# Isomer-Specific Antidiabetic Properties of Conjugated Linoleic Acid

## Improved Glucose Tolerance, Skeletal Muscle Insulin Action, and UCP-2 Gene Expression

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**Conjugated linoleic acid (CLA) isomers have a number of beneficial health effects, as shown in biomedical studies with animal models. Previously, we reported that a mixture of CLA isomers improved glucose tolerance in ZDF rats and activated peroxisome proliferator–activated receptor (PPAR)-γ response elements in vitro. Here, our aim was to elucidate the effect(s) of specific CLA isomers on whole-body glucose tolerance, insulin action in skeletal muscle, and expression of genes important in glucose and lipid metabolism. ZDF rats were fed either a control diet (CON), one of two CLA supplemented diets (1.5% CLA) containing differing isoforms of CLA (47% c9,t11; 47.9% c10,t12, 50:50; or 91% c9,t11, c9,t11 isomers), or were pair-fed CON diet to match the intake of 50:50. The 50:50 diet reduced adiposity and improved glucose tolerance compared with all other ZDF treatments. Insulin-stimulated glucose transport and glycogen synthase activity in skeletal muscle were improved with 50:50 compared with all other treatments. Neither phosphatidlyinositol 3-kinase activity nor Akt activity in muscle was affected by treatment. Uncoupling protein 2 in muscle and adipose tissue was upregulated by c9,t11 and 50:50 compared with ZDF controls. PPAR-γ mRNA was downregulated in liver of c9,t11 and pair-fed ZDF rats. Thus, the improved glucose tolerance in 50:50 rats is attributable to, at least in part, improved insulin action in muscle, and CLA effects cannot be explained simply by reduced food intake.** ***Diabetes*** **50:1149–1157, 2001**

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BAT, brown adipose tissue; CLA, conjugated linoleic acid; CON, control diet; ECL, enhanced chemiluminescence; EDL, extensor digitorum longus; G-6-P, glucose-6-phosphate; KHB, Krebs-Henseleit buffer; NEFA, nonesterified fatty acid; PI, phosphatidylinositol; PPAR, peroxisome proliferator–activated receptor; RT-PCR, reverse transcriptase–polymerase chain reaction; SSC, sodium chloride–sodium citrate; TBST, Tris-buffered saline plus Tween; TG, triglyceride; TZD, thiazolidinedione; UCP, uncoupling protein.

Conjugated linoleic acids (CLAs), geometric and positional isomers of linoleic acid, are potent cancer preventative agents in animal models of chemical-induced carcinogenesis (1). CLA isomers occur naturally in foods, are highest in ruminant meats and milks, and may be manipulated by animal husbandry (2,3). Recently, we reported that a mixture of dietary CLA isomers normalized impaired glucose tolerance and prevented/delayed the development of hyperglycemia in obese ZDF rats (4). ZDF rats spontaneously develop diabetes at 7–12 weeks due to β-cell decompensation; the development of hyperglycemia is secondary to obesity, because food restriction can prevent hyperglycemia in this model (5). We and others (4,6,7) have observed modest to significant reductions in food intake and/or adiposity with dietary CLA supplementation. Furthermore, data are emerging that different isomers of CLA are responsible for the antiobesity and anticancer effects (6,8,9).

In addition to antiobesity therapies, pharmaceutical agents that improve insulin-stimulated glucose uptake and utilization by skeletal muscle, such as the thiazolidinediones (TZDs), are able to improve whole-body glucose homeostasis and prevent/improve the hyperglycemic state in ZDF rats (10–12). It is possible that dietary CLA may improve glucose homeostasis by multiple mechanisms in ZDF rats. Thus, the aims of this work were to determine *1*) which CLA isomer(s) is responsible for the antidiabetic effects, *2*) if the CLA-induced antidiabetic effects are due to a reduction in food intake, *3*) whether CLA exerts an antidiabetic effect by improving insulin action in skeletal muscle, and *4*) whether CLA regulates expression of genes important in glucose and lipid homeostasis.

At least a portion of the CLA antidiabetic effects appears to be via activation of peroxisome proliferator–activated receptor (PPAR)-γ (4). We reported induction of aP2 gene expression in adipose tissue of CLA-treated ZDF rats and activation PPAR-γ response elements by CLA in vitro (4). It is not surprising that CLA can activate PPARs; the crystal structure of PPAR ligand binding domains reveals a large ligand binding pocket that explains the promiscuity of this receptor for multiple ligands, including fatty acids (13). PPAR-γ ligands, such as TZD, are potent insulin-sensitizing drugs that impact whole-body glucose utiliza-

tion, glucose uptake into insulin responsive tissues, whole-body lipid metabolism, and pancreatic β-cell function (rev. in 14,15). At least a portion of TZD effects is due to PPAR-γ induction of gene expression for genes important in glucose and lipid metabolism, such as the uncoupling proteins (UCPs) and PEPCK. Others (16) have shown that CLA isomers may also activate PPAR-α, an important regulator of lipid metabolism, in rodent liver. Thus, the activation of various PPARs by CLA may explain, at least in part, the complex regulation of glucose lipid metabolism observed with CLA treatment.

Skeletal muscle is the primary site of insulin-stimulated glucose uptake and, thus, is a possible target for CLA action. In most models of insulin resistance or diabetes, skeletal muscle glucose transport is not limited by glucose transporter expression (GLUT4) but is attributable to defects in GLUT4 translocation (rev. in 17,18). Thus, an important aim of this work was to determine whether various CLA isomers are able to improve insulin-stimulated glucose transport into slow- and fast-twitch skeletal muscle, and to determine whether alterations in glucose transport in skeletal muscle are due to CLA-induced changes in insulin signal transduction.

We provide evidence that certain CLA isomers are able to improve glucose tolerance, insulin-stimulated glucose transport into skeletal muscle, and insulin-stimulated glycogen synthase activity. In addition, dietary supplementation with CLA resulted in an upregulation in UCP2 gene expression in skeletal muscle and adipose tissue, suggesting a role in the regulation of lipid metabolism and perhaps thermogenesis in these tissues. Finally, we show that the antidiabetic effects of CLA could not be explained entirely by treatment-induced changes in food intake.

## RESEARCH DESIGN AND METHODS

**Animals and experimental design.** The institutional animal care and use committees at Purdue University or Karolinska Institute approved all studies. Male Zucker Diabetic Fatty rats (ZDF/GMI; *fa/fa*) and lean littermate controls were obtained at 6 weeks of age (Genetic Models, Indianapolis, IN). After a 7-day acclimation period, animals were screened for normoglycemia and subsequently assigned to dietary treatments ($n = 8$ lean controls, $n = 16$ fatty rats/treatment group). Initial body weights were higher ($P < 0.0001$) for ZDF fatty rats (mean for all treatments 166.6 ± 1.21 g) compared with lean controls (141.8 ± 3.1 g), but were not different among ZDF rats on the various treatments. Animals were maintained on treatment for 14 days. Fresh food was provided and food intake and body weights were monitored on alternate days throughout the treatment period. Water was provided ad libitum. On day 11, intraperitoneal glucose tolerance tests were administered to a subset of animals from each treatment. On day 15, animals were killed and blood, organs, and tissues were collected for further analyses (see below).

**Dietary treatments.** All diets were based on a complete powdered diet (Lactamin R36; Lactamin B, Stockholm, Sweden) that contained 4% total fat and 1% linoleic acid. The base diet was supplemented with either control fat (low CLA butter; 0.2% final CLA concentration; control treatment) or CLA fatty acid supplements to a final total fat concentration of 40% (wt/wt) for all experimental diets. Thus, all diets were balanced for total fat intake and are considered high-fat diets.

To test the differential effects of various CLA isomers, the high-CLA diets were formulated using two different CLA supplements (the final concentration of CLA in both high-CLA diets was 1.5%). The first supplement used was CLA-enriched butter (c9,t11) produced via dietary manipulation of dairy cows to alter CLA content of milk (2). The CLA-enriched butter contained predominantly c9,t11 isomer (90.5% c9,t11; 0.8% t10,c12; 4.6% c7 t9; 1.0% other c,t; 1.2% other c,c; 1.9% other t,t isomers) and was recently shown to be anticarcinogenic in a rat mammary tumor model (8). The second supplement used was the CLA-oil mixture that, as we previously reported, normalized glucose tolerance in ZDF rats (4). This CLA supplement (50:50) provided 47.0% c9,t11 and 47.9% t10,c12 isomers. The balance of CLA isomers in the 50:50 supplement include: 2.6% c,c9,11 + 10,12; 1.6% t,t9,11 + 10,12; and 0.8% other isomers. Because we previously observed a trend for reduced food intake when ZDF rats were fed the 50:50 diet (4), we included a pair-fed control group in which ZDF rats were fed the control diet at the same level of intake as the animals on the 50:50 diet.

**Glucose tolerance tests.** A glucose tolerance test was performed on day 11 of treatment. A subset of animals from each treatment ($n = 4$ lean controls; $n = 8$ ZDF rats/treatment) were fasted overnight (15 h) and D-glucose (1 g/kg body wt) was injected intraperitoneally into conscious animals. Blood samples were obtained from the tail vein for glucose analysis at times 0, 2, 5, 10, 15, 20, 40, 60, 120, and 180 min relative to glucose injection.

**Hormone and metabolite assays.** Glucose concentrations in whole blood were determined immediately upon collection using a One Touch glucose meter (Lifescan, Milpitas, CA). Plasma insulin and leptin concentrations were analyzed using species-specific radioimmunoassay kits (Linco Research, St. Charles, MO). Plasma triglycerides (TGs) and nonesterified fatty acids (NEFAs) were quantified using colorimetric kits (TG: Sigma, St. Louis, MO; NEFA: Wako Chemicals, Richmond, VA). Inter- and intra-assay coefficients of variation averaged <9% for each method.

**Muscle incubations.** Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt.). Isolated soleus and extensor digitorum longus (EDL) muscles were used for in vitro incubation. Each soleus and EDL muscle was split into two equal longitudinal portions before incubation. The procedure and suitability of the muscle preparation has been previously described (19,20). All incubation media were prepared from a stock solution of pregassed (95% $O_2$/5% $CO_2$) Krebs-Henseleit buffer (KHB), which contained 5 mmol/l HEPES and 0.1% bovine serum albumin (RIA Grade). The gas phase in the vials was maintained at 95% $O_2$/5% $CO_2$ throughout all incubations. Muscles were incubated (30°C) for 15 min in KHB supplemented with 2 mmol/l pyruvate and 38 mmol/l mannitol before addition of insulin. Thereafter, muscles were preincubated in the absence or presence of insulin (0.6, 2.4, and 120 nmol/l) for 6 min (signaling) or 20 min (glucose transport and glycogen synthase) as indicated in the figures.

**Glucose transport activity.** Glucose transport in EDL and soleus muscle was assessed using the glucose analogue 3-*O*-methylglucose as described by Wallberg-Henriksson et al. (20). Muscles were incubated at 30°C, in the absence or presence of insulin (0.6, 2.4, 120 nmol/l) for 10 min, in KHB containing 5 mmol/l 3-*O*-methyl[$^3$H]glucose (2.5 μCi/mmol/l), and 35 mmol/l [$^{14}$C]mannitol (26.3 μCi/mmol/l). Glucose transport activity is expressed as micromole glucose analog accumulated per milliliter of intracellular water per hour. A portion of the muscle was stored in liquid nitrogen for subsequent analysis of glycogen synthase activity and Akt phosphorylation. Thus, muscles for glucose transport, glycogen synthase activity, and Akt phosphorylation were exposed to insulin for a total of 30 min.

**Glycogen content.** Gastrocnemius muscle and liver were removed from anesthetized rats as described above and immediately frozen in liquid nitrogen. Glycogen content was measured fluorometrically on HCl extracts of muscle or liver as described by Lowry and Passonneau (21). Results are expressed as micromole glucose × gram wet weight$^{-1}$.

**Glycogen synthase activity.** A portion of the incubated soleus or EDL muscle was homogenized on ice, in buffer consisting of 20 mmol/l Tris-HCl, pH 8.0, 137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l $MgCl_2$, 0.5 mmol/l $Na_3VO_4$, 1% Nonidet P-40, 10% (wt/vol) glycerol, 10 μg/ml leupeptin, and 200 mmol/l phenylmethylsulfonyl fluoride. Glycogen synthase activity was measured on supernatants from homogenized muscle samples using a modification of the method by Thomas et al. (22). The concentration of UDP-glucose in the assay was 0.17 mmol/l. The dephosphorylated active form of the enzyme was determined in the presence of low concentration of glucose-6-phosphate (G-6-P) (0.3 mmol/l) and the total activity at a saturating level of G-6-P (6.0 mmol/l). The activity of the enzyme is expressed as percent of the active form in relation to the total.

**Tyrosine-associated phophatidylinositol 3-kinase activity.** A portion of soleus or EDL muscle was homogenized in 500 μl lysis buffer as described (23). Muscle proteins were solubilized by continuous stirring for 1 h at 4°C after centrifugation (12,000*g* for 4 min at 4°C). Protein was determined using a kit from Bio-Rad (Richmond, CA). The supernatant (500 μg) was immunoprecipitated overnight (4°C) with antiphosphotyrosine antibody coupled to protein A-Sepharose. Phosphatidylinositol (PI) 3-kinase activity was assessed directly on the protein A-Sepharose beads as described (24). The bands corresponding to PI-3-phosphate were quantified using a PhosphoImager (Bio-Rad).

**Akt phosphorylation.** Aliquots of soleus or EDL muscle lysate (20 μg) were mixed with Laemmli sample buffer, and proteins were separated by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Thereafter, membranes were blocked in Tris-buffered saline plus Tween (TBST) (10 mmol/l Tris, 100 mmol/l

NaCl, 0.02% Tween 20) containing 7.5% nonfat milk for 2 h at room temperature, washed with TBST for 10 min, and incubated overnight at 4°C with a phospho-Akt antibody (New England Biolabs, Beverly, MA) that recognizes Akt kinase when phosphorylated at Ser 473 (Akt kinase phosphorylation). Membranes were washed with TBST and incubated with appropriate secondary antibody for 1 h at room temperature. Phosphorylated proteins were visualized by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL) and quantified by densitometric scanning.

**Northern blot analysis.** Total RNA was extracted from snap frozen liver and gastrocnemius muscle with Tri-Reagent (Molecular Research Center, Cincinnati, OH). In the final step, RNA pellets were washed with 50% (vol/vol) isopropanol/salt solution (0.8 mol/l sodium citrate, 1.2 mol/l NaCl) to remove excess glycogen. Total RNA was extracted from frozen epididymal adipose tissue as described by Chomczynski and Sacchi (25). The yield and purity of RNA was determined by spectrophotometric absorption analysis at 260/280 nm. Total RNA (15–25 μg) was electrophoresed in 1.2% agarose containing 6% formaldehyde and transferred to Zeta-Probe GT Genomic Tested Blotting Membranes (liver analyses; Bio-Rad), as previously described (26), or to nitrocellulose (muscle and adipose tissue blots). RNA was crosslinked to the membrane by ultraviolet irradiation and baked dry at 80°C for 1 h. The membranes were incubated at 42°C in 5× hybridization buffer (5× Denharts solution, 50 mmol/l $KPO_4$ buffer, 750 mmol/l NaCl, 75 mmol/l $Na_3C_6H_5O_7$, 50 g/ml salmon sperm DNA, and 50% formamide). After a prehybridization period of 5 h, an excess of denatured radiolabeled cDNA probe (liver) or riboprobe (adipose tissue and muscle) was introduced to the buffer, and membranes were incubated at 42°C for an additional 16–20 h. The liver blots were washed three times with 2× sodium chloride–sodium citrate (SSC)/0.1% SDS, twice with 0.2× SSC/0.1% SDS, and exposed to a PhosphorImager Screen (Molecular Dynamics, Sunnyvale, CA) for 36 h. The images were scanned on a Phosphorimager Analyzer using ImageQuant Software (version 3.3). The adipose tissue and muscle blots were washed once with 1× SSC/0.5% SDS followed by a single wash with 0.1× SSC/0.1% SDS. Adipose tissue and muscle blots were analyzed by autoradiography using intensifying screens. Data were analyzed by scanning densitometry (Kodak Digital Science 1D Image Analysis Software; Eastman Kodak Company, Rochester, NY).

**Probe synthesis.** Radiolabeled cDNA probes were prepared by the random primed labeling method (Life Technologies-BRL, Grand Island, NY) using [α-$^{32}$P]dCTP (Amersham). Dr. Daniel Ricquier (INSERM, Meudon, France) kindly donated the murine UCP2 cDNA. A PEPCK cDNA subfragment (258 bp) was prepared from liver RNA by reverse transcriptase–polymerase chain reaction (RT-PCR) (Life Technologies, Gaithersburg, MD) using 5/3 primer pairs (AGG TGA GCC GCA AGG TAG ATC C/TGG TCT GGA CTT CTC TGC CAA G). The PPAR-$\gamma_2$ clone was kindly provided by Dr. Remy Burcelin (Institute of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland). An oligonucleotide (5′-CTTCCTCTAGATAGTCAAGTTCG-AC-CGTCT-3′) specific for 18S rRNA (GenBank accession no. X01117) was end-labeled using T4 polynucleotide kinase (Life Technologies-BRL) and [γ-$^{32}$P] dATP and used to correct variations in the amount of RNA loaded per lane.

For the UCP2 riboprobe synthesis, sense (CGCACTAGTGCCCCGAGCCT-TCTACAA) and antisense (GCGAATTCTCAGAARGGWGCC TCYCG) primers were used to generate a 153 cDNA subfragment from mouse gastrocnemius muscle by RT-PCR. The fragment was subsequently cloned into pBluescript and sequenced. A riboprobe was radioactively transcribed using a commercially available kit according to the manufacturer's protocol (Maxiscript kit; Ambion, Austin, TX).

**Western blotting.** PPAR-γ protein abundance in epididymal adipose tissue and gastrocnemius was determined by Western blotting using commercially available antisera as described previously (27). UCP1 protein in brown adipose tissue (BAT) was also quantified by Western blotting using a commercially available anti-peptide antibody corresponding to a 12–amino acid COOH-terminal peptide conserved in rat and mouse (Research Diagnostics, Flanders, NJ). Briefly, snap-frozen tissues were homogenized in HES buffer (1 mmol/l HEPES, 25 mmol/l sucrose, 1% EDTA, 10 μg/ml Leupeptin, 4 mmol/l PEFABLOC; Roche Molecular Biochemicals, Indianapolis, IN) and centrifuged (1,500*g*) to remove insolubles. Homogenate protein concentrations were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). Samples were electrophoresed in 12.5% SDS-PAGE gels, transferred to nitrocellulose, and blocked with 5% skim milk in phophate-buffered saline. Blots were incubated with either the UCP1 antibody (BAT only) or the PPAR-γ antiserum (Affinity Bioreagents, Golden, CO) that detects but does not distinguish between various PPAR-γ isoforms. Bands were visualized using ECL and quantified by scanning densitometry.

**Statistical analyses.** All data are presented as means ± SE. Data were analyzed by analysis of variance using StatView software (Abacus Concepts, Berkley, CA). Differences among means were determined (Student's *t* test) when main effects were found to be significant ($P < 0.05$).

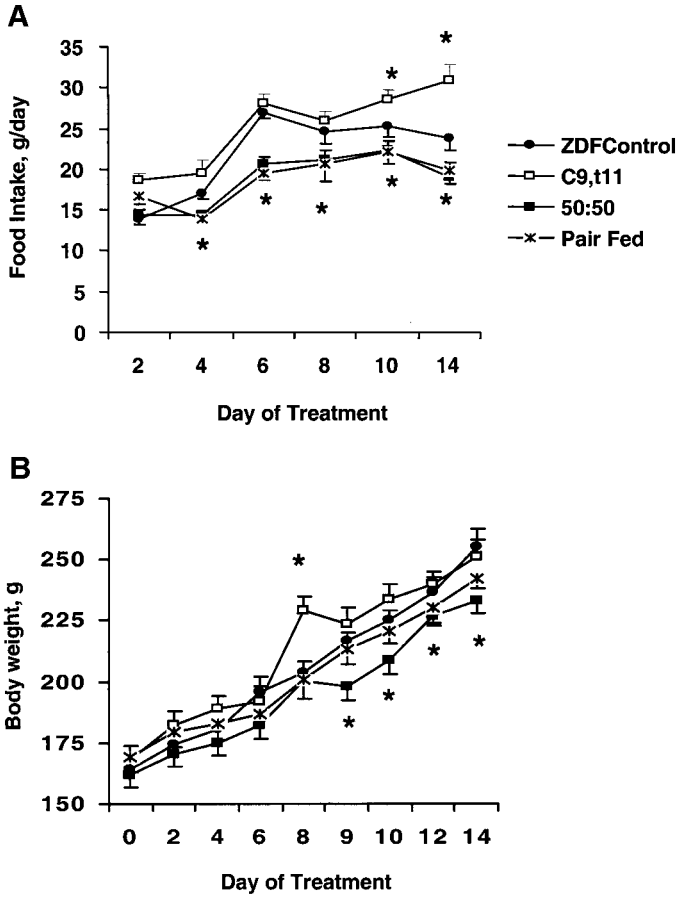


**FIG. 1. Effect of dietary treatments on food intake (*A*) and body weight gain in ZDF rats (*B*). Male normoglycemic ZDF rats ($n$ = 16 per treatment group, 7 weeks of age) were assigned to various dietary treatments as described in RESEARCH DESIGN AND METHODS. Body weight and food intake were quantified on alternate days throughout the study (14 days). *$P < 0.01$ vs. ZDF controls.**

## RESULTS

**Food intake and body weight.** Food intake and growth rate curves for ZDF fatty rats are illustrated in Fig. 1. Food intake (Fig. 1*A*) was similar among rats fed the control and c9,t11 diets until day 10, when food intake was significantly increased in c9,t11 rats and remained higher through the end of the study. Food intake was significantly lower in 50:50 vs. control and c9,t11 rats beginning on day 4 of treatment; the reduced food intake was maintained throughout the remainder of the study. By experimental design, food intake for pair-fed rats was identical to that of 50:50 animals.

Growth rates (Fig. 1*B*) were similar for control, c9,t11, and pair-fed rats throughout the study, with the exception of day 8, when growth rate was higher for c9,t11 ($P < 0.01$). However, beginning on day 9, growth rates were significantly slower ($P < 0.01$) for 50:50 compared with control and c9,t11 rats. Growth rates were slower for 50:50 vs. pair-fed rats on days 9 and 10 only ($P < 0.01$). Final body weights were highest for control and c9,t11 rats (239.3 ± 4.9 and 251.3 ± 5.7 g, respectively) and were significantly higher ($P < 0.001$) than that for 50:50 (231.5 ± 5.8 g) and pair-fed rats (234.0 ± 5.3 g).

The reduced food intake and slower growth rates for 50:50 animals were mirrored by smaller epididymal fat pads compared with ZDF control, c9,t11, and pair-fed animals (lean 6.7 ± 0.6; ZDF control 21.9 ± 0.8; c9,t11 20.5 ±

0.6; 50:50 18.1 ± 0.7; pair-fed 21.2 ± 1.3 mg/g body wt; $P <$ 0.01). Significant treatment effects on BAT mass were also observed. Specifically, scapular BAT depots (corrected for body weight) were significantly larger in ZDF rats compared with lean controls (lean 2.2 ± 0.3; ZDF control 2.9 ± 0.2; c9,t11 4.0 ± 0.4; 50:50 3.9 ± 0.3; pair-fed 4.1 ± 0.5 mg/g body wt). To determine whether the increase in BAT weight merely reflects an increase in white adipocyte content, content of the BAT-specific marker, UCP1, was quantified by Western blotting (data not shown). UCP1 content/mg BAT protein was significantly reduced in all ZDF rats compared with lean controls ($P < 0.001$), consistent with the notion of increased infiltration of white adipocytes within the depot. Among ZDF treatment groups, UCP1 content/mg protein was similar with the exception of pair-fed rats that had significantly lower UCP1 protein expression ($P < 0.05$), suggesting differential effects of fatty acid supplementation and caloric intake among fatty ZDF rats.

**Glucose homeostasis.** Postprandial blood glucose concentrations and whole-body glucose tolerance curves are illustrated in Fig. 2. By experimental design, all animals were determined normoglycemic before the onset of the study, as indicated by the initial blood glucose concentrations (Fig. 2*A*). However, by the end of the study, ZDF controls and c9,t11 animals were hyperglycemic ($P <$ 0.001) compared with lean controls. In contrast, 50:50 and pair-fed rats maintained normal glycemia throughout the course of the experiment. Results for 50:50 rats are consistent with our previous findings (4), and data from pair-fed rats suggest that protection from hyperglycemia may be due, at least in part, to reduced food intake in 50:50 compared with CON or c9,t11 animals.

Whole-body glucose tolerance curves are shown in Fig. 2*B*. Fasting blood glucose concentrations quantified just before glucose injection were greater in ZDF rats versus lean controls (3.04 ± 0.28 vs. 4.65 ± 0.51 mmol/l; $P < 0.01$) but were not different among fatty rats (data not shown). ZDF control rats were markedly glucose intolerant compared with lean controls (increased area under the curve; $P < 0.001$). Dietary supplementation with c9,t11 had no effect on glucose tolerance compared with ZDF controls ($P > 0.05$); however, 50:50 rats showed significantly improved glucose tolerance ($P < 0.01$), consistent with our previous report (4). Effects of the 50:50 diet could not be completely explained by reduced food intake, as pair-fed animals displayed an intermediate effect on glucose tolerance; tolerance was improved compared with ZDF controls ($P < 0.05$), but pair feeding was not as efficacious as the 50:50 diet (50:50 vs. pair feeding, $P < 0.05$).

**Hormones and metabolites.** Effects of dietary treatments on circulating concentrations of insulin, leptin, TGs, and NEFAs are illustrated in Table 1. ZDF rats, regardless of treatment, had significantly higher circulating concentrations of insulin, leptin, TGs, and NEFAs than lean controls ($P < 0.001$). The 50:50 CLA diet and pair-feeding, but not c9,t11, were equally efficacious in lowering plasma insulin concentrations compared with ZDF controls ($P < 0.05$).

Plasma leptin concentrations were markedly higher in ZDF rats compared with lean controls ($P > 0.001$), as would be expected given the genetic defect in ZDF leptin receptor, which results in leptin resistance. Only the 50:50 diet resulted in a significant reduction in plasma leptin

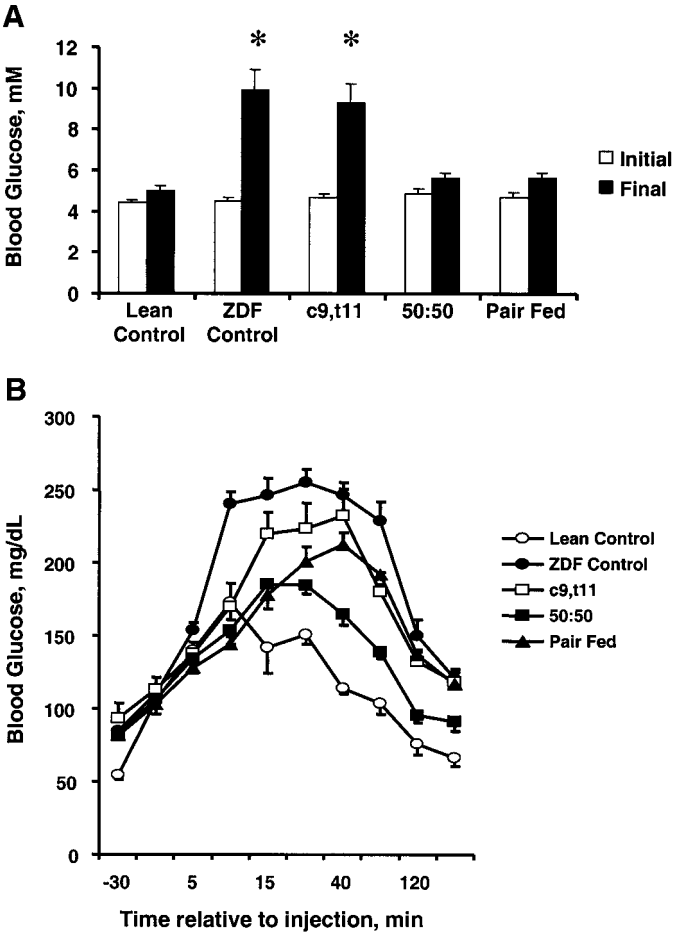


**FIG. 2. Effect of dietary treatments on blood glucose concentrations (*A*) and whole-body glucose tolerance (*B*) in ZDF rats. *A*: Blood glucose concentrations were determined in lean ($n = 8$) and ZDF ($n = 16$ per treatment) animals at 9:00 A.M. on day 0 (initial) and at the end of the treatment period (final), as described in RESEARCH DESIGN AND METHODS. *$P < 0.001$. *B*: On day 11, glucose tolerance was determined in a subset of animals ($n = 4$ lean controls and $n = 8$ ZDF per treatment group), as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for each time point. Area under the curve analysis indicates that glucose tolerance was improved in 50:50 vs. ZDF control rats ($P < 0.01$); pair-feeding had an intermediate effect ($P < 0.05$ vs. ZDF control and 50:50 rats).**

concentrations ($P < 0.03$ vs. ZDF controls, c9,t11, or pair-fed), presumably reflecting the treatment-induced reduction in adiposity.

Both CLA diets (c9,t11 and 50:50) significantly reduced ($P < 0.03$) plasma TG concentrations compared with ZDF controls (Table 1). However, neither CLA treatment normalized TG levels to those observed in lean controls ($P >$ 0.05). Pair-feeding was ineffective in lowering plasma TG concentrations. In contrast, the 50:50, but not the c9,t11 or pair feeding treatments, lowered plasma NEFA concentrations compared with ZDF controls ($P < 0.02$).

**Glucose transport into skeletal muscle.** Glucose transport into isolated EDL and soleus muscles in the absence (basal) and presence of insulin is depicted in Fig. 3. Basal glucose transport into isolated EDL and soleus muscles was unaltered by dietary treatment ($P > 0.05$). In lean control rats, insulin caused a 2.8-fold ($P < 0.01$) increase in glucose transport into EDL muscle (Fig. 3*A*). Insulin-stimulated glucose transport was reduced in ZDF versus lean control rats and was not improved by dietary treatment ($P > 0.05$).

TABLE 1
Effect of dietary treatment on plasma concentrations of hormones and metabolites

| | Treatment | | | | |
|---|---|---|---|---|---|
| | Lean control | ZDF control | c9,t11 | 50:50 | Pair-fed |
| Insulin (ng/ml) | 2.6 ± 0.28 | 12.2 ± 1.31 | 13.7 ± 1.83 | 9.4 ± 1.64* | 9.2 ± 1.43* |
| Leptin (ng/ml) | 1.3 ± 0.12 | 101.2 ± 15.80 | 90.1 ± 9.10 | 56.3 ± 7.10† | 78.9 ± 11.80 |
| Triglycerides (mg/dl) | 44.1 ± 5.11 | 250.5 ± 24.29 | 199.5 ± 18.75† | 175.9 ± 20.50† | 215.3 ± 20.10 |
| NEFA (mmol/l) | 0.096 ± 0.016 | 0.576 ± 0.042 | 0.547 ± 0.047 | 0.434 ± 0.048‡ | 0.626 ± 0.052 |

Data are means ± SE. *$P < 0.05$, †$P < 0.03$, and ‡$P < 0.02$ vs. ZDF control.

As in the case for EDL muscles, insulin stimulated a robust dose-dependent increase in 3-*O*-methylglucose transport into isolated soleus muscles of lean control rats ($P < 0.001$; Fig. 3*B*); this effect was significantly blunted in ZDF rats. The c9,t11 and control diets had no effect on insulin-stimulated glucose transport into soleus muscle ($P > 0.05$); however, the 50:50 diet caused a significant increase ($P < 0.01$) in glucose transport at the highest insulin dose, compared with ZDF control, c9,t11, or pair feeding.

**Glycogen content and synthesis.** Glycogen content in gastrocnemius muscle and liver (postprandial state) were not altered by dietary treatment ($P > 0.05$ vs. ZDF control; data not shown). Glycogen synthase activity in EDL and soleus muscles is illustrated in Fig. 4. There was no difference in basal glycogen synthase activity ratio among treatment groups ($P > 0.05$). In lean rats, insulin significantly increased glycogen synthase activity in both EDL (Fig. 4*A*) and soleus (Fig. 4*B*; $P < 0.05$) muscle. This insulin-stimulatory effect was undetectable in ZDF rats. The c9,t11 and pair-feeding treatments had no effect on glycogen synthase activity in response to insulin. However, the 50:50 diet was able to rescue this effect in both muscle fiber types ($P < 0.05$), thereby normalizing insulin-stimulated glycogen synthase activity ratios to those of lean controls.

**Insulin signaling via PI 3-kinase and Akt.** To examine whether the effects of the 50:50 diet to improve glucose tolerance, insulin-stimulated glucose transport into skeletal muscle, and insulin-stimulated glycogen synthase activity were due to treatment-induced changes in insulin signaling, we quantified PI 3-kinase and Akt activity in skeletal muscle in the absence and presence of insulin. Anti-phosphotyrosine-associated PI 3-kinase activity (Table 2) was significantly ($P < 0.01$) increased by insulin in EDL and soleus muscles from lean control rats but not ZDF rats. There was no effect of dietary treatment on insulin-stimulated PI-3 kinase in either EDL or soleus ($P > 0.05$).

Expression of total Akt protein and Akt phosphorylation in the absence and presence of insulin were quantified in EDL and soleus muscles (Table 2). Total Akt protein expression was unaltered by genotype (lean versus ZDF) or dietary treatment (data not shown). In EDL muscle, phosphorylated-Akt (phospho-Akt) expression in the basal state was significantly reduced in ZDF control and pair-fed rats compared with lean controls ($P < 0.01$). However, both CLA treatments prevented this downregulation. In the insulin-stimulated state, however, phospho-Akt expression was significantly reduced in ZDF rats compared with lean controls, and there was no effect of dietary treatment on phospho-Akt. As in the case of EDL muscle, basal phospho-Akt expression was significantly lower in ZDF control soleus muscle compared with lean control, c9,t11, 50:50, or pair-fed rats ($P < 0.004$). In the insulin-stimulated state, phospho-Akt expression was similar in soleus muscles from lean control, ZDF control, 50:50, and pair-fed rats but was significantly lower in soleus from c9,t11 rats ($P < 0.01$).

**Gene expression.** To determine the effects of dietary treatment on the expression of genes important in glucose and lipid metabolism, abundance of UCP2, PPAR-γ, and PEPCK mRNA was quantified. UCP2 mRNA expression was quantified in gastrocnemius muscle (Fig. 5*A*), epididymal adipose tissue (Fig. 5*B*), and liver (data not shown).

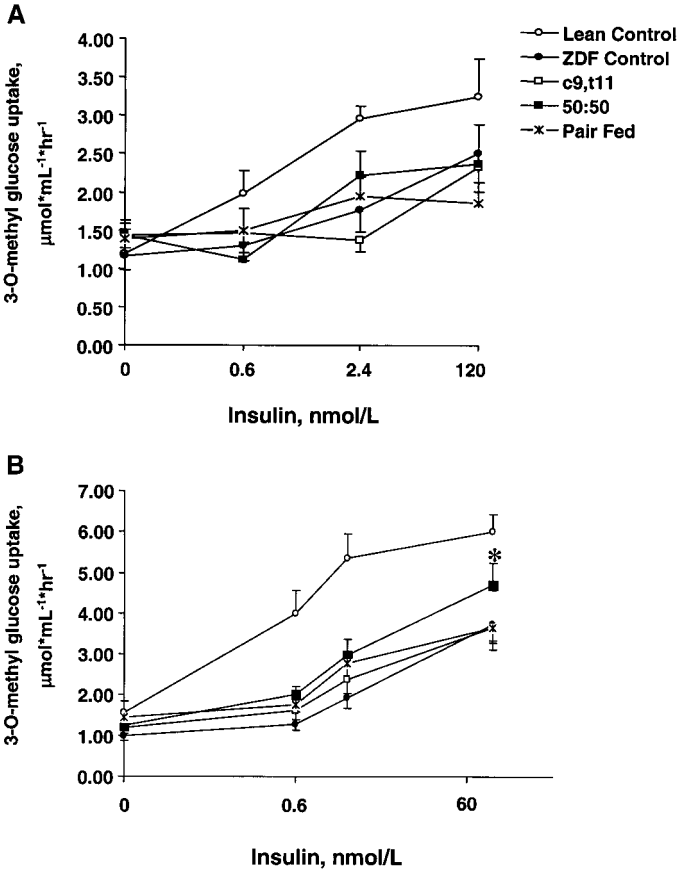


**FIG. 3. Effect of dietary treatments on glucose transport in isolated EDL (*A*) and soleus (*B*) muscles of ZDF rats. Lean and ZDF rats were maintained on experimental diets as described in RESEARCH DESIGN AND METHODS. At the culmination of the study, EDL and soleus muscles were dissected, and transport of 3-*O*-methyl[$^3$H]glucose was quantified in the absence (basal) and presence of increasing concentrations of insulin as described in RESEARCH DESIGN AND METHODS. Data are means ± SE. *A*: $n = 5$ lean controls; $n = 7$ ZDF controls; $n = 9$ c9,t11; $n = 4$ 50:50; $n = 5$ pair-fed rats. *B*: $n = 6$ lean controls; $n = 8$ ZDF controls; $n = 10$ c9,t11; $n = 5$ 50:50; $n = 5$ pair-fed rats. *$P < 0.01$ vs. ZDF control, c9,t11, and pair-fed rats.**

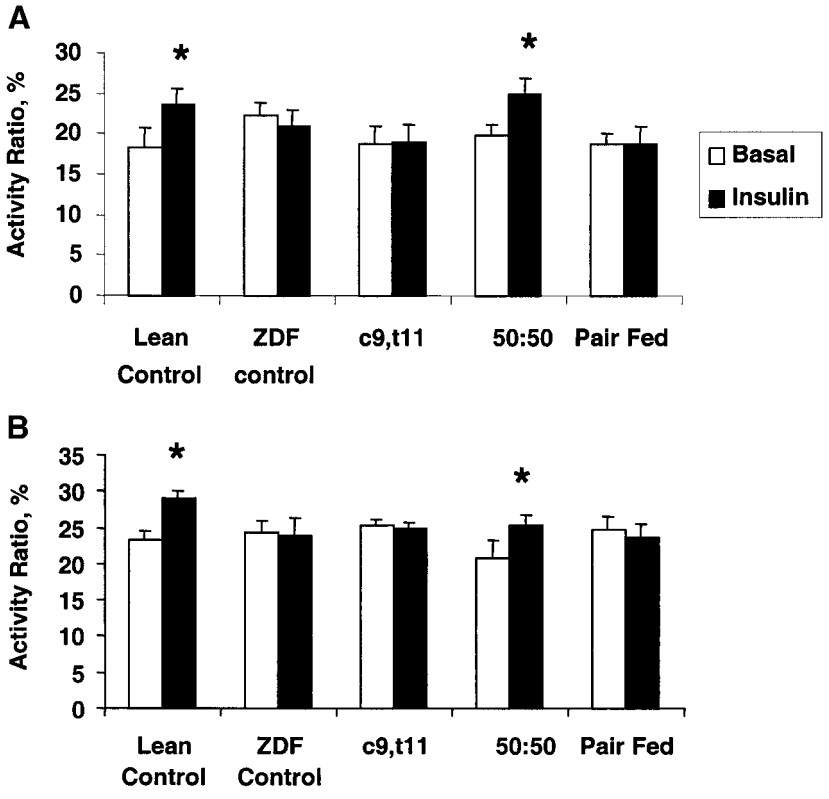


**FIG. 4. Effect of dietary treatments on glycogen synthase activity in isolated EDL (*A*) and soleus muscles from ZDF rats (*B*). Lean and ZDF rats were maintained on experimental diets as described in RESEARCH DESIGN AND METHODS. At the culmination of the study, EDL and soleus muscles were dissected, and glycogen synthase activity was quantified in the absence (basal) and presence of insulin, as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for $n = 6$ lean controls; $n = 6$ ZDF rats/treatment group. *$P < 0.05$ vs. ZDF control, c9,t11, and pair-fed rats.**

UCP2 mRNA abundance in gastrocnemius muscle was significantly downregulated in ZDF control and pair-fed rats compared with lean controls ($P < 0.001$; Fig. 5*A*). The downregulation was partially rescued by dietary CLA; both the c9,t11 and 50:50 diets increased UCP2 expression in gastrocnemius compared with ZDF control $P < 0.01$). Likewise, in epididymal white adipose tissue (Fig. 5*B*), UCP2 mRNA abundance was downregulated in ZDF rats compared with lean controls ($P < 0.001$). Pair-feeding ($P < 0.01$) and c9,t11 ($P < 0.001$) significantly increased UCP2 expression relative to ZDF controls. However, the 50:50 diet was the most efficacious ($P < 0.001$), as it normalized UCP2 expression to levels found in lean controls. In contrast, there was no effect of genotype or dietary treatment on UCP2 gene expression in liver (data not shown).

Protein expression of PPAR-γ in gastrocnemius was low and unaffected by any treatment (data not shown). Likewise, PPAR-γ expression in white adipose tissue was not regulated by CLA treatment (data not shown). Conversely, PPAR-γ mRNA expression in liver was reduced in c9,t11 ($P < 0.04$) and pair-fed ($P < 0.01$) treatments compared

TABLE 2
Effect of dietary CLA isomer supplementation on insulin signaling events in skeletal muscle

| | Treatment | | | | |
|---|---|---|---|---|---|
| | Lean control | ZDF control | c9,t11 | 50:50 | Pair-fed |
| PI 3-kinase activity (% of lean basal) | | | | | |
| EDL | | | | | |
| Basal | 100 | 91.5 ± 9.9 | 71.1 ± 12.1 | 74.0 ± 12.6 | 101.0 ± 41.8 |
| Insulin | 348.0 ± 78.8 | 158.0 ± 16.9 | 118.1 ± 30.4 | 127.0 ± 24.0 | 208.6 ± 23.1 |
| Soleus | | | | | |
| Basal | 100 | 170.0 ± 30.0 | 194.0 ± 17.0 | 141.0 ± 8.0 | 125.0 ± 18.0 |
| Insulin | 519.0 ± 140.0 | 307.2 ± 78.0 | 283.0 ± 33.0 | 270.0 ± 64.0 | 234.0 ± 100 |
| Phospho-Akt expression (% of lean basal) | | | | | |
| EDL | | | | | |
| Basal | 100 | 36.1 ± 6.48* | 78.0 ± 23.6 | 70.3 ± 18.2 | 37.3 ± 6.0* |
| Insulin | 881.4 ± 259.0 | 428.6 ± 145.0 | 466.5 ± 113.0 | 504.0 ± 109.0 | 456.5 ± 77.4 |
| Soleus | | | | | |
| Basal | 100 | 53.2 ± 12.4† | 79.3 ± 28.3 | 67.2 ± 20.2 | 68.1 ± 28.6 |
| Insulin | 256.4 ± 70.4 | 180.4 ± 46.3 | 102.5 ± 24.0‡ | 136.7 ± 36.0 | 240.4 ± 40.4 |

Data are means ± SE. *$P < 0.001$ vs. lean control; †$P < 0.004$ vs. lean control; ‡$P < 0.01$ vs. lean control and pair-fed.

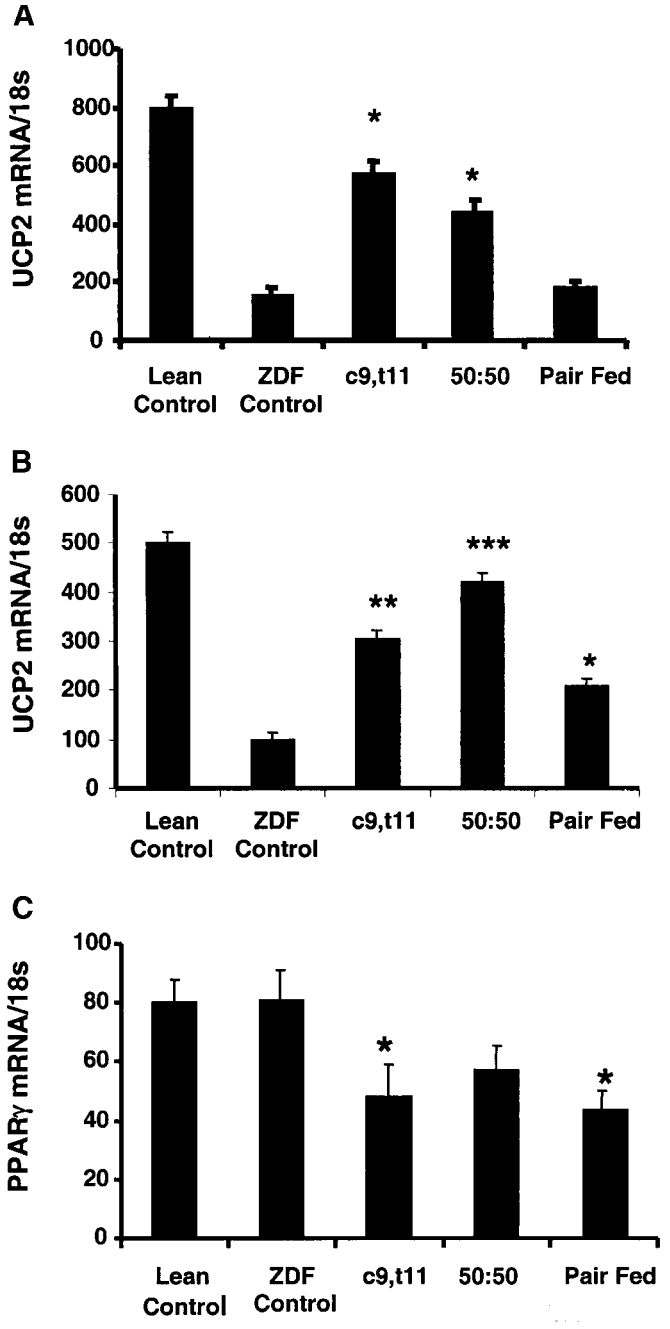


**FIG. 5. Effect of dietary treatments on UCP2 mRNA abundance in gastrocnemius muscle (*A*) or white adipose tissue (*B*) and on PPAR-γ mRNA abundance in liver (*C*) from ZDF rats. Lean and ZDF rats were maintained on experimental diets as described in RESEARCH DESIGN AND METHODS. At the culmination of the study, animals were killed, tissues were collected and processed for mRNA, and Northern analysis was performed as described in RESEARCH DESIGN AND METHODS. Bar graphs: data are means ± SE for $n = 4$ lean controls and $n = 6$ (*A* and *B*) or $n = 4$ (*C*) ZDF rats/treatment. *A*: *$P < 0.01$ vs. ZDF controls and pair-fed animals. *B*: *$P < 0.01$ vs. ZDF controls; **$P < 0.001$ vs. ZDF controls; ***$P < 0.0001$ vs. ZDF controls. *C*: *$P < 0.04$ vs. ZDF or lean controls; **$P < 0.01$ vs. ZDF or lean controls.**

with lean or ZDF controls (Fig. 5*C*). We observed no treatment effect on the expression of PEPCK mRNA in liver (data not shown).

## DISCUSSION

We provide evidence that dietary consumption of CLA significantly improves impaired glucose tolerance in ZDF rats and that the antidiabetic effects are mediated by specific CLA isoforms. This is the first study to examine the role of specific dietary CLA isomers on food intake, growth rate, adiposity, insulin action in skeletal muscle, and expression of genes thought to be important in glucose and lipid metabolism and, perhaps, thermogenesis. Consistent with improved glucose tolerance, we show insulin-stimulated glucose transport was improved in soleus muscle, and insulin-stimulated glycogen synthase activity was improved in soleus and EDL muscles of 50:50 rats. The fact that these effects were not observed in rats fed the c9,t11 diet suggests that the effects are specific to the 10,12 CLA isomer. Additionally, the pleotrophic effects of CLA isomers on glucose and lipid metabolism in the ZDF rat model are consistent with activation of PPARs.

Several groups have previously shown that consumption of mixtures of CLA isomers had significant effects on lipid metabolism. Specifically, CLA has been shown to affect fatty acid oxidation (28), lipolysis (29) and de novo lipogenesis (9), as well as regulate the expression of key enzymes involved in lipid metabolism (30,31). Evidence is also beginning to accumulate to implicate CLA activation of various PPARs in the regulation of expression of these enzymes (16,32). As regulation of whole-body lipid metabolism can have profound effects on insulin-stimulated glucose transport and utilization by skeletal muscle (33), it is likely that at least a portion of the antidiabetic effects of CLA that we report are attributable to alterations in lipid metabolism.

Insulin resistance is a feature of obesity and type 2 diabetes. Because skeletal muscle is a major site of insulin-stimulated glucose uptake, skeletal muscle is a major target for therapeutic intervention. It has been suggested that insulin sensitivity may be related to the fatty acid composition of the phospholipids within muscle membranes associated with insulin action (34). Consumption of CLA has been reported to increase the CLA content of skeletal muscle (35), adipose tissue (36,37), and plasma (36) in several species including humans. Thus, CLA uptake into insulin target tissues may have an impact on insulin action in those tissues.

High-fat diets or high-sucrose diets have been shown to increase visceral adiposity and impair whole-body insulin action and insulin signaling in skeletal muscle (38,39). In fact, when visceral adiposity is reduced surgically, hepatic insulin action is improved (40). In the present study, rats fed the 50:50 diet exhibited improved insulin action and reduced adiposity, despite high total dietary fat consumption, indicating that specific dietary fatty acid composition may have profound effects on adiposity and skeletal muscle insulin action. Because similar effects were not observed with the c9,t11 CLA diet, the improvements in insulin action must have been due to the 10,12 isomer. This finding differs from results from mammary cancer studies, in which the c9,t11 diet was effective in reducing tumor incidence in rats treated with the carcinogen methylnitrosourea (8). Our finding has important clinical implications because it suggests that diets enriched in the 10,12 CLA isomer may be efficacious in the management of obesity and insulin resistance.

The spontaneous diabetes observed in the ZDF rat is secondary to obesity (41); if prediabetic ZDF animals are

maintained on restricted caloric intake, the diabetes does not present (over the 12-week treatment period) (5). This phenomenon is thought to be due, at least in part, to the prevention of lipotoxicity (lipid accumulation in pancreatic β-cells) (41). In our study, pair-feeding was not as effective as the 50:50 diet in improving glucose tolerance and insulin action; thus, our data indicate that the effects of the 50:50 diet cannot be explained by a reduction in food intake alone. Nevertheless, a reduction in epididymal fat mass was observed in the 50:50 group, suggesting a reduction in whole-body adiposity. This observation confirms our previous finding (4) and is consistent with data that implicate the 10,12 isomer of CLA in reducing body fat in mice (6,42). Our data are also consistent with a recent report (7) that found that consumption of a mixture of CLA isomers (including the 10,12 and 9,11 isomers) by lean mice resulted in reduced adiposity due, at least in part, to increased apoptosis. Consistent with this notion, CLA-induced reduction in lipid accumulation in pancreatic islets could explain, at least in part, the prevention of diabetes in animals fed the 50:50 diet. Additionally, it is well known that small reductions in body weight can have profound effects on whole-body insulin action. Thus, although a reduction in food intake alone cannot explain the effects we observe, an increase in adipose tissue apoptosis (7) and subsequent reduction in adipose mass may play a role in the improved insulin action in ZDF rats fed the 50:50 diet. Additional studies are needed to determine whether long-term CLA treatment can completely prevent, or merely delay, the development of diabetes in this model.

In addition to stimulation of apoptosis, CLA-induced reduction in whole-body adiposity could be due to a combination of reduced food intake and increased thermogenesis in 50:50 but not pair-fed rats. UCPs, including UCP1 and UCP2 quantified in the present study, have been implicated in the regulation of thermogenesis as well as fatty acid metabolism in rodents (rev. in 43). Although thermogenesis was not quantified in the present study, we did observe an increase in BAT mass in CLA-fed ZDF rats as well as upregulation of UCP2 in muscle and epididymal adipose tissue. Others have reported that dietary CLA increases energy expenditure in rodents (28). A CLA-induced increase in BAT mass may be at least partially responsible for increased thermogenesis in CLA-fed animals, although the mechanism by which CLA stimulated BAT development in the ZDF rats is currently unknown. Additionally, although the precise role that UCP2 plays in the regulation of thermogenesis and fatty acid metabolism is still being elucidated (43), it is tempting to speculate that the partial rescue of UCP2 expression we observed in skeletal muscle and adipose tissue of CLA-fed ZDF rats plays a role in regulating adiposity and/or lipid metabolism in these animals.

We previously reported that a mixture of CLA isomers was able to activate PPAR-γ in vitro (4). Based on those data and the antidiabetic effects of specific CLA isomers, we proposed that the effects were at least partially attributable to activation of PPAR-γ. Subsequently, others (16) have shown that the 9,11 isomer preferentially activates PPAR-α, an important regulator of hepatic lipid metabolism. Our data are consistent with CLA activation of PPAR-α as both CLA dietary treatments resulted in reduced circulating lipids and fatty acids. However, the lack of effect of the 9,11 CLA isomer on glucose tolerance and insulin action indicates that it is likely that the 9,11 isomer does not significantly activate PPAR-γ. The relative potency of activation of PPAR-γ vs. PPAR-α for the 10,12 isomer has yet to be determined. PPAR-γ agonists, the thiazolidinediones, are potent insulin sensitizers and have been shown to ameliorate lipotoxicity and improve insulin secretion in ZDF rats (10–12). One mechanism underlying this improvement may be upregulation of UCPs in pancreatic islets and improved insulin secretion (44). Furthermore, UCP2 expression in tissues is upregulated with TZD treatment or with treatment with PPAR-α compounds (43,44). Although we saw no effect of CLA treatment on PPAR-γ expression in muscle or adipose tissue, our data are consistent with PPAR-γ activation in those tissues. Specifically, the CLA effects we observe with both the 50:50 and c9,t11 diets may be due to activation of PPAR-γ and/or PPAR-α by these isomers. However, given the lack of effect of c9,t11 on glucose homeostasis, it is unlikely that treatment-induced effects on UCP2 expression can completely explain the antidiabetic effects we observed.

Despite improved whole-body glucose tolerance and improved insulin action in skeletal muscle of rats fed the 50:50 diet, we observed no effect of dietary treatment on insulin signaling in skeletal muscle. The insulin-sensitizing effects of CLA on skeletal muscle may be secondary to reduced adiposity because visceral adiposity is correlated with reduced insulin action in skeletal muscle and liver (39, 40). We have previously reported that feeding high-fat diets to mice results in impaired insulin signaling, as compared with chow-fed animals (38); however, all diets used in the present study were high in dietary fat. Therefore, any isomer-specific effects may be too subtle for detection.

In conclusion, we provide the first evidence to show effects of specific isomers of CLA on whole-body glucose metabolism. Furthermore, we systematically examined and identified tissue-specific cellular mechanisms underlying the improved glucose tolerance observed in ZDF rats fed CLA. Specifically, we show that specific CLA isomers reduce adiposity and improve insulin action and signal transduction in skeletal muscle. Furthermore, CLA isomers regulate UCP2 gene expression in skeletal muscle and adipose tissue and downregulate PPAR-γ expression in liver. It is likely that the antidiabetic effects of CLA isomers in the ZDF rat are due to complex regulation of the expression of genes important in the regulation of adipogenesis, glucose and lipid metabolism, and, perhaps, whole-body thermogenesis. Further research is needed to determine specific effects of CLA isomers on regulation of PPAR-γ activation and insulin secretion in models of type 2 diabetes. Additionally, studies are needed to determine whether long-term CLA treatment prevents or merely delays development of the diabetic phenotype in the ZDF rat and other diabetic models. The ultimate goal is to determine the efficacy of CLA in the clinical treatment of obesity and type 2 diabetes.

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