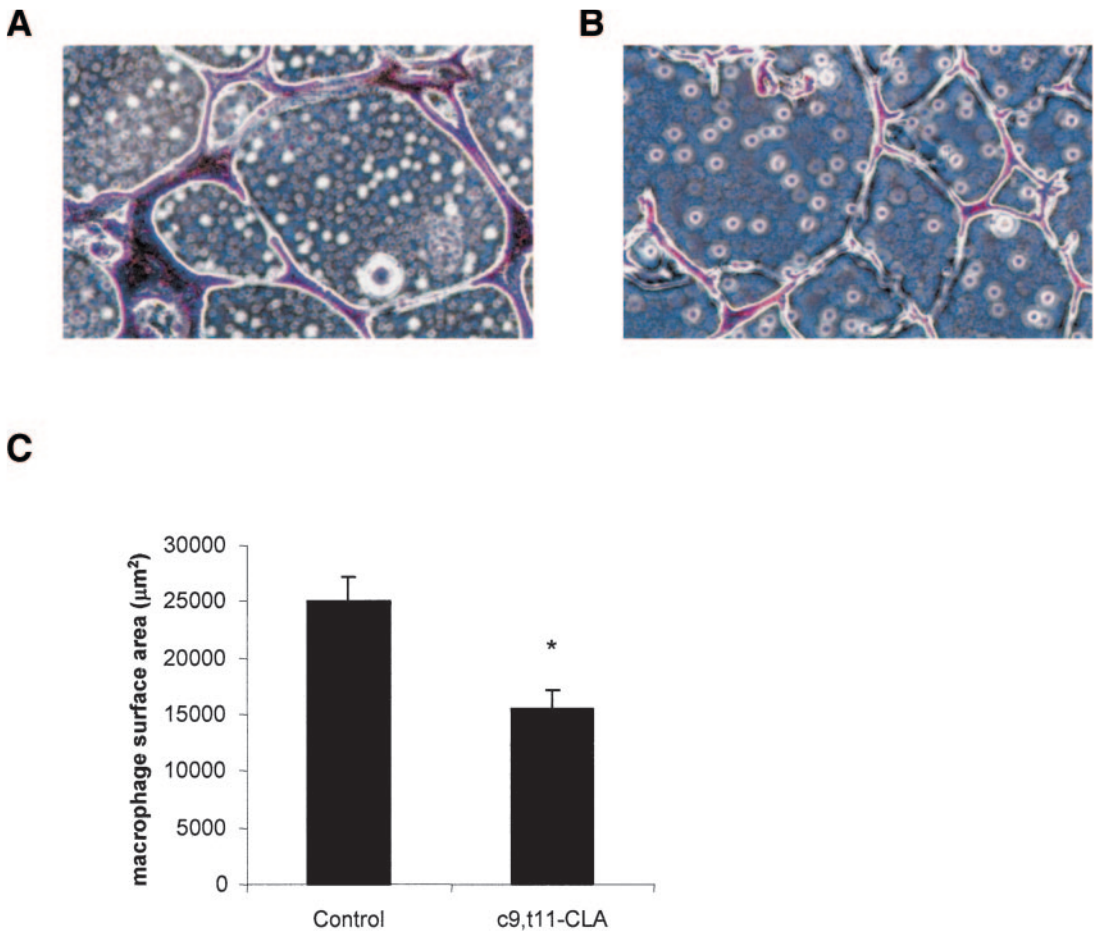

FIG. 2. Immunostaining of F4/80 in white adipose tissue sections from *ob/ob* mice fed control (linoleic acid) (n = 10) (*A*) and c9,t11-CLA–enriched diet (n = 10) (*B*). Staining is representative of three experiments at 40× magnification. *C*: F4/80 immunohistochemistry quantification representing macrophage surface area/total surface area in each dietary group (n = 10 per group). Data are means ± SE. *P < 0.05 vs. control diet.

some proliferator–activated receptor γ1 or γ2 mRNA were not affected by the dietary intervention (data not shown).

Improved insulin sensitivity is associated with less macrophages and an anti-inflammatory profile in adipose tissue. Immunohistochemical analysis of F4/80 clearly shows less macrophage infiltration in adipose tissue of c9,t11-CLA–fed mice (Fig. 2). The area occupied by macrophages (macrophage surface area/total surface area) was almost 40% less in the c9,t11-CLA–fed mice compared with the control group (15,566 ± 3,542 vs. 25,106 ± 1,397 μm^2, respectively). Macrophage infiltration and sub-acute inflammation in adipose tissue has been associated with attenuated insulin sensitivity in several animal models of obesity-induced insulin resistance and is characteristic of the metabolic syndrome in humans. Therefore, we determined the effect of the c9,t11-CLA diet on components of the NF-κB expression complex. The c9,t11-CLA diet reduced NF-κB p65 expression in adipose tissue ($P < 0.01$) (Fig. 3*A*). Cytosolic NF-κB p65 expression was also significantly decreased ($P < 0.05$) (Fig. 3*B*). IκBα expression was decreased in both nuclear and cytosolic fractions from white adipose tissue ($P < 0.001$) (Fig. 3*C* and *D*, respectively). To address the consequences of these alterations in NF-κB p65 and IκBα expression, NF-κB DNA binding was evaluated by electrophoretic mobility shift assay. Consistent with the anti-inflammatory hypothesis, the c9,t11-CLA diet markedly reduced NF-κB DNA binding ($P < 0.01$) in white adipose tissue (Fig. 3*G*). To further characterize the effect of c9,t11-CLA on NF-κB:DNA binding, we measured transcriptional activity of the individual NF-κB p65, p50, p52, c-Rel, and RelB subunits. This approach demonstrated comprehensive downregulation of NF-κB:DNA binding of the p65 ($P < 0.01$), p50 ($P < 0.001$), p52 ($P < 0.001$), c-Rel ($P < 0.01$), and Rel-B ($P < 0.02$) subunits of the transcriptional complex in the nuclear fraction of adipose tissue from the c9,t11-CLA–fed *ob/ob* mice compared with the control linoleic acid–fed mice (Fig. 4). Downregulation of all components of the NF-κB:DNA binding complex after the c9,t11-CLA dietary treatment probably reflects less macrophages in adipose tissue as shown in Fig. 2.

The effect of c9,t11-CLA on markers of insulin sensitivity and inflammation in 3T3-L1 adipocytes. It was important to determine whether the c9,t11-CLA isomer had a direct effect on molecular markers of insulin sensitivity in adipose tissue and if the fatty acid could rescue an inflammatory-induced insulin-resistant state. Therefore, we investigated the effect of c9,t11-CLA treatment on classic markers of insulin sensitivity in the well-characterized murine 3T3-L1 adipocyte cell line. Figure 5*A* demonstrates that culturing 3T3-L1 adipocytes in media supplemented with 50 μmol/l c9,t11-CLA significantly increased GLUT4 mRNA expression compared with vehicle control and control fatty acid (linoleic acid)-treated cells ($P < 0.001$).

The insulin desensitizing effect of TNF-α treatment in

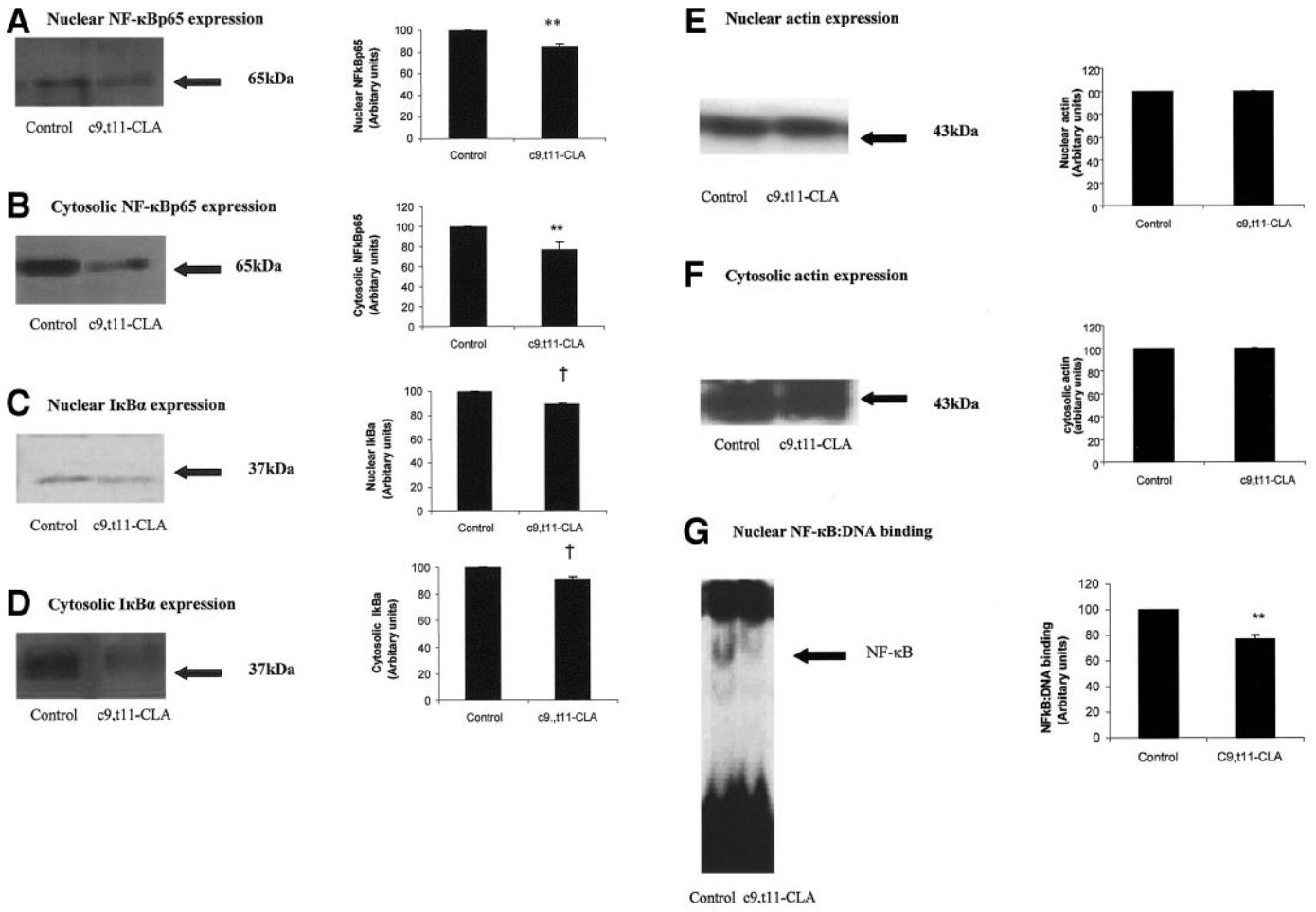

FIG. 3. Nuclear and cytosolic NF-κB p65 and IκBα expression and NF-κB:DNA binding in white adipose tissue after a control (linoleic acid) or c9,t11-CLA–enriched diet (n = 3 per group). NF-κB p65 expression (*A*), cytosolic NF-κB p65 expression (*B*), nuclear IκBα expression (*C*), cytosolic IκBα expression (*D*), nuclear actin expression (*E*), cytosolic actin expression (*F*), and NF-κB:DNA binding (*G*). Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are group means ± SE. Western blots are normalized to the actin control. Significantly different from the control diet, **$P < 0.01$, †$P < 0.001$.

3T3-L1 adipocytes is well characterized. Therefore, we also determined whether c9,t11-CLA could prevent the insulin desensitizing effect of TNF-α treatment in vitro. TNF-α treatment (3 ng/ml for 3 days) significantly reduced 3T3-L1 adipocyte GLUT4 and IRS-1 mRNA expression, by 90 and 58%, respectively ($P < 0.001$) (Fig. 5*B*). Interestingly, the presence of c9,t11-CLA during TNF-α treatment increased both GLUT4 mRNA and IRS-1 mRNA expression ($P < 0.05$) (Fig. 5*B*). Also, TNF-α mRNA levels were substantially lower (70%) in 3T3-L1 cells treated with c9,t11-CLA, particularly in the TNF-α–treated insulin-resistant adipocytes ($P < 0.05$) (Fig. 5*A* and *B*). Also, TNF-α levels were significantly reduced in the cell culture media of c9,t11-CLA–treated normal adipocytes and TNF-α–treated insulin-resistant 3T3-L1 adipocytes compared with control cells ($P < 0.05$) (Fig. 5*C*). We measured glucose uptake in 3T3-L1 cells cultured in the presence of c9,t11-CLA, then exposed to TNF-α (data not shown). TNF-α treatment significantly reduced basal and insulin-stimulated glucose uptake ($P < 0.001$). Interestingly, c9,t11-CLA treatment increased insulin-stimulate glucose transport in normal and TNF-α–induced insulin-resistant 3T3-L1 adipocytes ($P < 0.05$ and $P < 0.01$); however, this effect was only evident at the lower insulin concentration (0.1 nmol/l). Thus, c9,t11-CLA displayed the potential to attenuate TNF-α–induced insulin-resistant profile in 3T3-L1 adipocytes.

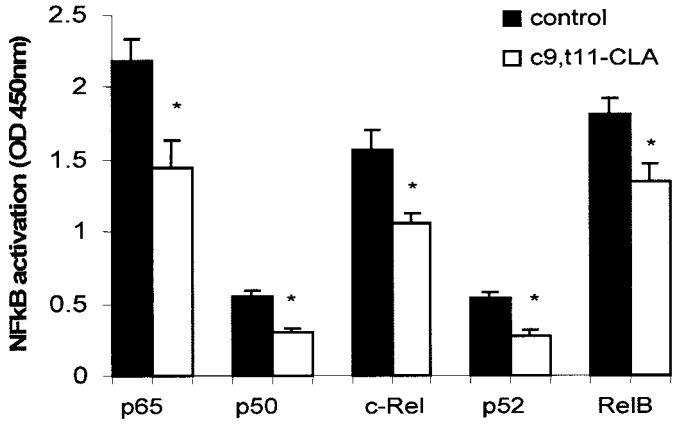

FIG. 4. Effect of feeding a control (linoleic acid) or c9,t11-CLA–enriched diet for 4 weeks on transcriptional activity of p65, p50, c-Rel, p52, and RelB in white adipose tissue of fasting male *ob/ob* mice (n = 8 per group). Values represent group means ± SE. OD, odds density. *$P < 0.05$ vs. linoleic acid diet.

DISCUSSION

Obesity-induced insulin resistance is the principal etiological factor of the metabolic syndrome and type 2 diabetes.

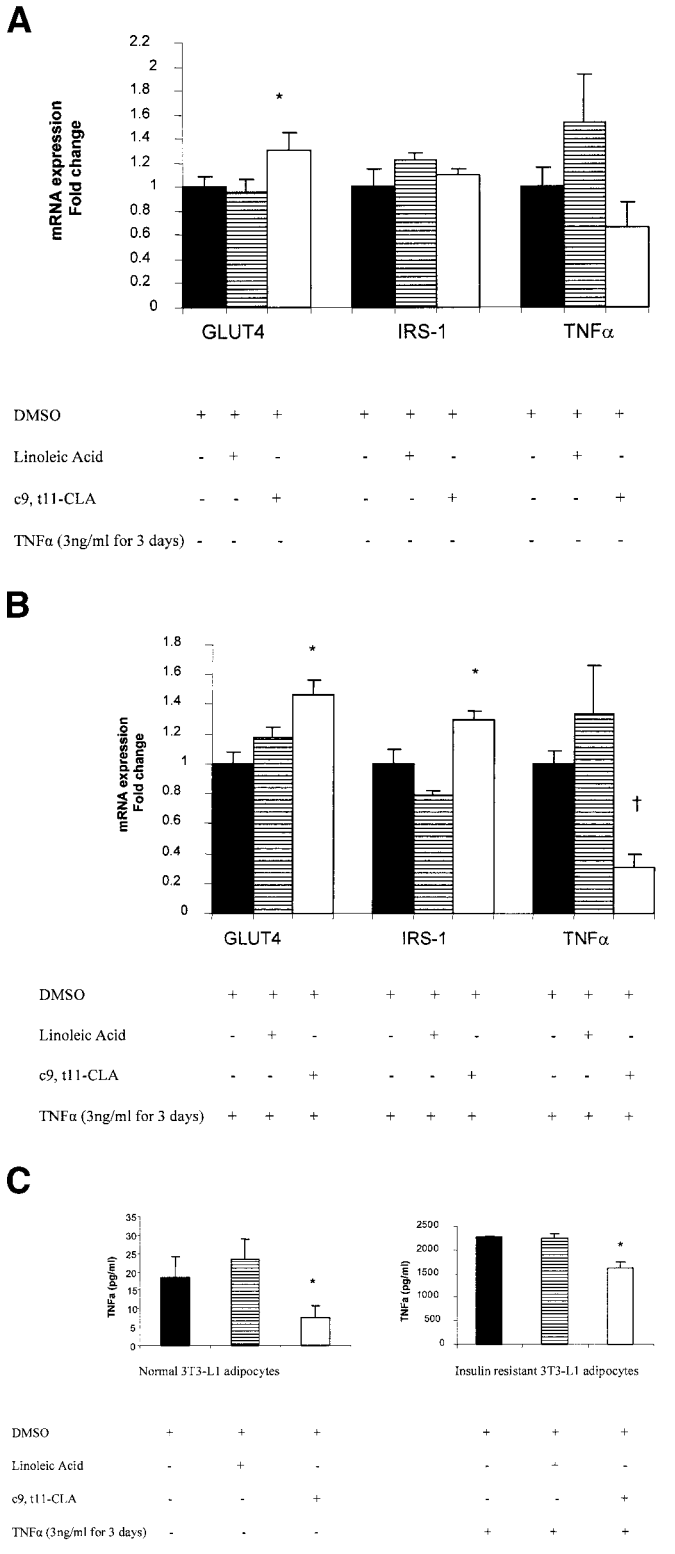

FIG. 5. Effect of linoleic acid and c9,t11-CLA on GLUT4, IRS-1, and TNF-α mRNA expression in 3T3-L1 adipocytes (*A*) and TNF-α–induced insulin-resistant 3T3-L1 adipocytes (*B*). *C*: TNF-α concentration (pg/ml) in the culture media of TNF-α–induced insulin-resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalized to GAPDH and expressed relative to DMSO. Values represent group means ± SE of six independent experiments ($n = 6$ per group). In normal adipocytes, GLUT4 significantly increased (*$P < 0.001$) by c9,t11-CLA compared with DMSO and linoleic acid. In TNF-α–treated insulin-resistant adipocytes, c9,t11-CLA significantly increased GLUT4 and IRS-1 mRNA (*$P < 0.05$) and decreased TNF-α mRNA expression (†$P < 0.05$) and TNF-α levels in the cell culture media compared with DMSO and linoleic acid–treated cells (*B*).

Given the increasing prevalence of obesity, it would be advantageous to identify potential therapeutic nutrients/functional foods to improve insulin sensitivity within the context of obesity. Interestingly, this article suggests that a dietary fatty acid, c9,t11-CLA, mediates its insulin-sensitizing effects by reducing macrophage infiltration and attenuating the inflammatory profile of obese adipose tissue. Several lines of evidence suggest that low-grade inflammation in adipose tissue predisposes to the development and augments the severity of whole-body insulin resistance. Knocking out various components of the inflammatory response (TNF-α, IKK-β, or Jnk1) or pharmacological anti-inflammatory treatment protects against obesity-induced insulin resistance (14–16,35). Recent research shows that obesity is associated with progressive infiltration of macrophages into adipose tissue, which may be the principal source of proinflammatory insulin desensitizing cytokines in obesity-related insulin resistance (7,8). Also C-C motif chemokine receptor-2 (CCR2) deficiency, an important mediator of macrophage-dependent inflammatory response, attenuated the development of a proinflammatory expression profile in adipose tissue of obese animals, reduced adipose tissue macrophage number, and improved insulin sensitivity (36).

This study demonstrated that intervention with a c9,t11-CLA–enriched diet significantly reduced plasma insulin and glucose concentrations, decreased the HOMA-IR index of insulin resistance, and improved the revised QUICKI indicator of insulin sensitivity in the well-characterized *ob/ob* mouse model that displays an obese insulin-resistant phenotype. The improved metabolic profile may be partly ascribed to enhanced GLUT4 expression in adipose tissue after the c9,t11-CLA diet. GLUT4 is selectively reduced in adipocytes of insulin-resistant obese and type 2 diabetic subjects (37). The central role of adipose tissue GLUT4 in glucose homeostasis has been demonstrated by tissue-specific knockout studies. Adipocyte-specific GLUT4$^{-/-}$ mice show a 50% reduction in whole-body glucose uptake and systemic hyperinsulinemia (38,39). Also, adipose-specific GLUT4 overexpression reverses insulin resistance in mice lacking GLUT4 in muscle (40).

Consistent with the anti-inflammatory hypothesis, our group has shown that c9,t11-CLA attenuates the proinflammatory phenotype of LPS-stimulated THP-1 macrophages and primary murine dendritic cells (41,22). The present study demonstrated that adipose tissue macrophage infiltration was lower, and TNF-α and CD68 mRNA expression was halved after the c9,t11-CLA diet, coupled with important immunoregulatory changes on several components of the NF-κB expression complex. The c9,t11-CLA diet markedly downregulated NF-κB p65 expression. IκBα expression was also attenuated, which probably reflected the absence of several components of the macrophage inflammatory system. The net effect of these alterations was demonstrated by electrophoretic mobility shift assay, which indicated significantly reduced NF-κB DNA binding in adipose tissue, with coordinated reduction in the transcriptional activity of all components (p65, p50, p52, c-Rel, and RelB subunits) of the NF-kB complex. Other inflammatory cytokines (interleukin-6 and MCP-1 mRNA) were reduced in adipose tissue of the c9,t11-CLA–fed group, albeit to a lesser extent. Consistent with our hypothesis that dietary fatty acids can modulate the inflammatory profile of adipocytes, two recent studies determined the effects of other fatty acids in vitro. The first showed that

the saturated fatty acid palmitate activated NF-κB activity and induced TNF-α and interleukin-6 expression in 3T3-L1 adipocytes (42). The second extensive investigation demonstrated that a mixture of saturated and unsaturated free fatty acid treatments impaired insulin signaling at multiple sites, decreased insulin-stimulated GLUT4 translocation and glucose transport, and activated the stress/inflammatory c-Jun NH_2-terminal kinase pathway in 3T3-L1 adipocytes (43). Furthermore, Poirier et al. (19) demonstrated that feeding t10,c12-CLA promoted macrophage infiltration into adipose tissue, inducing inflammation in adipose tissue that was coupled with an insulin-resistant state. Also, it has been shown that the t10,c12-CLA isomer promotes insulin resistance in human adipocytes, at least in part through NF-κB activation and subsequent induction of interleukin-6 (44), which is consistent with the observed effect of t10,c12-CLA on insulin and glucose metabolism in vivo (17,19).

Despite the attenuation in inflammatory stressors, feeding a c9,t11-CLA–enriched diet did not reduce the phosphorylation of IRS-1 at Ser^{307}, contrary to our hypothesis. TNF-α increases phosphorylation at this site, and the salicylates are known to exert an insulin-sensitizing effect, at least in part through a reduction in serine phosphorylation at this site through reduced IκB kinase-β expression/activation (45). In view of the attenuation in TNF-α mRNA expression, a reduction in IRS-1 Ser^{307} phosphorylation was expected. Nevertheless, it would be worthwhile to investigate the impact of c9,t11-CLA on IRS-1 Ser^{307} phosphorylation in the fed state, when insulin stimulates glucose uptake, rather than in the background quiescent state that was investigated in this study.

In this study, it was important to discern whether the apparent anti-inflammatory effect of c9,t11-CLA in adipose tissue was a direct effect of the nutrient or reflected another metabolic change. Therefore, we investigated the interaction between c9,t11-CLA and TNF-α in 3T3-L1 adipocytes. TNF-α impeded insulin-stimulated glucose uptake, which corroborates previous research that showed that TNF-α downregulates many adipocyte-abundant genes that determine insulin responsiveness (9,12,46,47). Consistent with our anti-inflammatory insulin-sensitizing hypothesis, pretreating 3T3-L1 adipocytes with c9,t11-CLA lessened the impact of TNF-α–induced reduction in GLUT4 and IRS-1 expression. Also, c9,t11-CLA promoted insulin-stimulated glucose transport, and this effect was most evident in the insulin-resistant TNF-α–treated adipocytes. This evidence suggests that it may be possible to reduce the impact of obesity-induced insulin resistance with nutrient-based anti-inflammatory strategies. Further work is required to characterize the interactions between macrophages and adipocytes in vivo, to understand which part of the adipocyte-macrophage interaction is most responsive to nutritional interventions. In the present study, this approach was limited by technical difficulties associated with the standard isolation of primary adipocytes that triggers proinflammatory gene expression (47). In terms of defining the implications of CLA to human nutrition, our group and others have shown that the presence of t10,c12-CLA in generic CLA supplements induces a proinflammatory insulin-resistant state (19,20,48). Therefore, the impact of feeding pure forms of c9,t11-CLA to obese insulin-resistant subjects needs to be addressed to determine if anti-inflammatory dietary fatty acid manipulations could modulate obese adipose tissue biology in humans.

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