Conjugated Linoleic Acid Supplementation Reduces Adipose Tissue by Apoptosis and Develops Lipodystrophy in Mice

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Conjugated linoleic acid (CLA) is a naturally occurring group of dienoic derivatives of linoleic acid found in beef and dairy products. CLA has been reported to reduce body fat. To examine the mechanism(s) of CLA reduction of fat mass, female C57BL/6J mice were fed standard semipurified diets (10% fat of total energy) with or without CLA (1% wt/wt). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) and DNA fragmentation analysis revealed that fat-mass decrease by CLA was mainly due to apoptosis. Tumor necrosis factor (TNF)-α and uncoupling protein (UCP)-2 mRNA levels increased 12- and 6-fold, respectively, in isolated adipocytes from CLA-fed mice compared with control mice. Because it is known that TNF-α induces apoptosis of adipocytes and upregulates UCP2 mRNA, a marked increase of TNF-α mRNA with an increase of UCP2 in adipocytes caused CLA-induced apoptosis. However, with a decrease of fat mass, CLA supplementation resulted in a state resembling lipoatrophic diabetes: ablation of brown adipose tissue, a marked reduction of white adipose tissue, marked hepatomegaly, and marked insulin resistance. CLA supplementation decreased blood leptin levels, but continuous leptin infusion reversed hyperinsulinemia, indicating that leptin depletion contributes to the development of insulin resistance. These results demonstrate that intake of CLA reduces adipose tissue by apoptosis and results in lipodystrophy, but hyperinsulinemia by CLA can be normalized by leptin administration. *Diabetes* 49:1534–1542, 2000

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ACC, acetyl-CoA carboxylase; ACS, acyl-CoA synthetase; BAT, brown adipose tissue; CLA, conjugated linoleic acid; FAS, fatty acid synthase; LPL, lipoprotein lipase; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PSL, phosphostimulated luminescence; SREBP, sterol regulatory element-binding protein; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; UCP, uncoupling protein; WAT, white adipose tissue.

Conjugated linoleic acid (CLA) is a group of positional and geometric isomers of conjugated dienoic derivatives of linoleic acid. The major dietary sources of CLA for humans are beef and dairy products (1). There is a great interest in CLA because of its anticarcinogenic and antiatherogenic properties and its ability to reduce body fat while enhancing lean body mass (2). Reduction of body fats by CLA was observed in pigs (3), mice (4,5), and hamsters (6).

In this study, we investigated the mechanism(s) of CLA-mediated reduction of fat mass in mice. Reduction of fat mass by CLA was due to apoptosis. Initially, we expected the beneficial effect of CLA-induced fat mass decrease, such as improvement of insulin resistance and reduction of blood triglyceride concentration. Unexpectedly, however, rapid reduction of fat mass by CLA resulted in marked insulin resistance and hepatomegaly. These metabolic abnormalities are characteristics of lipodystrophy (7,8). During this study, Delany et al. (9) also reported that CLA reduced body fat but resulted in hyperinsulinemia and liver enlargement in AKR/J male mice. To understand the mechanism(s) of CLA-induced lipodystrophy, we examined expression levels of several genes important in energy expenditure and lipid and carbohydrate metabolism in adipose tissues and isolated adipocytes. Furthermore, effects of leptin infusion on CLA-induced insulin resistance were also examined.

RESEARCH DESIGN AND METHODS

Experimental protocols. Female C57BL/6J mice were obtained from Tokyo Laboratory Animals Science (Tokyo) at 7 weeks of age and fed a normal laboratory diet (CE2; Clea, Tokyo) for 1 week to stabilize the metabolic conditions. Mice were exposed to a 12-h light/12-h dark cycle and maintained at a constant temperature of 22°C. Mice were fed a standard semipurified diet (10% fat of total energy) with or without CLA (1% wt/wt) for 4 days to 8 months. Each cage contained 4–5 mice. The semipurified diet was a low-fat diet and on a calorie basis contained 63% carbohydrate, 11% safflower oil, and 26% protein. Safflower oil was used as a source of fat. Safflower oil (high-oleic type) contained 46% oleic acid (18:1 n-9) and 45% linoleic acid (18:2 n-6) from total fatty acids. CLA was prepared as a free fatty acid at Rinoru Oil Mills (Nagoya, Japan) and stored frozen in plastic bottles blanketed with nitrogen. Linoleic acid was isomerized to CLA with isomers (34% c9, t11/t9, and c11; 36% t10 and c12; 3% c9, c11/c10, and c12; 2% t9, t11/t10, and t12 from total fatty acids). In the CLA-fed group, to keep fat intake constant in the 2 groups, 25% of the safflower oil was replaced with CLA. Other materials and methods of the diet preparation and those of estimation of energy intake were the same as those used in our previous study (10). Oxygen consumption (energy expenditure) was measured as in our previous study (11). Energy intake was measured dur-

ing the 13- to 14-week feeding, whereas energy expenditure was measured during the 10- to 11-week and 19- to 20-week feedings. During these studies, animals were anesthetized at ~10:00 A.M. by intraperitoneal injection of pentobarbital sodium (0.08 mg/g body wt) (Nembutal; Abbot, Chicago) under feeding conditions. Liver, gastrocnemius, parametrial white adipose tissue (WAT), and brown adipose tissue (BAT) were isolated immediately, weighed, and homogenized in guanidine-thiocyanate, and RNA was prepared by the method described by Chirgwin et al. (12).

Histological analysis and morphometry. Adipose tissues from mice fed a diet with or without CLA were fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydrated, and embedded in epon. The sections were stained with toluidine blue for quantitation of size of adipocytes, which have osmiophilic black-stained fat droplets. The sizes of adipocytes in a fixed area (450,000 μm^2) were quantitated. Liver from mice fed diet with or without CLA or with CLA plus leptin treatment was fixed with formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin to detect steatosis.

For analysis of DNA fragmentation, Mebstain apoptosis kit (MBL, Japan) was used for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) (13). Mice were fed CLA (1% wt/wt) in a standard semipurified diet and killed before and 7 and 20 days after CLA supplementation under pentobarbital sodium anesthesia (0.08 mg/g body wt). Parametrial WAT and BAT were fixed with 100% ethanol and embedded in paraffin. Deparaffinized sections of 4 μm thickness were incubated with proteinase K (40 mg/ml) at 37°C for 30 min. After washing with 0.01 mol/l phosphate-buffered saline, the sections were reacted with biotinylated dUTP in the TdT reacting buffer at 37°C for 60 min. They were then visualized with streptavidin–fluorescein isothiocyanate conjugate and Proppidium Iodide (Molecular Probes, Eugene, OR) for counterstain and were analyzed by laser-scanning microscope units. For the negative control, sections were treated by the same procedure without TdT.

DNA extraction and analysis. DNA was extracted from the samples using a standard phenol/chloroform/isoamyl alcohol technique (14). Electrophoretic gels were run using 2% agarose with TAE (0.04 mol/l Tris-acetate, 0.001 mol EDTA) as the running buffer. A total of 5 μg DNA was loaded per lane. Gels were run for 40 min, stained with SYBR Green (Molecular Probes), and photographed under ultraviolet light.

Preparation of cDNA probe and Northern blot. cDNA clones containing the coding sequence of mouse uncoupling protein (UCP)-1 and human UCP2 and UCP3 were obtained by polymerase chain reaction (PCR) amplification as described previously (15). The cDNA fragments for mouse sterol regulatory element-binding protein (SREBP)-1 and lipoprotein lipase (LPL) were obtained by PCR from first-strand cDNA using mouse liver total RNA as described previously (16). The cDNA probes for rat acyl-CoA synthetase (ACS) were provided by Dr. T. Yamamoto at Tohoku University, rat acetyl-CoA carboxylase (ACC) and rat fatty acid synthase (FAS) by Dr. N. Iritani at Tezukayama Gakuin College, peroxisome proliferator-activated receptor (PPAR)-γ by Dr. T. Osumi at the Himeji Institute of Technology, and tumor necrosis factor (TNF)-α by Dr. Y. Hosokawa at the National Institute of Health and Nutrition. These cDNAs were used as probes for Northern blots. A portion of RNA (15 μg per lane) was denatured with glyoxal and dimethyl sulfoxide and analyzed by electrophoresis in 1% agarose gels. After transfer to nylon membranes (DuPont-NEN, Boston, MA) and ultraviolet cross-linking, RNA blots were stained with methylene blue to locate 28S and 18S rRNAs and to ascertain the amount of loaded RNAs (17). The blots were hybridized overnight at 42°C with cDNAs that had been labeled with [^{32}P]dCTP (DuPont-NEN) by a random prime labeling kit (Amersham Pharmacia Biotech). The filters were washed several times with 1 × sodium chloride–sodium citrate, 0.1% SDS at room temperature, washed twice at 50°C, and then exposed to X-ray film at –80°C. The amounts of each mRNA were quantitated with an image analyzer (BAS 2000; Fuji Film, Tokyo) and expressed as the intensity of phosphostimulated luminescence (PSL).

Isolation of adipocytes and nonadipocytes from adipose tissues. Adipocytes and nonadipocytes were prepared by the collagenase method (18) from parametrial adipose tissue from 12–18 mice. The cells were fractionated by brief centrifugation (350*g* for 20 s) in Krebs-Henseleit 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer, pH 7.4, supplemented with 20 mg/ml of bovine serum albumin (fraction V) and 2 mmol/l glucose. Floated cells were adipocytes, whereas the pellet was a nonadipocyte.

Oral glucose and insulin tolerance test. For oral glucose tolerance testing, 17 weeks after CLA supplementation, D-glucose (1 mg per g body wt, 10% [wt/vol] glucose solution) was administered after an overnight fast by a stomach tube. Blood samples were obtained by cutting the tail tip before and 30, 60, and 120 min after glucose administration. For insulin tolerance testing, 9 weeks after CLA supplementation, human insulin (Humulin R; Eli Lilly Japan K.K., Kobe, Japan) was injected intraperitoneally (0.75 mU/g body wt) in fed animals. Blood glucose was measured on samples obtained from the tail tip before and 15, 30, 60, 90, and 120 min after insulin injection. Blood glucose concentrations were measured using the TIDEX glucose analyzer (Sankyo, Tokyo).

Leptin treatment. After 8 months of feeding with CLA, each group of mice was divided into 2 subgroups, and an Alzet micro-osmotic pump (model 1002, Alza) was inserted subcutaneously in the back of each mouse (19). The pumps delivered 0 or 5 μg mouse recombinant leptin (Linco) per day in a total volume of 0.1 ml saline. All mice were killed 12 days after inserting the pumps.

Other analyses. Blood samples were obtained by cutting the tail end under fasting and feeding conditions. Immunoreactive insulin was measured by an insulin assay kit (Morinaga, Kanagawa, Japan), and leptin was measured by a mouse leptin assay kit (Morinaga).

Statistical analysis. Comparisons of data from 2 experimental groups were made by an unpaired Student's *t* test. The glucose and insulin tolerance curve of each group was compared by repeated-measures analysis (Super ANOVA; Abacus Concepts). Statistical significance was defined as $P < 0.05$. Values are means ± SE.

RESULTS

Gross changes in fat and liver of CLA-fed mice. Figure 1 shows a picture of mice fed standard semipurified diets in the presence and absence of CLA for 5 months. Although there was no difference in body weight, CLA-fed mice showed ablation of BAT and a marked decrease of subcutaneous WAT (Fig. 1*A*). In Table 1, wet weights of several WAT depots and major organs in the 2 groups are shown. There was little renal, retroperitoneal, and subcutaneous WAT or BAT in CLA-fed mice. Parametrial WAT was less sensitive to CLA than other WATs, but it weighed only 27% as much as control mice. Histological analysis revealed that the mean diameters of adipocytes in CLA-fed mice were 41% smaller than those in control mice (79.1 ± 4.8 μm, $n = 46$, in control mice; 46.4 ± 3.8 μm, $n = 72$, in CLA-fed mice). Thus, decreased cell size partly contributed to fat mass decrease. However, the liver was massively enlarged and very pale, suggesting deposition of fat (Fig. 1*B* and *C*). CLA-fed mice showed 3.6-fold ($P < 0.001$) and 1.6-fold (NS) enlargement of liver and spleen, respectively. Histological analysis of liver revealed that there was panlobular macrovesicular steatosis but no increased hepatic inflammation (Fig. 6*C*). Liver enlargement was manifested 14 days after CLA supplementation (data not shown). No enlargement of the kidney, heart, or skeletal muscles was noted. Despite these marked phenotypic changes, the average energy intake in the 2 groups was not significantly different (7.4 ± 0.5 and 7.7 ± 0.9 kcal · mouse^{-1} · day^{-1} in control and CLA-fed mice, respectively; $n = 5$).

CLA-induced apoptosis in adipose tissues. To examine whether decrease of fat mass by CLA was also due to a decrease in adipocyte number, change of wet weight of parametrial WAT and BAT and rate of apoptosis were estimated by TUNEL (13) and DNA analysis (14) before and 7 and 20 days after CLA supplementation. The TUNEL assay detects DNA fragmentation and is commonly used to detect apoptosis. Decreases in wet weight of parametrial WAT and BAT were manifested 4 days after CLA supplementation (Fig. 2*A*). TUNEL$^+$ cells were rare both in parametrial WAT and BAT before CLA supplementation but were clearly observed 7 days after CLA supplementation (see green stain in Fig. 2*B*). The number of TUNEL$^+$ cells increased further after CLA supplementation. Biochemical evidence of apoptosis was obtained by DNA analysis. Electrophoresis of DNA from WAT and BAT in control mice showed no cleavage, whereas that of DNA in CLA-fed mice for 7 days displayed a ladder pattern, indicative of the internucleosomal cleavage characteristic of apoptosis (Fig. 2*C*). Together, these data indicated that both cell death and decreased cell size contributed to decreased WAT and BAT mass.

Increased TNF-α and UCP2 mRNA expression in adipose tissues. To elucidate the mechanism(s) of WAT and BAT mass decreases, mRNA levels of TNF-α and UCPs were examined.

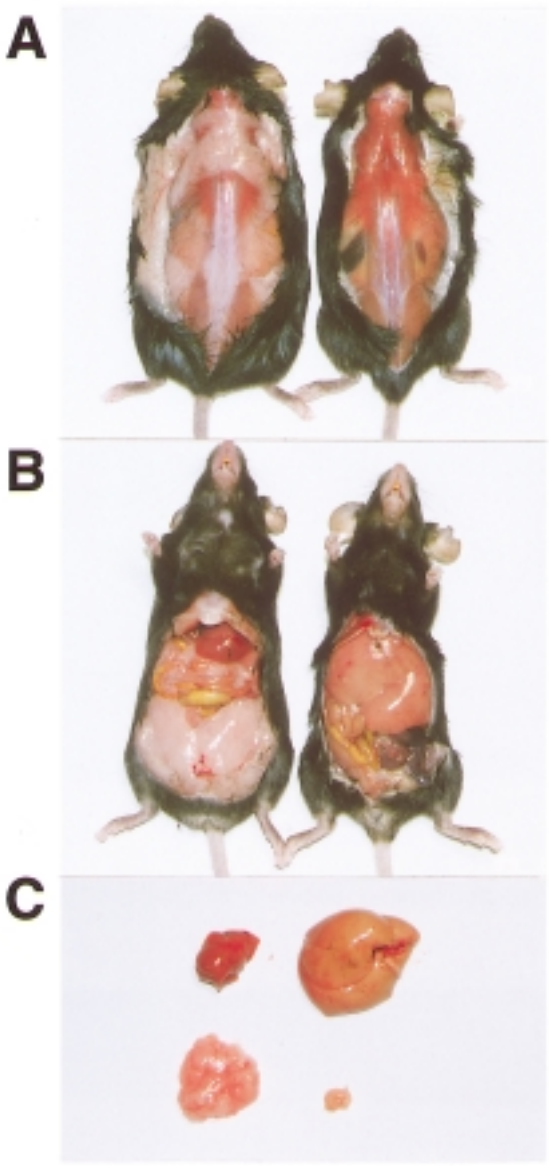

FIG. 1. Photograph of gross change in mice fed standard semipurified diet (left) and CLA-supplemented diet (right). Mice were fed for 5 months in the absence (left) and presence (right) of CLA. *A*: Exposed dorsal view of the mice illustrating ablation of BAT and a marked decrease of subcutaneous WAT. *B*: Exposed ventral view of the mice illustrating the enlarged fatty liver in the mouse fed CLA-supplemented diet. *C*: Liver and parametrial WAT from these mice.

TABLE 1
Body weight and tissue weight in each group of mice

	Experimental diet	
	CLA^-	CLA^+
Initial body weight (g)	17.5 ± 0.2 (14)	17.6 ± 0.2 (14)
Final body weight (g)	29.3 ± 1.1 (13)	27.6 ± 0.9 (13)
WAT		
Parametrial weight (g)	1.33 ± 0.14 (14)	0.36 ± 0.05 (14)*
Renal weight (g)	0.42 ± 0.10 (5)	0.03 ± 0.01 (5)†
Retroperitoneal weight (g)	0.29 ± 0.06 (5)	ND (5)
Abdominal subcutaneous weight (g)	1.11 ± 0.21 (5)	0.04 ± 0.01 (5)*
Dorsal subcutaneous weight (g)	0.76 ± 0.16 (5)	0.02 ± 0.00 (5)†
BAT weight (g)	0.14 ± 0.01 (14)	ND (14)
Liver weight (g)	1.24 ± 0.07 (14)	4.44 ± 0.43 (14)*
Spleen weight (g)	0.10 ± 0.01 (5)	0.16 ± 0.02 (5)
Kidney weight (g)	0.27 ± 0.01 (5)	0.28 ± 0.01 (5)
Heart weight (g)	0.13 ± 0.00 (5)	0.15 ± 0.01 (5)
Skeletal muscles		
Gastrocnemius weight (g)	0.23 ± 0.01 (5)	0.24 ± 0.01 (5)
Quadriceps weight (g)	0.24 ± 0.02 (5)	0.25 ± 0.02 (5)

Data are means ± SE of 5–14 mice (n). *$P < 0.001$ and †$P < 0.01$, the mark on standard semipurified diets plus CLA in comparison with standard semipurified diets without CLA by unpaired Student's t test. ND, not able to determine because of ablation of tissue.

TNF-α is a cytokine that leads to apoptosis of adipocytes (20). UCPs are mitochondrial transporters that have the capacity to dissipate the proton gradient and thereby produce heat (21). Thus, overexpression of UCPs may lead to a decrease of cellular ATP and then cause apoptosis (22). TNF-α mRNA levels in WAT increased 8-fold in CLA-fed mice, whereas those in skeletal muscles decreased (Fig. 3*A*). UCP is a family protein; UCP1 is expressed exclusively in BAT, UCP2 is expressed in multiple tissues, and UCP3 is expressed at high levels in skeletal muscle as well as BAT (23). CLA supplementation increased UCP2 mRNA by 5-fold in parametrial WAT and by 2-fold in liver (Fig. 3*B*). There were no significant differences of UCP2 (Fig. 3*B*) and UCP3 (data not shown) mRNA levels in gastrocnemius. Because UCP2 is a predominant uncoupling protein in WAT (23), a 5-fold increase of UCP2 may contribute to increased energy expenditure by CLA feeding. Indeed, oxygen consumption at night was 8% higher in CLA-fed mice than control mice ($P < 0.05$, $n = 6$, data not shown).

To examine the relationship between decreases of adipose tissue mass and TNF-α and UCPs, a time course study of CLA supplementation was conducted. Mice were killed before and 4, 7, 14, and 28 days after CLA supplementation, and TNF-α, UCP1, and UCP2 mRNA levels in parametrial WAT and BAT were measured. Increases in TNF-α mRNA in parametrial WAT and BAT were observed 4 days after CLA supplementation, and their increases were larger during the first week, and thereafter they declined (Fig. 3*C*). Similar changes of UCP2 mRNA were also observed. Upregulation of UCP2 was observed 4 days after CLA supplementation (Fig. 3*C*) and then declined slightly, whereas downregulation of UCP1 in BAT was observed 4 days after CLA supplementation (data not shown). Because UCP1 was weakly expressed in parametrial WAT but CLA supplementation upregulated UCP2, downregulation of UCP1 mRNA in BAT may be an adaptive response to UCP2 upregulation. These data suggest that upregulation of TNF-α and UCP2 mRNA in WAT and BAT might be related to the reduction of WAT and BAT mass.

Marked upregulation of TNF-α and UCP2 mRNA in isolated adipocytes. To investigate whether increased expressions of TNF-α and UCP2 mRNA observed in adipose tissues were due to increased mRNA in adipocytes or in nonadi-

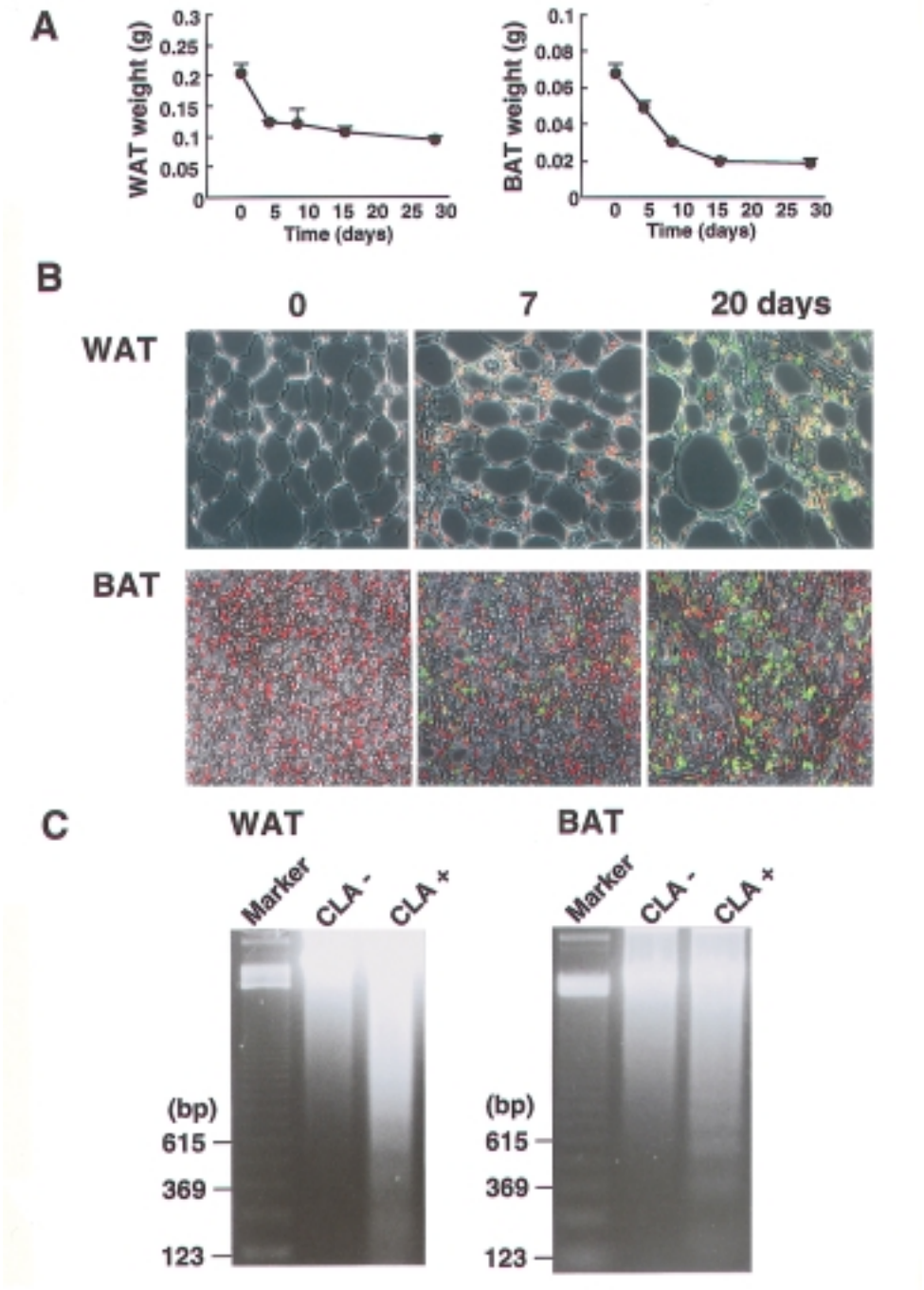

FIG. 2. CLA supplementation–induced apoptosis in adipose tissues. *A*: Effects of CLA on WAT and BAT weight. Tissue weight was measured at 0, 4, 7, 14, and 28 days after CLA supplementation. Each data point represents the mean ± SE of 3–4 mice. Statistical differences are omitted for the sake of clarity. *B*: Apoptotic nuclei stained by the TUNEL technique. All sections were cut to a thickness of 4 μm. Nuclei were stained red, and apoptotic nuclei were stained green. Photomicrographs from representative parametrial WAT and BAT in mice fed a CLA-supplemented diet for 0, 7, and 20 days are shown. *C*: Agarose gel electrophoresis of DNA extracted from adipose tissues. DNA was extracted from parametrial WAT and BAT in mice fed diets in the absence and presence of CLA for 7 days. The DNA ladder marker was loaded as a molecular weight standard.

pocytes such as blood cells, fibroblasts (including preadipocytes), and macrophages, adipose tissues were digested by collagenase and then separated into adipocytes and nonadipocytes by brief centrifugation, and TNF-α and UCP2 mRNA levels on each fraction were measured. As shown in Fig. 4, nonadipocytes had 5-fold larger expressions of TNF-α and UCP2 than adipocytes. GLUT4 was substantially expressed in adipocytes but not in nonadipocytes, suggesting that there was no significant contamination of nonadipocyte cell fractions by adipocytes. After 11 days, CLA supplementation resulted in 12- and 6-fold increases of TNF-α and UCP2 mRNA in adipocytes and in only 3- and 2-fold increases of these mRNAs in nonadipocytes, respectively (Fig. 4). Thus, CLA supplementation induced TNF-α and UCP2 mRNA expression both in adipocytes and nonadipocytes, but its effect was prominent in adipocytes.

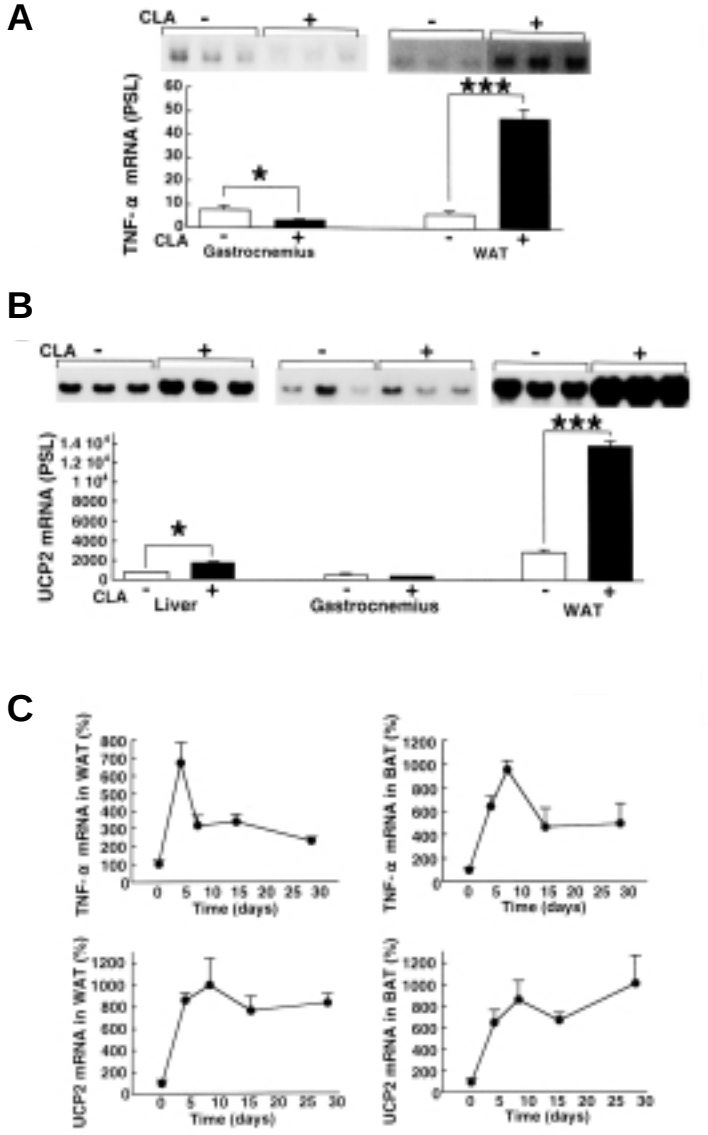

FIG. 3. TNF-α and UCP2 mRNA levels from mice fed a CLA-supplemented diet. *A*: Expression of TNF-α mRNA in gastrocnemius and WAT by CLA supplementation. Gastrocnemius and WAT from mice fed a standard semipurified diet with or without CLA were used for preparation of total RNA. RNA transferred membrane sheets were probed with ^{32}P-labeled human TNF-α cDNA. These mRNA levels were quantified using an image analyzer. Typical autoradiograms of TNF-α mRNA in gastrocnemius (1-week exposure) and WAT (1-week exposure) and its PSL levels (2-h exposure) are shown. In the autoradiogram, each lane represents a sample from an individual mouse. Each data point represents the mean ± SE of 3–4 mice. Statistical differences are shown as *P < 0.05 and ***P < 0.001. *B*: Expression of UCP2 mRNA in liver, gastrocnemius, and parametrial WAT by CLA supplementation. RNA transferred membrane sheets were probed with ^{32}P-labeled human UCP2 cDNA. These mRNA levels were quantified using an image analyzer. Typical autoradiograms of liver, skeletal muscle, and parametrial WAT UCP2 mRNA (16-h exposure) and PSL levels (1-h exposure) are shown. In the autoradiogram, each lane represents a sample from an individual mouse. Each data point represents the mean ± SE of 4 mice. Statistical differences are shown as *P < 0.05 and ***P < 0.001. *C*: Effects of CLA on mRNA expression of TNF-α and UCP2. Parametrial WAT and BAT from CLA-fed mice (0, 4, 7, 14, and 28 days) were used for preparation of total RNA. RNA transferred membrane sheets were probed with ^{32}P-labeled cDNA. These mRNA levels were quantified using an image analyzer. PSL levels (1-h exposure) are shown as relative percentage. Each data point represents the mean ± SE of 3–4 mice. Statistical differences are omitted for the sake of clarity.

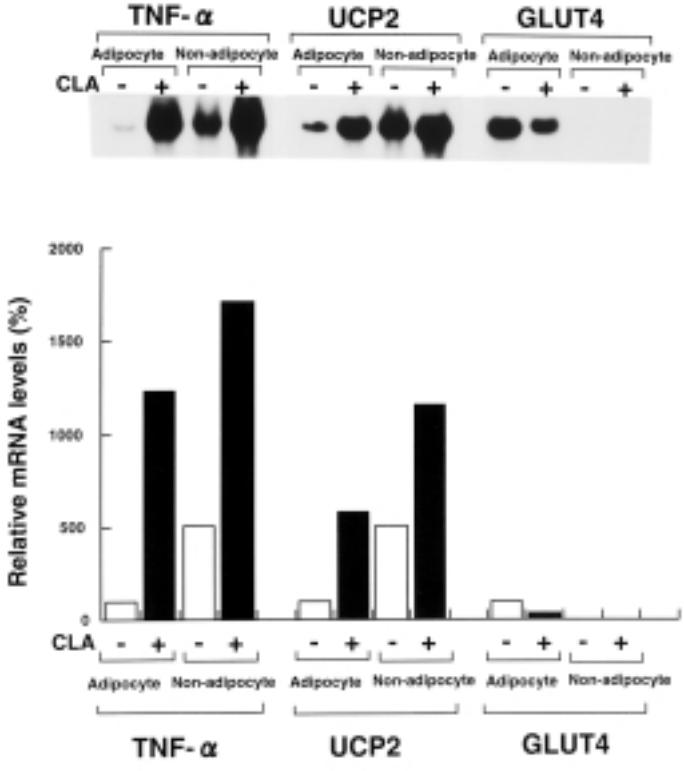

FIG. 4. TNF-α, UCP2, and GLUT4 mRNA expression in adipocytes and nonadipocytes. Mice were fed with or without CLA for 11 days and then killed. Adipose tissue pooled from 12–18 mice was digested by collagenase and then separated into adipocytes and nonadipocytes by brief centrifugation; TNF-α, UCP2, and GLUT4 mRNA levels of each fraction were measured. Autoradiograms and their relative PSL levels are shown. The mRNA levels of each gene are expressed as the percentage of that of each gene from adipocytes of control mice (without CLA).

Expression of mRNAs for lipid-related genes in fat. To elucidate the mechanism of decrease of WAT size, we examined the mRNA levels of several enzymes involved in lipogenesis (FAS and ACC) in fatty acids uptake (LPL) and in triglyceride formation (ACS) (Table 2). Mice supplemented with 1% CLA for 5 months showed marked decreases of FAS by 88% and ACC by 72%. CLA feeding did not have a statistically significant effect on LPL and ACS mRNA levels. Because expression of lipogenic enzymes is regulated by the transcription factor SREBP-1 (24), the effect of CLA on SREBP-1 expression was also examined. SREBP-1 mRNA abundance showed a tendency to decrease with CLA feeding (P = 0.054, n = 4). PPAR-γ, another important transcription factor for adipogenesis (25), was also downregulated by CLA supplementation.

Development of insulin resistance from CLA-fed mice. Although there was no significant alteration of blood glucose concentration after oral glucose tolerance testing (Fig. 5*A*), insulin tolerance testing clearly showed a marked insulin resistance in CLA-fed mice (Fig. 5*B*). Indeed, CLA-fed mice showed increases of fasting and feeding insulin concentrations by 4-fold (P < 0.05) and 8-fold (P < 0.001), respectively (Fig. 5*C*). This result suggested that insulin resistance might develop in CLA-fed mice. To elucidate mechanism(s) of insulin resistance, blood leptin concentration and GLUT4 mRNA levels in parametrial WAT and

TABLE 2
Relative levels of various mRNAs in parametrial WAT from each group of mice

	Experimental diet	
	% CLA$^-$	% CLA$^+$
PPAR-γ	100 ± 6 (4)	33 ± 3 (4)*
SREBP-1	100 ± 16 (4)	55 ± 9 (4)
FAS	100 ± 9 (3)	12 ± 1 (4)*
ACC	100 ± 23 (4)	28 ± 7 (3)†
ACS	100 ± 6 (4)	81 ± 11 (4)
LPL	100 ± 14 (4)	84 ± 2 (4)

Data are means ± SE of 3–4 mice (*n*). *P < 0.001 and †P < 0.05, the mark on standard semipurified diets plus CLA in comparison with standard semipurified diets without CLA by unpaired Student's *t* test.

skeletal muscles were investigated. Leptin enhanced insulin-mediated stimulation of glucose disposal (26,27). GLUT4 protein concentration on plasma membranes was a rate-limiting step for glucose metabolism in skeletal tissue and WAT (28). Both fasting and feeding plasma leptin levels decreased in CLA-fed mice by 49% (P < 0.01) and 79% (P < 0.001), respectively (Fig. 5*C*). CLA supplementation markedly downregulated GLUT4 mRNA by 91% (P < 0.001) in parametrial WAT, whereas it increased GLUT4 mRNA 1.5-fold (P < 0.05) in gastrocnemius (Fig. 5*D*). Decreases of GLUT4 mRNA in parametrial WAT and BAT were also observed 4 days after CLA supplementation (data not shown). Thus, the marked insulin resistance observed in CLA-fed mice may be partly explained by leptin deficiency and a decrease of GLUT4 in adipocytes.

Leptin normalizes blood insulin concentration and decreases fat accumulation in liver. Insulin resistance in a mouse model of lipodystrophy, a transgenic mouse expressing the truncated nuclear protein SREBP-1c, could be overcome by continuous systemic infusion of leptin (29). Thus, it is conceivable that CLA-induced insulin resistance was also due to leptin depletion. To test this theory, using the same protocol as in the previous study (29), effects of leptin infusion on CLA-fed mice were examined. Figure 6 presents changes in feeding plasma leptin and insulin concentrations in CLA-fed mice that were infused with saline or leptin (5 μg/day) for 12 days. CLA-fed mice had very low plasma leptin, which was restored to normal levels by leptin infusion (Fig. 6*A*). CLA-increased feeding insulin concentration was somewhat increased after saline treatment but became almost normal after leptin treatment (Fig. 6*B*). The livers of CLA-fed mice showed massive vacuolization due to fat deposition (Fig. 6*C*), but this result was reversed partially by leptin treatment (Fig. 6*D*). Parallel with this morphological change, leptin treatment decreased liver weight by 30% (data not shown).

DISCUSSION

Human congenital or acquired lipodystrophies are a heterogeneous group of diseases characterized by decreased fat mass; insulin resistance; organomegaly of the liver, spleen, pancreas, and kidney; and increased metabolic rate (7,8). In this study, intake of CLA, a naturally occurring substance, in a low-

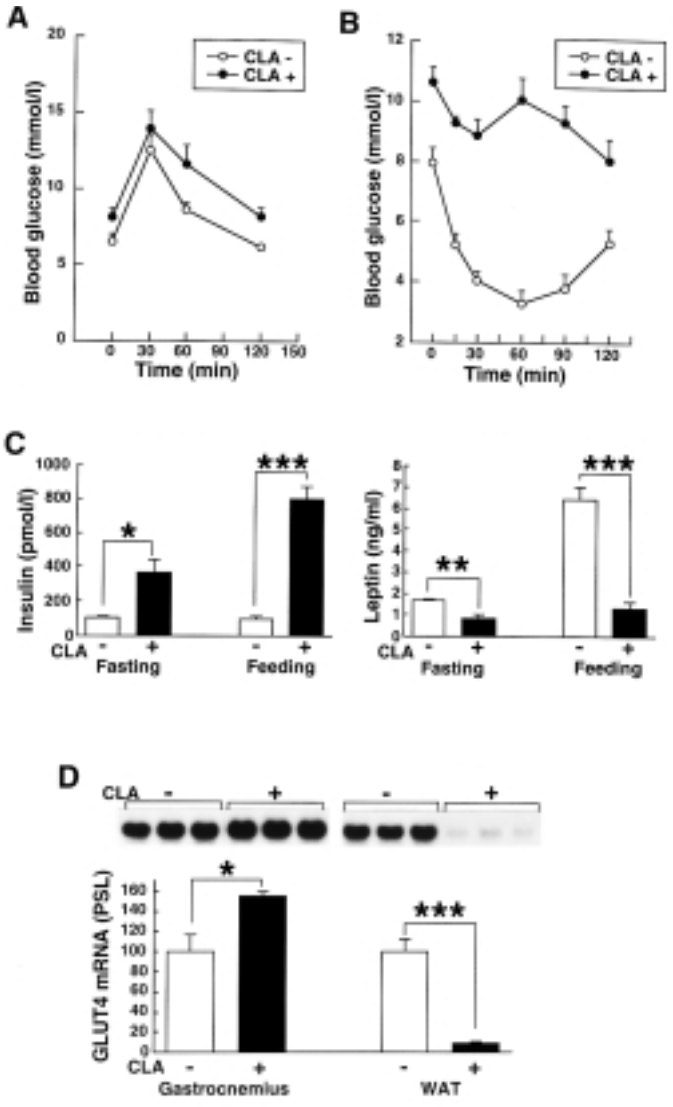

FIG. 5. CLA supplementation induced insulin resistance. *A*: Oral glucose tolerance test results in mice fed diet with or without CLA. Mice were fasted overnight and given D-glucose (1 mg/g body wt) orally by a stomach tube. Blood glucose levels were determined at the times indicated. Each data point represents the mean ± SE for 4–5 mice. P = 0.07, in mice fed a standard semipurified diet with or without CLA, by repeated-measures analysis of variance. *B*: Insulin tolerance test results in mice fed a standard diet with or without CLA. Mice were fed freely and then given 0.75 mU of human insulin per gram of body weight. Blood glucose levels were measured at the indicated time points. Each data point represents the mean ± SE for 4–5 mice. P < 0.0001, in mice fed a standard semipurified diet with or without CLA, by repeated-measures analysis of variance. *C*: Comparison of plasma insulin and leptin levels in mice fed diet with or without CLA. Under fasting and feeding conditions, plasma insulin and leptin was measured at 19 and 15 weeks, respectively. Each data point represents the mean ± SE of 4–5 mice. Statistical differences are shown as *P < 0.05, **P < 0.01, and ***P < 0.001. *D*: Expression of GLUT4 mRNA in gastrocnemius and WAT by CLA supplementation. Gastrocnemius and parametrial WAT in mice fed a standard semipurified diet with or without CLA was used for preparation of total RNA. RNA transferred membrane sheets were probed with ^{32}P-labeled mouse GLUT4 cDNA. These mRNA levels were quantified using an image analyzer. A typical autoradiogram of gastrocnemius (6-h exposure) and WAT (12-h exposure) GLUT4 mRNA and its PSL levels (1-h exposure) are shown. In the autoradiogram, each lane represents a sample from an individual mouse. Each data point represents the mean ± SE of 3–4 mice. Statistical differences are shown as *P < 0.05 and ***P < 0.001.

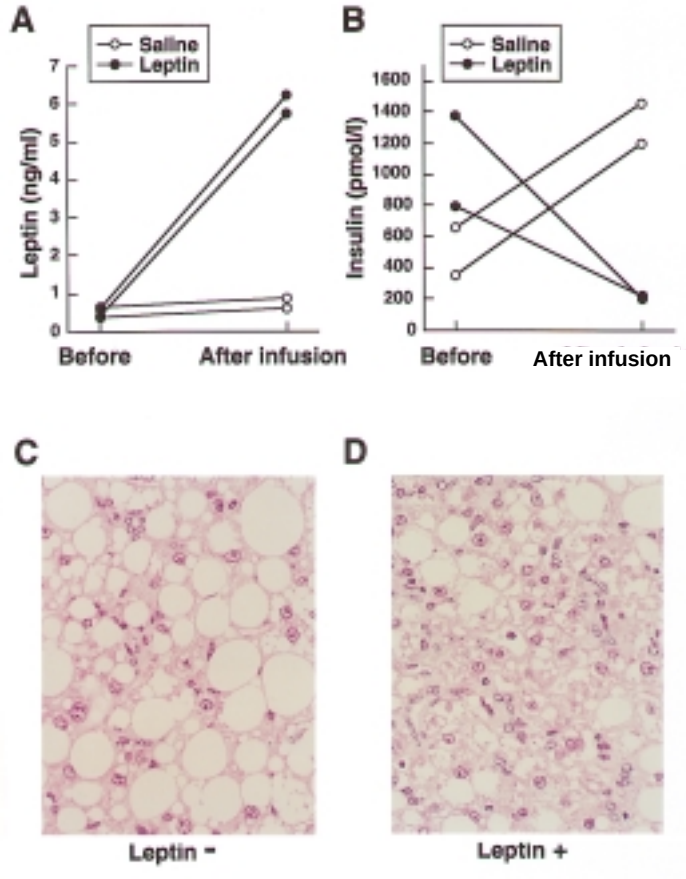

FIG. 6. Metabolic parameters in CLA-fed mice after continuous infusion of recombinant leptin (5 μg/day for 12 days) or saline. On day 12, the mice were killed and blood and liver were removed. Each value denotes an individual mouse for groups of 2 mice. The change in feeding leptin (*A*) and insulin (*B*) concentrations and representative histological sections of liver (hematoxylin and eosin stain) treated with either saline (*C*) or leptin (*D*) are shown. Original magnification ×400.

fat diet (10% of total energy) induced lipodystrophy. Apoptosis in adipocytes by CLA induced a marked increase of TNF-α and may account for the phenotypic characteristics of lipodystrophy, such as a decrease of WAT and BAT mass, lipid accumulation in the liver, and insulin resistance. At present, several mouse models of lipoatrophic diabetes have been established. With use of genetic manipulation targeted to WAT and BAT by using the 5′ regulatory region of the adipocyte P2 (aP2) gene, 3 types of mouse models of lipoatrophic diabetes were made. Ross et al. (30) expressed an attenuated diphtheria toxin A chain in WAT and BAT, Moitra et al. (31) expressed a dominant-negative protein (A-ZIP/F) that prevents DNA binding of B-ZIP transcription factors of both C/EBP and Jun families, and Shimomura et al. (32) expressed the nuclear form of SREBP-1/adipocyte determination and differentiation factor 1. Because all these mice models lack adipose tissue and develop the phenotype of lipodystrophy, adipose tissues themselves are considered to be important in maintaining glucose tolerance. Similar to CLA-fed mice, SREBP-1 overexpressing mice showed a marked elevation of TNF-α in BAT and WAT (29). It will be interesting to determine whether expression levels of TNF-α in adipose tissue increase in other lipoatrophic models of mice and patients.

It has been shown directly that exposure to TNF-α causes apoptosis in human adipocyte cultures (20). In addition, TNF-α inhibits the synthesis of LPL (33), ACC (34), and FAS (34), all of which are involved in fat synthesis. These changes in enzyme activity favor a decrease in fat cell size. In liver, TNF-α stimulated lipogenesis in vivo (35). TNF-α secreted from adipose tissue plays a crucial role in the systemic insulin resistance of type 2 diabetes (36). In addition, obesity and improved insulin sensitivity were demonstrated in mice rendered TNF-α–deficient by a targeted null mutation in the gene encoding TNF-α or that encoding TNF-α receptors (37). All these studies support the idea that increased expression of TNF-α may induce body fat decrease, hepatomegaly, and insulin resistance.

Apoptosis induced by CLA supplementation may also be due to a marked upregulation of UCP2 in WAT and BAT. Because UCP2 is the predominant uncoupling protein under normal conditions in WAT, a 6-fold increase in adipocytes may have a functional role for CLA-mediated increased energy expenditure. Indeed, 2-fold overexpression of UCP2, especially in adipose tissue, using the 422/aP2 promoter resulted in a moderate decrease of fat mass under high-fat diet conditions (N.T.-K., O.E., unpublished data). If UCP2 induction reduced the efficiency of ATP synthesis, a decrease of ATP may lead to global loss of cell membrane integrity and cell death by necrosis and/or apoptosis. Decreased GLUT4 expression that restricts glucose entry into the cells may contribute to a further decrease of ATP. Recently, repeated injection of TNF-α in rats was shown to upregulate UCP2 in BAT and WAT and downregulate UCP1 in BAT (38), indicating that increased expression of TNF-α by CLA supplementation may cause upregulated UCP2 in adipose tissues.

Leptin deficiency by CLA supplementation may also contribute to development of insulin resistance. It has been reported that leptin can enhance insulin-mediated stimulation of glucose disposal (26,27). In addition, Ogawa et al. (39) reported that transgenic skinny mice that showed a marked increase of leptin expression in liver demonstrated increased insulin sensitivity, irrespective of ablation of WAT and BAT. Shimomura et al. (29) reported that insulin resistance in lipoatrophic SREBP-1c–overexpressed mice could be overcome by an infusion of 5 μg leptin per mice. However, infusion of leptin into another lipoatrophic A-ZIP/F-1 mouse model at the same rate as that in the Shimomura study had no effect on serum glucose and insulin concentrations (40). These differences between aP2-SREBP-1c and A-ZIP/F-1 mice are considered to be because of their different amount of fat. A-ZIP/F-1 mice have an almost complete lack of WAT, whereas aP2-SREBP-1c mice have more residual adipose tissues. These data suggest that leptin is not the sole or principal cause of insulin resistance. In CLA-supplemented mouse, leptin infusion reversed CLA-induced hyperinsulinemia and ameliorated liver lipid accumulation (Fig. 6). Because CLA-supplemented mice have residual fat in parametrial and renal regions (Table 1) similar to aP2-SREBP-1c mice, this mouse model of lipoatrophic mice might respond well to leptin treatment. In Fig. 7, possible mechanisms of CLA-induced insulin resistance are presented.

In addition to leptin deficiency, a marked decrease of GLUT4 in adipose tissue may also contribute to insulin resistance. Transgenic mice overexpressing GLUT4 selectively in fat have enhanced glucose disposal in vivo and massively

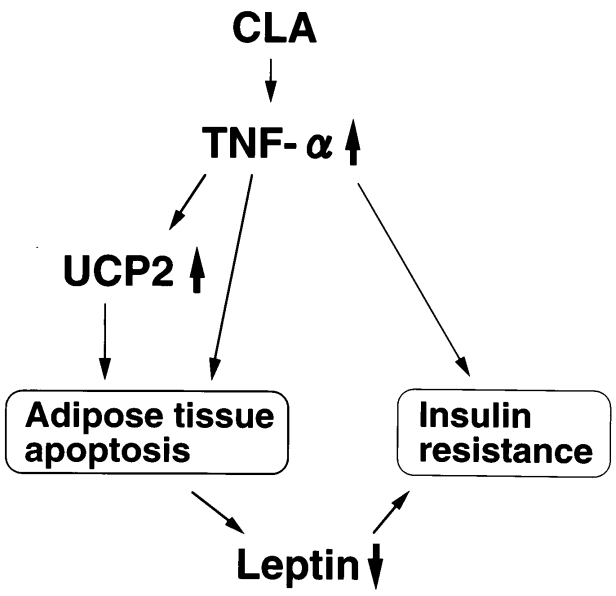

FIG. 7. Possible mechanisms of CLA-induced insulin resistance.

increased glucose transport in adipocytes (41). In addition, this GLUT4 transgene in adipose tissue ameliorated insulin resistance in streptozotocin diabetic mice (42). The decrease of GLUT4 expression by CLA supplementation is also explained by an increase of TNF-α expression. In 3T3-L1 cells, TNF-α was reported to decrease GLUT4 mRNA by transcriptional repression (43).

So why does CLA upregulate TNF-α, which leads to apoptosis in adipose tissue? Unfortunately, we cannot answer this question. CLA may bind unknown transcription factor(s) that stimulate TNF-α expression. CLA-induced apoptosis was also reported in another cell. Ip et al. (44) found that CLA inhibited growth of rat mammary epithelial cell organoids both by a reduction in DNA synthesis and a stimulation of apoptosis. Because upregulation of TNF-α mRNA was also observed in nonadipocytes in adipose tissue, it is possible that some cell types are very sensitive to CLA and that these effects are related to inhibitory effects of CLA on mammary carcinogenesis.

This article reports the first observation that a dietary component causes lipodystrophy and suggests that some agents that decrease fat mass may lead to lipodystrophy. Furthermore, we found that a fat mass decrease from CLA supplementation is due to apoptosis and that leptin treatment can reverse CLA-induced lipodystrophy.

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