

Maintenance of adiponectin attenuates insulin resistance induced by dietary conjugated linoleic acid in mice

Aparna Purushotham, Angela A. Wendel,¹ Li-Fen Liu,¹ and Martha A. Belury²

Department of Human Nutrition, Ohio State University, Columbus, OH 43210

Abstract Conjugated linoleic acid (CLA) causes insulin resistance and hepatic steatosis in conjunction with depletion of adipokines in some rodent models. Our objective was to determine whether the maintenance of adipokines, mainly leptin and adiponectin, by either removing CLA from diets or using an adiponectin enhancer, rosiglitazone (ROSI), could attenuate CLA-induced insulin resistance. Male C57BL/6 mice were consecutively fed two experimental diets containing 1.5% CLA mixed isomer for 4 weeks followed by a diet without CLA for 4 weeks. CLA significantly depleted adiponectin but not leptin and was accompanied by hepatic steatosis and insulin resistance. These effects were attenuated after switching mice to the diet without CLA along with restoration of adiponectin. To further elucidate the role of adiponectin in CLA-mediated insulin resistance, ROSI was used in a subsequent study in male *ob/ob* mice fed either control (CON) or CLA diet. ROSI maintained significantly higher adiponectin levels in CON- and CLA-fed mice and prevented the depletion of epididymal adipose tissue and the development of insulin resistance. In conclusion, we show that insulin resistance induced by CLA may be related more to adiponectin depletion than to leptin and that maintaining adiponectin levels alone either by removing CLA or using ROSI can attenuate these effects.—Purushotham, A., A. A. Wendel, L-F. Liu, and M. A. Belury. Maintenance of adiponectin attenuates insulin resistance induced by dietary conjugated linoleic acid in mice. *J. Lipid Res.* 2007. 48: 444–452.

Supplementary key words hepatic steatosis • adipokines • hepatic steatosis • rosiglitazone

Type 2 diabetes is characterized by impaired glucose and lipid metabolism and is associated with obesity (1). Adipose tissue not only stores excess energy but also has important endocrine functions. Proteins secreted from the adipose tissue, known as adipokines, have important functions in regulating whole body metabolism (2). In particular, the adipokine adiponectin was identified in the adipose tissue and plasma of humans and rodents (3–6) and is inversely associated with obesity and type 2 diabetes

(7, 8). Administration of adiponectin attenuates insulin resistance by decreasing tissue triglyceride (TG) levels as a result of increased fatty acid oxidation (9, 10). In addition, adiponectin decreases hyperglycemia by suppressing hepatic glucose production along with increasing glucose uptake by the skeletal muscle (8, 9).

Conjugated linoleic acid (CLA) consists of positional and geometric isomers of octadecadienoate that are naturally found in foods such as beef, lamb, milk, and other dairy products (11). It is well established that *cis*9,*trans*11 (*c9t11*)-CLA and *trans* 10,*cis* 12 (*t10c12*-CLA) have unique effects on lipid metabolism (12), and it is the *t10c12*-CLA isomer that is mainly associated with decreases in body fat in experimental rodent models (13–16) as well as in some but not all human studies (17–19), independently of energy intake (20).

Feeding CLA to mice is associated with lipodystrophy and worsening of insulin sensitivity (16, 21–24). These effects have been attributed to a rapid and significant reduction of adipose tissue and a sharp decline in insulin-sensitizing adipokines such as adiponectin and leptin (23). Although feeding dietary CLA has been shown previously to deplete adipokines and cause hyperinsulinemia (23), it is unknown whether removing CLA from the diet can restore the level of adipokines and attenuate insulin resistance, showing that there is, in fact, a causal link between adipokine depletion by feeding dietary CLA and the development of hepatic steatosis and insulin resistance in mice. Furthermore, dietary CLA has been shown to induce insulin resistance in *ob/ob* mice, which lack functional leptin (16), raising the possibility that adipokines other than leptin may be more important in CLA-mediated insulin resistance in mice. Thus, we further investigated the role of adiponectin in insulin resistance mediated by CLA in leptin-deficient *ob/ob* mice. To this end, we used the peroxisome proliferator-activated receptor γ (PPAR γ) agonist rosiglitazone (ROSI) in conjunction with CLA and

Abbreviations: AOX, acetyl-coenzyme A oxidase; CLA, conjugated linoleic acid; *c9t11*, *cis*9,*trans*11; CON, control; FBG, fasting blood glucose; IL-6, interleukin-6; PPAR γ , peroxisome proliferator-activated receptor γ ; ROSI, rosiglitazone; TG, triglyceride; TNF- α , tumor necrosis factor- α .

¹A. A. Wendel and L-F. Liu contributed equally to this work.

²To whom correspondence should be addressed.

e-mail: belury.1@osu.edu

Manuscript received 31 August 2006 and in revised form 3 October 2006 and in re-revised form 5 October 2006.

Published, JLR Papers in Press, October 18, 2006.
DOI 10.1194/jlr.M600393-JLR200

hypothesized that maintaining serum adiponectin in *ob/ob* mice, which lack functional leptin, attenuates the effects of CLA on insulin resistance and hyperglycemia.

RESEARCH DESIGN AND METHODS

Materials

Diet components were purchased from Research Diets (New Brunswick, NJ) and Bio-Serv (Frenchtown, NJ) for studies 1 and 2, respectively. CLA-mixed TGs (39.2% *c9t11*-CLA and 38.5% *t10c12*-CLA) were obtained from Cognis (Cincinnati, OH). ROSI was obtained from Cayman Chemical (Ann Arbor, MI).

Animals

Eleven week old male C57BL/6 mice and 6 week old male *ob/ob* mice were purchased from Harlan (Indianapolis, IN) and Charles River Laboratories, Inc. (Wilmington, MA), respectively. Mice were housed four per cage at $22 \pm 0.5^\circ\text{C}$ on a 12 h day/night cycle. Mice received standard chow for 1 week while adjusting to their new environment. All procedures were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of Ohio State University.

Study 1: depletion-repletion of adipokines

To determine the role of adipokine depletion by CLA in the development of insulin resistance and hepatic steatosis, 12 week old male C57BL/6 mice ($n = 10$) were consecutively fed two different experimental diets. For the first 4 weeks, mice were fed a 1.5% CLA experimental diet, which contained 5% soybean oil plus 1.5% CLA-TG mix by weight, for a total of 6.5% fat. This dose of CLA provided $\sim 0.6\%$ (by weight) each of the *c9t11*-CLA and *t10c12*-CLA isomers. Supplementation with either 0.5% purified *t10c12*-CLA isomer or 1% CLA mixture has been shown to effectively reduce adipose tissue in mice and produce liver steatosis (15, 23, 24). At the end of the first 4 week diet period, half of the mice were euthanized, and the remaining mice were switched to a diet without CLA (chow diet containing 4–5% total fat). All mice had free access to food and water. Body weights were measured at the indicated time points.

Study 2: sustenance of adiponectin

Six week old male *ob/ob* mice were randomized by body weight and fed experimental diets containing 6.5% total fat for 4 weeks. The diets contained 6.5% soybean oil [control (CON) diet; $n = 8$] by weight. Additionally, 10 mice were maintained on the CON diet for the first 2 weeks, after which 6 mice were switched to the CLA diet and 4 mice were continued on the CON diet for the last 2 weeks of the study period. During the last 2 weeks, mice received daily intraperitoneal injections of PBS (vehicle control: 10% DMSO and 90% PBS solution) or 10 mg/kg body weight/day ROSI (25, 26). Body weights were measured weekly.

Insulin tolerance test and fasting blood glucose

Insulin sensitivity was determined for studies 1 and 2 using an insulin tolerance test at the indicated times. Mice were fasted overnight and received intraperitoneal injections of insulin [Humulin R (Eli Lilly, Inc.)] at doses of 0.75 U/kg body weight for C57BL/6 mice and 1.5 U/kg body weight for *ob/ob* mice. Insulin-stimulated glucose clearance was determined by tail vein bleeding at 0, 15, 30, 45, 60, 90, and 120 min after insulin injection. Insulin sensitivity was determined by calculating the areas under the curves, and individual baselines were used to normalize

data. Fasting blood glucose (FBG) levels were measured at baseline, 2 weeks, and 4 weeks for study 2. Mice were fasted overnight for 12 h, and tail vein blood was used to analyze FBG using a One Touch Basic glucose analyzer (Lifescan, Milpitas, CA).

Necropsy (studies 1 and 2)

To avoid the effects of injected insulin on gene expression, mice were anesthetized by isoflurane in the fasted state 2 days after the insulin tolerance test. Blood was collected by heart puncture, centrifuged at 1,500 *g* at 4°C to isolate serum, and stored at -80°C for hormone and metabolite analyses. Liver, epididymal adipose, and gastrocnemius muscle tissues were weighed, snap-frozen in liquid nitrogen, and stored at -80°C for further analysis.

Serum hormone and metabolite determination

Time course depletion-repletion of adipokines from study 1 was determined using 4 h fasted serum from retro-orbital eye bleeds at the indicated time points. Mice were anesthetized using isoflurane. Additionally, fasted serum insulin, adiponectin, and resistin levels from study 2 were determined using ELISAs (LINCO Research, St. Charles, MO). Fasting serum TGs and NEFAs from study 2 were measured using spectrophotometric assays from Sigma (St. Louis, MO) and Wako Chemicals (Richmond, VA), respectively.

Tissue TG analysis (studies 1 and 2)

Liver and muscle tissues were homogenized and lysed in $10\times$ Tris (v/v) buffer containing 20 mM Trizma base, 1% Triton X-100, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, and protease inhibitors. TGs were extracted with 2:1 (v/v) chloroform-methanol, final extracts were dissolved in 3:1:1 (v/v/v) tert-butanol-methanol-Triton X-100 (27), and TGs were quantitatively measured with an enzymatic colorimetric kit (Sigma). Values are expressed as percentage tissue weight.

Real-time RT-PCR (study 2)

Sections from liver and muscle tissue were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA), and RNA was isolated using the manufacturer's protocol. RNA from adipose tissue was isolated using the RNeasy lipid extraction kit (Qiagen, Valencia, CA). RNA was diluted in RNase-free water and quantified by spectrophotometry. RNA integrity was assessed by electrophoresis using agarose gel and ethidium bromide staining. The first transcripts were reverse-transcribed using reverse transcriptase (Invitrogen), and cDNA was amplified using real-time PCR with FAM-labeled TaqMan gene expression assays (Applied Biosystems, Foster City, CA). In short, 5 ng of the reverse transcription reaction was amplified in a total reaction volume of 25 μl using pre-designed and validated primers for liver fatty acid synthase (FAS), the fatty acid transporter CD36, acetyl-coenzyme A oxidase (AOX), and tumor necrosis factor- α (TNF- α) using universal cycling conditions. Target gene expression was normalized to Vic-labeled 18S, which was used as an endogenous control and amplified in the same reaction as the target gene.

Statistical analysis

All data are presented as means \pm SEM. Data were analyzed using MINITAB (version 14). Data from study 2 were analyzed by one-way ANOVA. Posthoc analysis was performed using Tukey's test. Other comparisons were analyzed by Student's *t*-test as appropriate. Weight gain and serum adipokine concentrations over time were analyzed by repeated-measures ANOVA using SAS (version 9.1; Cary, NC). Differences were considered significant at $P < 0.05$.

TABLE 1. Body weights and organ weights, study 1

Variable	Baseline	+CLA	-CLA
Body weight (g)	28.08 ± 0.73	27.211 ± 0.90 ^a	30.33 ± 1.60 ^b
Liver weight (% body weight)	—	7.56 ± 0.83	4.28 ± 0.08 ^b
Epididymal adipose (g)	—	0.233 ± 0.03	0.38 ± 0.01 ^b
Insulin-stimulated glucose uptake (area under the curve)	-6272.14 ± 793.51	-1,864.69 ± 581.99 ^a	-4,735.00 ± 514.13 ^b
Liver TG (% liver weight)	—	12.4089 ± 0.4968	2.8534 ± 1.1637 ^b

CLA, conjugated linoleic acid; TG, triglyceride. Body and organ weights of male C57BL/6 mice at baseline (n = 10), after 4 weeks of feeding diet with (+) CLA (n = 5), and after an additional 4 weeks of feeding diet without (-) CLA (n = 5). Values represent means ± SEM. Superscripts represent significant differences between treatments.

^a P < 0.05 versus baseline.

^b P < 0.05 versus +CLA.

RESULTS

Study 1

Body weights and organ weights. Body weights and weight gain were reduced significantly in C57BL/6 mice after 4 weeks of supplementation with dietary CLA (Table 1, Fig. 1A). Supplementation with dietary CLA significantly decreased epididymal adipose mass and increased liver weight. When CLA was removed from the diets, body weight and adipose tissue mass significantly increased (Table 1). Concomitant with increased body weight and adipose tissue weight, liver weights decreased significantly after 4 weeks on the diet without CLA (Table 1).

Serum metabolites. Levels of adiponectin decreased over time in C57BL/6 mice on the diet containing CLA. Significant differences were observed at day 6, and adiponectin levels continued to decrease over time (Fig. 2B). Switching mice to the diet without CLA significantly increased adiponectin levels; however, levels remained significantly lower (50% of baseline) than in C57BL/6 mice. In contrast, leptin levels were less responsive to dietary CLA and were not significantly different compared with baseline (Fig. 2A).

Insulin tolerance. Insulin sensitivity was significantly worsened in C57BL/6 mice after 4 weeks on the CLA diet (area under the curve) (Table 1, Fig. 3A). Switching mice to the diet without CLA significantly improved insulin sensitivity. The improvement in insulin sensitivity was significant at 2 weeks after the switch to the diet without CLA.

Liver and muscle TG. Corresponding to increased liver weights, 4 weeks of feeding dietary CLA significantly increased hepatic TG in C57BL/6 mice. Switching to the diet without CLA for 4 weeks significantly attenuated hepatic steatosis (Table 1). Muscle TG levels were not significantly different between the two diet groups (data not shown).

Study 2

Body weights and organ weights. ROSI administration for 2 weeks prevented weight loss in male *ob/ob* mice fed dietary CLA, and weight gain was comparable to that in

CON-PBS mice in both ROSI-treated groups (Fig. 1B). Epididymal adipose mass was also not significantly different in the CLA-ROSI group compared with the CON-ROSI and CON-PBS groups (Table 2).

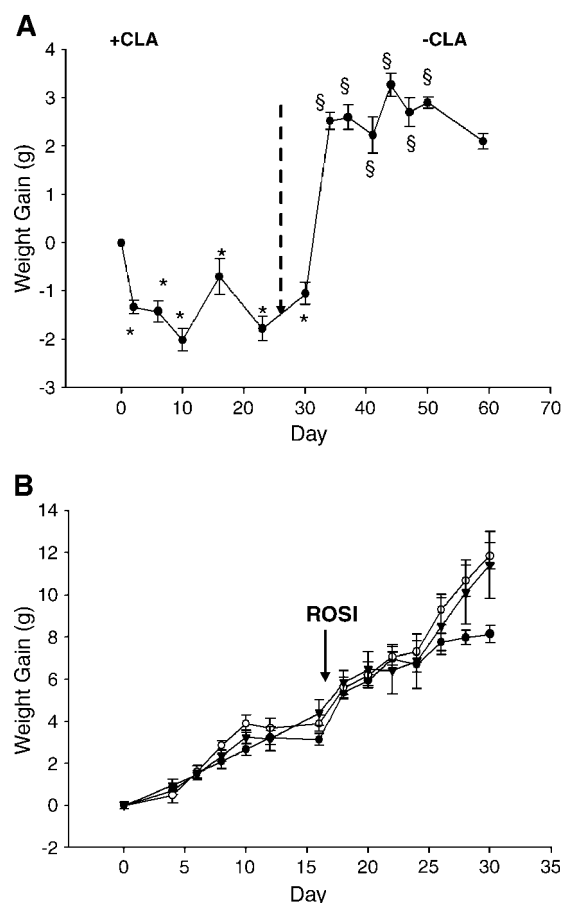


Fig. 1. Effect of dietary conjugated linoleic acid (CLA) on weight gain from studies 1 and 2. A: Male C57BL/6 mice were fed a diet containing 1.5% CLA (+CLA; n = 10) for 4 weeks followed by 4 weeks without CLA (-CLA; n = 5). * P < 0.05 versus baseline; § P < 0.05 versus the last time point on the diet containing CLA. B: Male *ob/ob* mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either PBS or rosiglitazone (ROSI) for 2 weeks by intraperitoneal injection daily. Closed circles, CON-PBS (n = 8); open circles, CON-ROSI (n = 4); closed triangles, CLA-ROSI (n = 6). Data shown are means ± SEM.

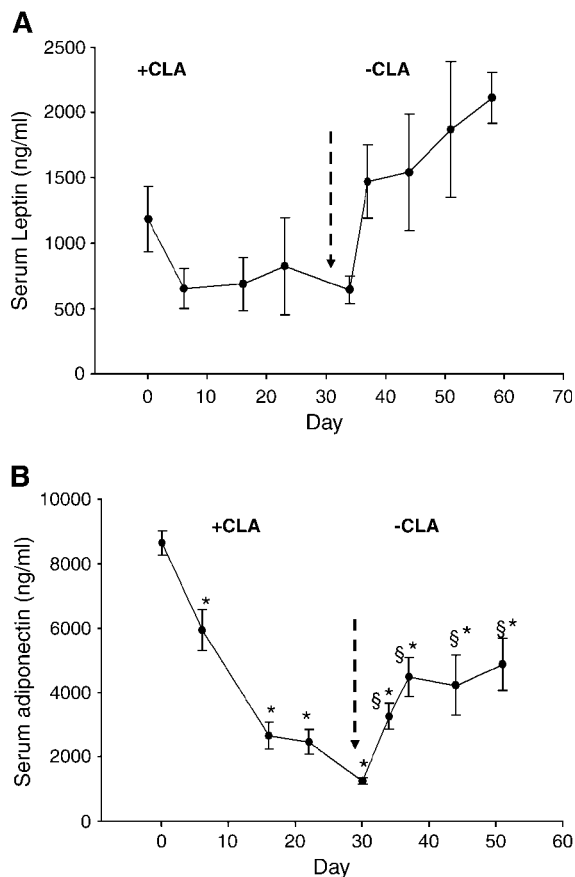


Fig. 2. Effect of CLA on time-course depletion/repletion of adipokines from study 1. Male C57BL/6 mice were fed a diet containing 1.5% CLA (+CLA; $n = 10$) for 4 weeks followed by 4 weeks without CLA (-CLA; $n = 5$). A: Serum leptin in male C57BL/6 mice. B: Serum adiponectin in male C57BL/6 mice. Adipokine concentrations were determined at the indicated times. * $P < 0.05$ versus baseline; [§] $P < 0.05$ versus the last time point on the diet containing CLA. Data shown are means \pm SEM.

Serum metabolites. Two weeks of treatment with ROSI not only prevented increases in both glucose and insulin in mice fed dietary CLA (CLA-ROSI vs. CON-ROSI and CON-PBS) but also significantly decreased FBG levels compared with CON-PBS mice (Table 2). ROSI administration significantly increased adiponectin levels in mice fed the CON or CLA diet compared with mice fed the CON-PBS diet (Table 2). Serum resistin levels were significantly higher in CON-PBS mice compared with CON-ROSI mice; however, ROSI administration did not have an effect on resistin levels in mice fed CLA. Furthermore, ROSI administration significantly decreased serum levels of TG and NEFA in both CON- and CLA-fed mice compared with the CON-PBS group (Table 2).

Insulin tolerance. Compared with the CON-PBS group, ROSI significantly improved insulin sensitivity in both CON- and CLA-fed *ob/ob* mice, and CLA-ROSI-fed mice had insulin sensitivity comparable to CON-ROSI mice (area under the curve) (Table 2, Fig. 3B).

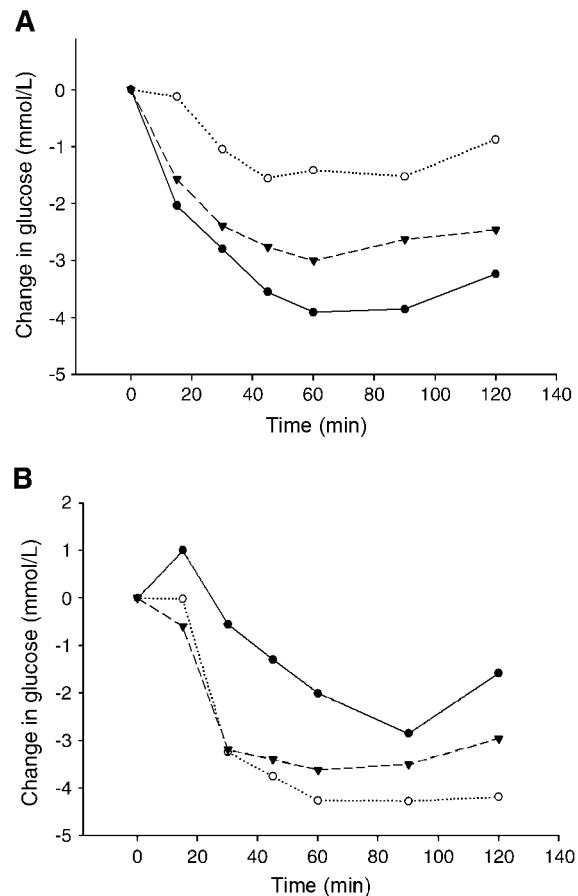


Fig. 3. Effect of dietary CLA on insulin sensitivity. A: Male C57BL/6 mice from study 1. Insulin sensitivity was measured at baseline ($n = 10$; closed circles), after 4 weeks on dietary CLA (+CLA; $n = 10$; open circles), and after 2 weeks on the diet without CLA (-CLA; $n = 5$; closed triangles). B: Male *ob/ob* mice from study 2. Insulin sensitivity was measured after 2 weeks on either CON or CLA-supplemented diets and injected with PBS or ROSI. Closed circles, CON-PBS ($n = 8$); open circles, CON-ROSI ($n = 4$); closed triangles, CLA-ROSI ($n = 6$). Significance was determined using area under the curve (Tables 1, 2).

Liver and muscle TG. Although ROSI treatment did not decrease hepatic lipids in CON-fed mice, interestingly, there was a significant reduction in liver TG in the CLA-ROSI group compared with the CON-PBS and CON-ROSI groups after 2 weeks of ROSI treatment (Table 2). There was no effect of diet or treatment on muscle TG in *ob/ob* mice (data not shown).

Liver mRNA expression. Because the combination of CLA with ROSI led to significantly lower liver TG, we measured mRNA levels of genes indicative of lipid oxidation and lipid synthesis in the liver. ROSI treatment significantly increased mRNA levels of liver AOX in both CON- and CLA-fed mice compared with CON-PBS mice (Fig. 4A). There were no significant differences in the mRNA levels of PPAR α and carnitine palmitoyl transferase between the groups (data not shown). Although ROSI treatment significantly increased mRNA levels of FAS in the CON-fed mice, interestingly, the combination of CLA with ROSI led

TABLE 2. Body weights, organ weights, and serum metabolites, study 2

Variable	CON-PBS	CON-ROSI	CLA-ROSI
Final body weight (g)	42.20 ± 1.5	47.58 ± 2.12	46.5 ± 1.73
Liver weight (% body weight)	6.50 ± 0.35 ^a	6.91 ± 0.49 ^{a,b}	8.35 ± 0.41 ^b
Epididymal adipose (g)	2.96 ± 0.18	3.00 ± 0.27	3.15 ± 0.22
Serum insulin (pg/ml)	3,099.64 ± 440.20 ^a	2,180.24 ± 525.05 ^{a,b}	1,015.95 ± 80.95 ^b
Resistin (ng/ml)	22.14 ± 1.02 ^a	14.72 ± 1.35 ^b	20.31 ± 1.54 ^{a,b}
Adiponectin (ng/ml)	10,751.00 ± 1530.2 ^a	55,996.00 ± 1459.0 ^b	56,023 ± 1349.6 ^b
Fasting blood glucose (mmol/l)	6.82 ± 0.66 ^a	3.93 ± 0.98 ^b	4.70 ± 0.80 ^b
Insulin-stimulated glucose uptake (area under the curve)	-1,735 ± 1088.03 ^a	-6,760.00 ± 962.85 ^b	-5,576.25 ± 920.97 ^b
Serum TG (mg/dl)	88.13 ± 11.85 ^a	33.89 ± 3.89 ^b	44.27 ± 8.84 ^b
NEFA (mEq/l)	0.90 ± 0.09 ^a	0.40 ± 0.12 ^b	0.51 ± 0.09 ^b
Liver TG (% liver weight)	18.1527 ± 1.9909 ^a	18.2921 ± 2.3035 ^a	10.2850 ± 0.5803 ^b

ROSI, rosiglitazone. Male *ob/ob* mice were fed experimental diets containing 6.5% soybean oil (CON diet; n = 8) for 4 weeks. Additionally, 10 mice were maintained on the CON diet for the first 2 weeks, after which 6 mice were switched to the CLA diet containing 5% soybean oil plus 1.5% CLA-mixed TG and 4 mice were continued on the CON diet for the last 2 weeks of the study period and injected with either PBS (CON-PBS) or ROSI (CON-ROSI and CLA-ROSI). Values represent means ± SEM. Superscripts represent significant differences between treatments. Differences between means were calculated using one-way ANOVA, and values were considered significant at $P < 0.05$.

to significantly lower levels of FAS mRNA comparable to CON-PBS mice (Fig. 4B).

Muscle mRNA expression. Although muscle lipid content did not differ between diet groups, we measured mRNA levels of the fatty acid transporter CD36 and AOX, which have been shown previously to be upregulated by adiponectin and have important functions in lipid metabolism (10). ROSI treatment significantly increased CD36 and AOX mRNA levels in mice fed dietary CLA (Fig. 4C, D) compared with mice fed the CON diet.

Adipose mRNA expression. ROSI administration significantly decreased TNF- α mRNA levels in both CON- and CLA-fed mice compared with PBS-injected mice fed the CON diet (Fig. 4E).

DISCUSSION

The effects of CLA on insulin sensitivity are controversial and vary depending on the species and level of dietary fat. Preliminary data from male and female *ob/ob* mice showed that feeding dietary CLA (1.5% mixed isomer) as part of a 6.5% total fat diet for 4 weeks resulted in a significant decrease in body weight gain and adiposity along with an increase in hepatic steatosis (data not shown). The decrease in body weight measurements was significant by day 7 in mice fed dietary CLA. Along with the decrease in weight gain, dietary CLA feeding resulted in a significant decrease in serum adiponectin and an increase in FBG levels by 2 weeks (data not shown). Serum levels of resistin were not increased by dietary CLA in *ob/ob* mice after 4 weeks. Furthermore, adipose mRNA levels of inflammatory cytokines [e.g., TNF- α and interleukin-6 (IL-6)] were also not significantly different between the CON and CLA groups after 4 weeks (data not shown).

In this study, we show that CLA causes rapid changes in weight and adiposity in mice. The depletion in adipose tissue is accompanied by worsened insulin sensitivity and

the development of hepatomegaly (likely attributable, in part, to increased hepatic lipids). These results are in accordance with other studies using both mixed isomers of CLA and the $\Delta 10c12$ isomer alone (16, 21–24). Previously, it was shown that CLA causes a time course depletion of adiponectin and leptin and is associated with the development of insulin resistance in female C57BL/6J mice (23). Here, we show for the first time that removing CLA from the diet results in the reversal of depressed adipokines, insulin resistance, and hepatic steatosis.

In study 1, the level of adiponectin was depleted significantly at 6 days in male C57BL/6 mice supplemented with dietary CLA. These results are in accordance with a study conducted by Poirier et al. (23). However, in contrast to their study, in this study, using comparable levels of dietary CLA, leptin levels were not significantly lower than baseline. These differences may be attributable to differences in gender in the two studies. Associated with the depletion of adiponectin, there was a significant decrease in the phosphorylation of key mediators of glucose and lipid metabolism pathways, such as AMP-activated protein kinase and acetyl-CoA carboxylase, in the livers of C57BL/6 mice, and levels of these phosphoproteins were restored by removing CLA from the diet (data not shown). The observed decrease in phosphorylation state of AMP-activated protein kinase and acetyl-CoA carboxylase may be indicative of decreased lipid oxidation in the liver and impaired insulin responsiveness (9, 28–30). Switching mice to the diet without CLA resulted in a significant improvement in serum adiponectin levels as early as 7 days. Of interest here, although insulin resistance and hepatic TG were significantly attenuated when CLA was removed from the diet, adiponectin levels were only partially restored, suggesting that the presence of leptin may compensate for insufficient adiponectin levels and that the overall adipokine status may influence the response to dietary CLA in different animal models. The concomitant repletion of adipokines and reversal of insulin resistance by removing CLA from the diets is novel and further supports a strong association between the dysregulation of adipokine syn-

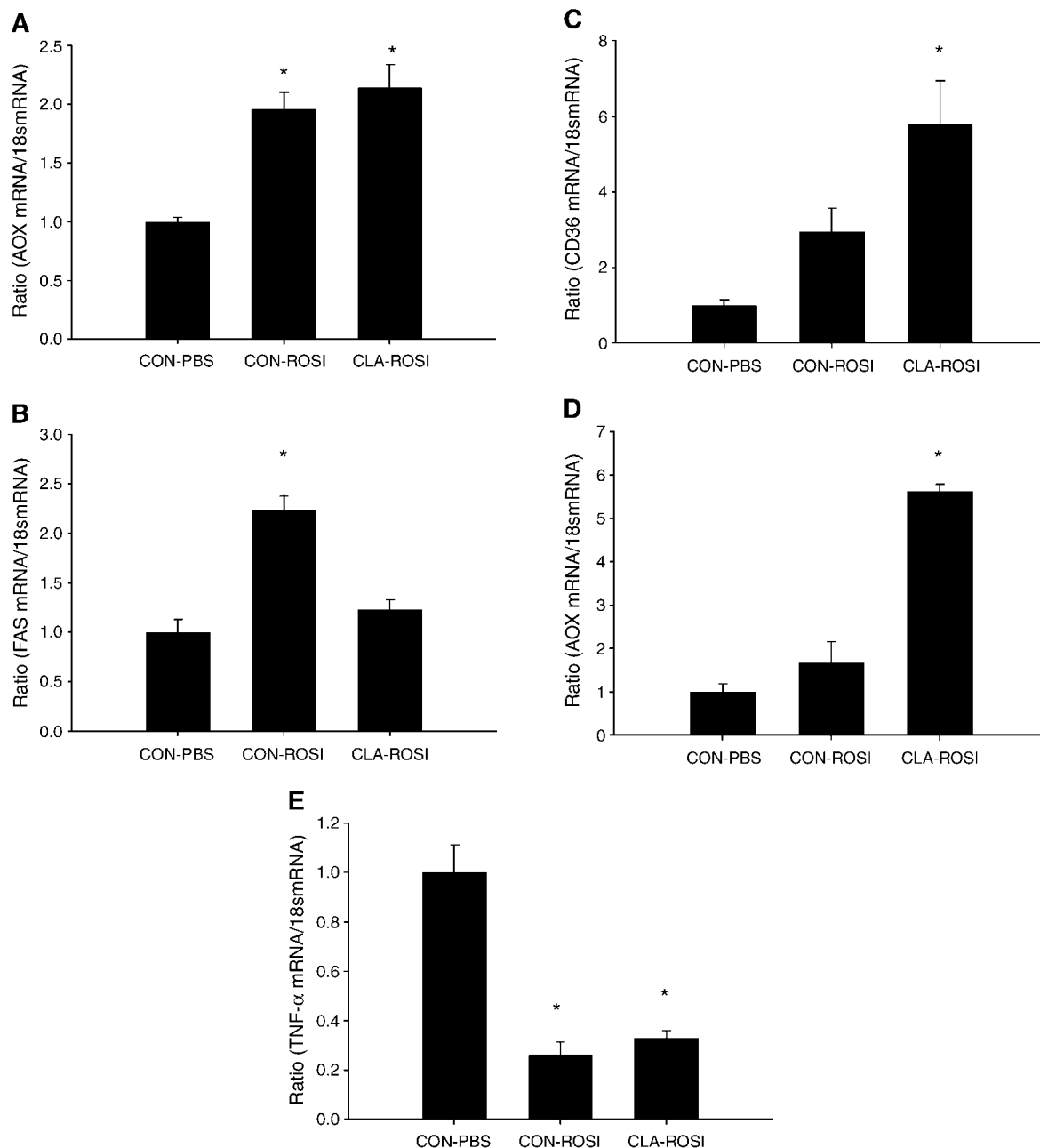


Fig. 4. Effect of CLA on mRNA expression of genes involved in lipid metabolism from study 2. Male *ob/ob* mice were fed either CON or CLA-supplemented diets and received either PBS or ROSI for 2 weeks by intraperitoneal injection daily. A: CON-PBS (n = 8), CON-ROSI (n = 4), and CLA-ROSI (n = 6) hepatic acetyl-coenzyme A oxidase (AOX) mRNA. B: Hepatic FAS mRNA. C: Muscle CD36. D: Muscle AOX. E: Epididymal adipose tumor necrosis factor- α (TNF- α). Vertical bars represent means \pm SEM. * $P < 0.05$.

thesis by dietary CLA and the worsening of insulin sensitivity in mice.

Previously, it was demonstrated that leptin infusion into C57BL/6 mice reverses insulin resistance and hepatic steatosis when adipose stores are not completely ablated (21). However, leptin has no effect when infused into A-ZIP/F-1 fatless mice (31). These data suggest that leptin insufficiency alone is not the principal cause of lipodystrophy-associated insulin resistance caused by CLA (31). The effects of CLA on insulin resistance and hepatic steatosis have been demonstrated previously in *ob/ob* mice by

Roche et al. (16). Preliminary findings from our study are in agreement with the study conducted by Roche et al. (16). In addition, we measured serum adiponectin levels over time in female *ob/ob* mice fed CLA and found that levels were significantly and maximally depressed by 2 weeks (data not shown). Furthermore, mice developed significant hepatic steatosis and insulin resistance along with the depletion of adiponectin. Because *ob/ob* mice lack functional leptin, these data in conjunction with our data from study 1 suggest that the worsening of insulin sensitivity caused by dietary CLA may be more strongly associated with the

depletion of adiponectin or overall adipokine status than leptin alone. To further examine the role of adiponectin in CLA-induced insulin resistance, we injected leptin-deficient male *ob/ob* mice with an adiponectin enhancer, ROSI, for 2 weeks.

ROSI treatment significantly increased serum concentrations of adiponectin in mice fed either the CON or CLA diet compared with CON-PBS-treated mice. These effects of ROSI on serum adiponectin are consistent with other studies (10, 32, 33). Because higher circulating levels of serum resistin are often associated with insulin resistance and diabetes in rodents (32), we measured serum levels of resistin. Preliminary data from male *ob/ob* mice fed dietary CLA for 4 weeks did not show significantly increased resistin levels compared with CON-PBS mice (data not shown). Furthermore, ROSI treatment did not have an effect on serum resistin levels in *ob/ob* mice fed dietary CLA. The lack of effect of ROSI on serum resistin levels in CLA-fed mice further demonstrates that CLA-mediated insulin resistance is related more to the depletion of adiponectin. The increase in serum adiponectin in the CLA-fed mice was accompanied by significantly lower levels of fasting glucose, insulin, insulin sensitivity, serum TG, and NEFA, similar to those in CON-ROSI-treated mice. Cotreatment of CLA with ROSI for 2 weeks prevented lipodystrophy and the associated increases in serum levels of glucose and insulin that are usually associated with the supplementation of dietary CLA in mice (21–24). Additionally, ROSI treatment also prevented significant body weight loss, which is often reported with treatment of CLA in mice. This may be attributable to the maintenance of adipose mass, as ROSI, a PPAR γ agonist, often increases adipose mass (25, 26). These data suggest that when adipose mass and adiponectin levels are maintained, the lipodystrophic effects associated with dietary CLA supplementation are attenuated.


In this study, increased adiponectin levels were associated with higher levels of hepatic AOX mRNA in CON- and CLA-fed mice, but only the CLA-ROSI group had significantly lower hepatic FAS mRNA levels and hepatic TG. To our knowledge, the interactive effect of the combination of CLA and ROSI on hepatic TG is novel and suggests a complementary effect of these two agents for restoring normal lipid levels in the liver. Furthermore, there were significant increases in the levels of the lipid transporter CD36 and the lipid oxidative enzyme AOX in the muscle of the CLA-ROSI group. Administration of adiponectin has similar effects on CD36 and AOX mRNA in mice fed high-fat diets (10), suggesting that at least part of the effects of ROSI are mediated through adiponectin. Although it is unclear why the combination of CLA with ROSI has additional effects in liver and muscle, it may be attributable to increased adiponectin levels with ROSI administration. It is possible that in the presence of adequate adiponectin, CLA increases lipid utilization and decreases lipid accumulation in tissues, similar to observations from rat studies (33–35).

It has been shown previously that supplementation with dietary CLA significantly decreases adipokines and in-

duces hyperinsulinemia in female C57BL/6J mice by 6 days (23). In this study, ROSI was administered for 2 weeks. In this short-term treatment, ROSI prevented CLA-induced insulin resistance by maintaining adequate adiponectin levels in *ob/ob* mice. Although we cannot speculate on the effects of ROSI if treatment had been prolonged in this model, these results are in accordance with a recent study conducted in C57BL/6 mice (M. A. Belury, unpublished data) that were administered the combination of CLA-ROSI for 6 weeks.

Because other adipokines secreted from the adipose tissue, such as TNF- α , IL-6, and resistin, are known to modulate insulin sensitivity in addition to leptin and adiponectin, the overall adipokine status and not any particular adipokine alone may influence CLA's effects on insulin resistance and hepatic steatosis. It was recently shown that short-term *l10c12*-CLA administration induced adipose IL-6 and TNF- α mRNA without affecting serum levels (36). In contrast to these findings, preliminary data from male *ob/ob* mice fed mixed isomers of CLA for 4 weeks did not show significantly higher mRNA levels of TNF- α and IL-6 compared with CON mice (data not shown). Thus, whereas in this study, adiponectin alone seems to be more important than other adipokines in insulin resistance mediated by CLA, future studies using adiponectin-deficient mice are necessary to make conclusions regarding the relative importance of this adipokine. ROSI treatment significantly decreased adipose tissue TNF- α mRNA levels in both CON- and CLA-fed mice compared with CON-PBS-treated mice. These data are in accordance with previous reports (37, 38). Because ROSI is a thiazolidinedione known to improve insulin sensitivity, possibly through multiple pathways, the increased synthesis of adiponectin is not the only possible explanation for our findings. In fact, ROSI may have additional effects in the adipose tissue and may decrease insulin resistance mediated by CLA by directly or indirectly modulating inflammatory cytokines such as TNF- α and IL-6.

In conclusion, we show that removing CLA from the diet of mice reverses insulin resistance and restores adiponectin, establishing a link between depletion of this adipokine and the development of hepatic steatosis and insulin resistance by dietary CLA in mice. We further show that in the absence of significant changes in leptin levels (male C57BL/6 mice fed dietary CLA) or in mice lacking functional leptin (male *ob/ob* mice), adiponectin depletion alone results in worsened insulin sensitivity by CLA. In addition, restoration of adiponectin (by either removal of CLA from the diet or administration of ROSI) is sufficient for the reversal of these effects. Thus, in this study, we show that adiponectin is an important factor responsible for insulin resistance caused by CLA provided as mixed isomer oil at 1.5% of the diet. We based our dose (1.5% CLA equaling approximately 0.6% *l10c12*-CLA) on studies by others showing this dose to be effective for adipose suppression in mice. We observed that C57BL/6 mice weighing an average \sim 30 g, ate \sim 2.0 g diet per day (and *ob/ob* mice weighing \sim 45 g, consumed \sim 5.0 g diet per day). If this were translated to a human of \sim 55 kg, our

dose of CLA could equal ~44 g CLA (or 21.2 g of t10-CLA). With this estimate, it seems an unrealistic choice for humans to use and achieve such a rapid loss of body fat with foods at this time. However, there are other factors to consider between species (mice versus humans) including rate of metabolism of CLA isomers, rapidity of adipose tissue metabolism, and specific to this design, differences in adipose catabolism. Nevertheless, we consider these preclinical studies to be important for improving our understanding of mechanisms of anti-adipose effects of CLA. 

The authors thank Cognis (Cincinnati, OH) for the CLA-mixed TG used in this study. The authors thank Dr. Neile Edens (Ross Products Division, Abbott Laboratories) and members of the Belury laboratory for their assistance with editing the manuscript. This work was supported by funds from the Carol S. Kennedy Professorship and the Anita R. McCormick Fellowship.

REFERENCES

- Kahn, B. B., and J. S. Flier. 2000. Obesity and insulin resistance. *J. Clin. Invest.* **106**: 473–481.
- Matsuzawa, Y., T. Funahashi, and T. Nakamura. 1999. Molecular mechanism of metabolic syndrome X: contribution of adipocytokines adipocyte-derived bioactive substances. *Ann. N. Y. Acad. Sci.* **892**: 146–154.
- Maeda, K., K. Okubo, I. Shimomura, T. Funahashi, Y. Matsuzawa, and K. Matsubara. 1996. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (adipose most abundant gene transcript 1). *Biochem. Biophys. Res. Commun.* **221**: 286–289.
- Scherer, P. E., S. Williams, M. Fogliano, G. Baldini, and H. F. Lodish. 1995. A novel serum protein similar to Clq, produced exclusively in adipocytes. *J. Biol. Chem.* **270**: 26746–26749.
- Hu, E., P. Liang, and B. M. Spiegelman. 1996. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J. Biol. Chem.* **271**: 10697–10703.
- Nakano, Y., T. Tobe, N. H. Choi-Miura, T. Mazda, and M. Tomita. 1996. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J. Biochem. (Tokyo)*. **120**: 803–812.
- Hotta, K., T. Funahashi, Y. Arita, M. Takahashi, M. Matsuda, Y. Okamoto, H. Iwahashi, H. Kuriyama, N. Ouchi, K. Maeda, et al. 2000. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler. Thromb. Vasc. Biol.* **20**: 1595–1599.
- Berg, A. H., T. P. Combs, X. Du, M. Brownlee, and P. E. Scherer. 2001. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat. Med.* **7**: 947–953.
- Yamauchi, T., J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, et al. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med.* **8**: 1288–1295.
- Yamauchi, T., J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, et al. 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat. Med.* **7**: 941–946.
- Pariza, M. W., and Y. L. Ha. 1990. Conjugated dienoic derivatives of linoleic acid: a new class of anticarcinogens. *Med. Oncol. Tumor Pharmacother.* **7**: 169–171.
- Brown, J. M., M. S. Boysen, S. S. Jensen, R. F. Morrison, J. Storkson, R. Lea-Currie, M. Pariza, S. Mandrup, and M. K. McIntosh. 2003. Isomer-specific regulation of metabolism and PPARgamma signaling by CLA in human preadipocytes. *J. Lipid Res.* **44**: 1287–1300.
- Hargrave, K. M., C. Li, B. J. Meyer, S. D. Kachman, D. L. Hartzell, M. A. Della-Fera, J. L. Miner, and C. A. Baile. 2002. Adipose depletion and apoptosis induced by trans-10,cis-12 conjugated linoleic acid in mice. *Obes. Res.* **10**: 1284–1290.
- Park, Y., J. M. Storkson, K. J. Albright, W. Liu, and M. W. Pariza. 1999. Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids.* **34**: 235–241.
- Ryder, J. W., C. P. Portocarrero, X. M. Song, L. Cui, M. Yu, T. Combatsiaris, D. Galuska, D. E. Bauman, D. M. Barmano, M. J. Charron, et al. 2001. Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. *Diabetes.* **50**: 1149–1157.
- Roche, H. M., E. Noone, C. Sewter, S. McBennett, D. Savage, M. J. Gibney, S. O'Rahilly, and A. J. Vidal-Puig. 2002. Isomer-dependent metabolic effects of conjugated linoleic acid: insights from molecular markers sterol regulatory element-binding protein-1c and LXRalpha. *Diabetes.* **51**: 2037–2044.
- Blankson, H., J. A. Stakkestad, H. Fagertun, E. Thom, J. Wadstein, and O. Gudmundsen. 2000. Conjugated linoleic acid reduces body fat mass in overweight and obese humans. *J. Nutr.* **130**: 2943–2948.
- Belury, M. A., A. Mahon, and S. Banni. 2003. The conjugated linoleic acid (CLA) isomer, t10c12-CLA, is inversely associated with changes in body weight and serum leptin in subjects with type 2 diabetes mellitus. *J. Nutr.* **133 (Suppl.)**: 257–260.
- Terpstra, A. H. 2004. Effect of conjugated linoleic acid on body composition and plasma lipids in humans: an overview of the literature. *Am. J. Clin. Nutr.* **79**: 352–361.
- DeLany, J. P., F. Blohm, A. A. Truett, J. A. Scimeca, and D. B. West. 1999. Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake. *Am. J. Physiol.* **276**: R1172–R1179.
- Tsuboyama-Kasaoka, N., M. Takahashi, K. Tanemura, H. J. Kim, T. Tange, H. Okuyama, M. Kasai, S. Ikemoto, and O. Ezaki. 2000. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes.* **49**: 1534–1542.
- Clement, L., H. Poirier, I. Niot, V. Bocher, M. Guerre-Millo, S. Krief, B. Staels, and P. Besnard. 2002. Dietary trans-10,cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *J. Lipid Res.* **43**: 1400–1409.
- Poirier, H., C. Rouault, L. Clement, I. Niot, M. C. Monnot, M. Guerre-Millo, and P. Besnard. 2005. Hyperinsulinaemia triggered by dietary conjugated linoleic acid is associated with a decrease in leptin and adiponectin plasma levels and pancreatic beta cell hyperplasia in the mouse. *Diabetologia.* **48**: 1059–1065.
- Poirier, H., I. Niot, L. Clement, M. Guerre-Millo, and P. Besnard. 2005. Development of conjugated linoleic acid (CLA)-mediated lipotrophic syndrome in the mouse. *Biochimie.* **87**: 73–79.
- Chaput, E., R. Saladin, M. Silvestre, and A. D. Edgar. 2000. Fenofibrate and rosiglitazone lower serum triglycerides with opposing effects on body weight. *Biochem. Biophys. Res. Commun.* **271**: 445–450.
- Carmona, M. C., K. Louche, M. Nibbelink, B. Prunet, A. Bross, M. Desbazeille, C. Dacquet, P. Renard, L. Casteilla, and L. Penicaud. 2005. Fenofibrate prevents rosiglitazone-induced body weight gain in ob/ob mice. *Int. J. Obes.* **29**: 864–871.
- Danno, H., Y. Jincho, S. Budyanto, Y. Furukawa, and S. Kimura. 1992. A simple enzymatic quantitative analysis of triglycerides in tissues. *J. Nutr. Sci. Vitaminol. (Tokyo)*. **38**: 517–521.
- Hardie, D. G., and D. A. Pan. 2002. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem. Soc. Trans.* **30**: 1064–1070.
- Lochhead, P. A., I. P. Salt, K. S. Walker, D. G. Hardie, and C. Sutherland. 2000. 5-Aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. *Diabetes.* **49**: 896–903.
- Stein, S. C., A. Woods, N. A. Jones, M. D. Davison, and D. Carling. 2000. The regulation of AMP-activated protein kinase by phosphorylation. *Biochem. J.* **345**: 437–443.
- Gavrilova, O., B. Marcus-Samuels, L. R. Leon, C. Vinson, and M. L. Reitman. 2000. Leptin and diabetes in lipotrophic mice. *Nature.* **403**: 850–851.
- Steppan, C. M., and M. A. Lazar. 2002. Resistin and obesity-associated insulin resistance. *Trends Endocrinol. Metab.* **13**: 18–23.
- Purushotham, A., G. Shrode, A. Wendel, L-F. Liu, and M. Belury.

2006. Dietary conjugated linoleic acid attenuates hepatic steatosis by modifying stearoyl-CoA desaturase-1 mRNA and activity in high-fat-fed rats. *FASEB J.* **20**: A574.

34. Nagao, K., N. Inoue, Y. M. Wang, B. Shirouchi, and T. Yanagita. 2005. Dietary conjugated linoleic acid alleviates nonalcoholic fatty liver disease in Zucker (fa/fa) rats. *J. Nutr.* **135**: 9–13.
35. Wang, Y. M., K. Nagao, N. Inoue, Y. Ujino, Y. Shimada, T. Nagao, T. Iwata, T. Kamegai, Y. Yamauchi-Sato, and T. Yanagita. 2006. Isomer-specific anti-obese and hypolipidemic properties of conjugated linoleic acid in obese OLETF rats. *Biosci. Biotechnol. Biochem.* **70**: 355–362.
36. Poirier, H., J. S. Shapiro, R. J. Kim, and M. A. Lazar. 2006.

Nutritional supplementation with trans-10,cis-12-conjugated linoleic acid induces inflammation of white adipose tissue. *Diabetes.* **55**: 1634–1641.

37. McTernan, P. G., A. L. Harte, L. A. Anderson, A. Green, S. A. Smith, J. C. Holder, A. H. Barnett, M. C. Eggo, and S. Kumar. 2002. Insulin and rosiglitazone regulation of lipolysis and lipogenesis in human adipose tissue in vitro. *Diabetes.* **51**: 1493–1498.
38. Singh, A. H., S. Liu, D. L. Crombie, M. Boehm, M. D. Leibowitz, R. A. Heyman, C. Depre, L. Nagy, P. Tontonoz, and P. J. Davies. 2001. Differential effects of rexinoids and thiazolidinediones on metabolic gene expression in diabetic rodents. *Mol. Pharmacol.* **59**: 765–773.