Conjugated linoleic acid fails to worsen insulin resistance but induces hepatic steatosis in the presence of leptin in ob/ob mice

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Abstract **Conjugated linoleic acid (CLA) induces insulin resistance preceded by rapid depletion of the adipokines leptin and adiponectin, increased inflammation, and hepatic steatosis in mice. To determine the role of leptin in CLA-mediated insulin resistance and hepatic steatosis, recombinant leptin was coadministered with dietary CLA in ob/ob mice to control leptin levels and to, in effect, negate the leptin depletion effect of CLA. In a 2 × 2 factorial design, 6 week old male ob/ob mice were fed either a control diet or a diet supplemented with CLA and received daily intraperitoneal injections of either leptin or vehicle for 4 weeks. In the absence of leptin, CLA significantly depleted adiponectin and induced insulin resistance, but it did not increase hepatic triglyceride concentrations or adipose inflammation, marked by interleukin-6 and tumor necrosis factor-α mRNA expression. Insulin resistance, however, was accompanied by increased macrophage infiltration (F4/80 mRNA) in adipose tissue. In the presence of leptin, CLA depleted adiponectin but did not induce insulin resistance or macrophage infiltration. Despite this, CLA induced hepatic steatosis. In summary, CLA worsened insulin resistance without evidence of inflammation or hepatic steatosis in mice after 4 weeks. In the presence of leptin, CLA failed to worsen insulin resistance but induced hepatic steatosis in ob/ob mice.**—Wendel, A. A., A. Purushotham, L-F. Liu, and M. A. Belury. **Conjugated linoleic acid fails to worsen insulin resistance but induces hepatic steatosis in the presence of leptin in ob/ob mice.** ***J. Lipid Res.*** **2008.** 49: **98–106.**

Supplementary key words adipokine • lipodystrophy • inflammation

Obesity contributes to the etiologies of a variety of comorbid conditions, such as cardiovascular disease, hypertension, and type 2 diabetes. In addition to storing lipid for energy, adipose secretes a variety of adipokines, many of which affect metabolism and inflammation in adipose and nonadipose tissues. Modulation of the endocrine functions of adipose tissue can contribute to a chronic state of inflammation, which leads to the pathogenesis of associated disorders, specifically insulin resistance (1). Conjugated linoleic acid (CLA) is a group of dietary fatty acids that modulate adiposity and adipokine levels (2–5). CLA exists as positional isomers and stereoisomers of octadecadienoic acid (18:2) and is found naturally in foods derived from ruminants, such as beef, lamb, and dairy products. Commercially, mixed isomer CLA is marketed as a weight-loss supplement (e.g., Tonalin™). Different isomers of CLA have varied biological functions, such as reducing carcinogenesis, decreasing adipose mass, and modulating immune function and type 2 diabetes (6). Although CLA, specifically the *trans*-10,*cis*-12 isomer (7), significantly decreases body weight primarily through a reduction of adipose tissue in a variety of species (8–10), CLA also induces hyperinsulinemia and insulin resistance, primarily in mice (2, 11–14).

In mice, insulin resistance induced by CLA develops in parallel with lipodystrophy (i.e., decreased adipose mass, significant and rapid depletion of the adipokines leptin and adiponectin, and increased hepatic steatosis) (2, 4). However, the mechanism by which CLA causes lipodystrophy in mice and the reason this effect is species-specific are not completely understood. Results from several studies emphasize the importance of leptin and adiponectin in the development of CLA-induced insulin resistance: leptin levels and adipose mass were partially preserved when CLA was fed as part of a high-fat diet in C57BL/6J mice. The preservation of leptin may have contributed to improvements in plasma insulin and liver weight also observed in these mice (15). In lean mice supplemented with CLA, adipokines decreased rapidly and before a significant reduction of adipose mass. Furthermore, hyperinsulinemia and increased hepatic lipid concentration accompanied the time-dependent depletion of adiponectin and leptin (4). These findings indicate that the initial reduction of adipokines by

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Abbreviations: CCL2, CC chemokine ligand 2; CLA, conjugated linoleic acid; CON, control diet; HOMA, homeostasis model assessment; IL-6, interleukin-6; ITT, insulin tolerance test; PPARα, peroxisome proliferator-activated receptor-α; SREBP-1, sterol-regulatory element binding protein-1; TNF-α, tumor necrosis factor-α; WAT, epididymal white adipose tissue.

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CLA may be independent of reduced adipose mass. In a subsequent study, the reduction of leptin and adiponectin induced by CLA coincided with a proinflammatory state marked by an increased expression of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and macrophage infiltration in adipose tissue, likely contributing to increased insulinemia (16). These results suggest that the deleterious effects of CLA on insulin sensitivity and insulinemia may be dependent on the alteration of adipokines and the induction of inflammation in adipose tissue.

Recent studies show that CLA worsens hyperinsulinemia and insulin resistance in ob/ob mice, which have nonfunctional leptin as a result of a gene mutation (3, 11). Consequently, insulin resistance induced by CLA cannot be attributed solely to the depletion of leptin. However, because both CLA-fed and ob/ob mice are leptin-deficient, insulin resistance and the other effects of CLA may not necessarily be completely independent of leptin depletion. In this study, we controlled the level of leptin in the ob/ob mouse model with chronic administration of recombinant leptin. By controlling leptin levels and, in effect, negating the leptin depletion effect of feeding CLA, we aimed to determine whether the effects of CLA on insulin resistance, hepatic steatosis, and inflammation occur in a leptin-dependent manner.

RESEARCH DESIGN AND METHODS

Experimental animals and design

Six week old male B6.V-Lep^{ob}/OlaHsd (ob/ob) mice were obtained through Harlan (Indianapolis, IN) and housed four per cage at 22 ± 0.5°C on a 12 h light/dark cycle. Mice were maintained on isocaloric, modified AIN-93G powdered diets (Bio-Serv, Frenchtown, NJ) containing 6.5% fat by weight. Diets contained either 6.5% soybean oil (CON) or 5% soybean oil and 1.5% CLA mixed triglycerides (CLA). CLA mixed triglycerides (Tonalin™ TG 80; Cognis Corp., Cincinnati, OH) were ~80% CLA composed of 39.2% *cis*-9,*trans*-11- and 38.5% *trans*-10,*cis*-12-CLA isomers. In a 2 × 2 factorial design, mice were randomized by body weight and fed either the CON or CLA diet and received intraperitoneal injections of either 1 mg/kg body weight recombinant mouse leptin (R&D Systems, Minneapolis, MN) (CON+ or CLA+) or a similar volume of the vehicle (PBS) (CON− or CLA−) for 4 weeks (n = 8 mice per group). Mice were injected every day, 2 h before the onset of the dark cycle. The leptin dose was based on the lowest dose that induced a reduction in body weight gain and fat and rescued serum insulin levels in ob/ob mice (17). Four weeks was chosen as the end point because this duration allows time for the development of insulin resistance and hepatic steatosis induced by CLA supplementation in mice (11, 12, 18) and, independently, the correction of metabolic abnormalities of ob/ob mice by leptin (17, 19). Body weights were measured every other day. At 4 weeks, after an overnight (12 h) fast, mice were anesthetized with isoflurane and blood was collected via cardiac puncture. After clotting, blood was centrifuged at 1,500 *g* for 20 min and sera were used for analyses. Tissues were quickly harvested, weighed, snap-frozen with liquid nitrogen, and stored at −80°C until analyses. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of Ohio State University.

Fasting glucose and insulin tolerance test

Glucose levels were measured after an overnight (12 h) fast immediately before (baseline) and at 2 and 4 weeks of experimental treatments via tail vein blood using a One Touch Basic glucometer (Lifescan, Milpitas, CA). An insulin tolerance test (ITT) was conducted 3 days before necropsy. After an overnight (12 h) fast, mice were injected intraperitoneally with 1.5 U/kg body weight insulin (Humulin® R; Eli Lilly and Co., Indianapolis, IN). Tail vein blood was used to measure glucose immediately before injection (time 0) and at 15, 30, 45, 60, 90, and 120 min after the injection. Area under the curve was calculated as the net area contained between individual baselines (set by the glucose value at time 0) and curves using the trapezoidal rule (20). Homeostasis model assessment (HOMA) values were calculated according to Matthews et al. (21) as insulin $(\text{mU/l})/22.5e^{-\ln \text{ glucose (mM)}}$.

Serum hormones

Fasted serum levels of insulin, IL-6, and TNF-α were measured by the LINCOplex Mouse Serum Adipokine Immunoassay kit (Linco Research, Inc., St. Charles, MO). Adiponectin and resistin serum concentrations were determined by ELISA (Linco Research, Inc.) according to the manufacturer's directions.

Analysis of hepatic triglycerides

Lipids were extracted from a section of liver with 2:1 (v/v) chloroform and methanol. Final extracts were solubilized in 3:1:1 (v/v/v) tert-butanol, methanol, and Triton X-100 (22). Tissue lipid extracts were analyzed for triglycerides by colorimetric enzymatic hydrolysis (Triglyceride, Free-Glycerol reagents; Sigma, St. Louis, MO). Data are expressed as equivalent triolein concentrations.

Real-time RT-PCR

RNA was extracted from epididymal adipose tissue using the RNeasy® Lipid Tissue Mini kit (Qiagen, Valencia, CA) and from liver using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturers' protocols. RNA was reverse-transcribed with the High-Capacity cDNA Archive kit (ABI, Foster City, CA) according to the directions. cDNA was amplified by real-time PCR in a total reaction volume of 25 μl with TaqMan Gene Expression Assays (ABI) using predesigned and validated primers (FAM probes) from ABI under universal cycling conditions defined by ABI. Target gene expression was normalized to the endogenous control 18S rRNA (VIC probe) amplified in the same reaction and expressed as $2^{-\Delta\Delta ct}$ relative to the CON− group (23).

Statistical analyses

Data are expressed as least square means ± SEM. The main and interaction effects of diet (CON or CLA) and treatment (leptin or vehicle) were analyzed by two-way ANOVA using a complete model in the GLM procedure of the Statistical Analysis System (SAS version 9.1; SAS Institute, Inc., Cary, NC). Body weights over time, fasting glucose levels, and ITT curves were analyzed as repeated measures. Differences of $P < 0.05$ were considered significant.

RESULTS

Effects of CLA and leptin on body and tissue weights

Before beginning the experimental diets and treatments, all groups had similar average body weights (32.4–

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32.7 g). After 4 weeks on the experimental diets and treatments, leptin and CLA, both in the absence and presence of leptin, significantly reduced body weights compared with the CON diet alone (**Table 1**). The reduction in body weight by CLA was significantly more than that by leptin alone; however, there was no significant additive or synergistic effect of CLA and leptin on body weight. Although leptin alone decreased weight gain, CLA, regardless of leptin, induced significant weight loss from initial body weights. Body weights over time are shown in **Fig. 1**. Significant differences in weight between mice fed CLA, regardless of leptin treatment, and vehicle-treated mice fed CON were first observed on day 9. On day 15, weights of CLA-fed mice were first significantly different from leptin-treated mice fed CON. A significant difference in weight between vehicle-treated and leptin-treated mice fed CON was first detected on day 17. All weights among the aforementioned comparisons remained significantly different through the end of the study. CLA-fed groups, whether in the absence or presence of leptin, never differed significantly from each other over the duration of the study. Differences in body weight were reflected by differences in epididymal adipose mass (Table 1). Leptin and CLA, in the absence and presence of leptin, significantly reduced epididymal adipose mass compared with the CON diet alone, and the reduction of epididymal adipose mass by CLA was significantly greater than that of leptin alone. Leptin treatment, regardless of diet, significantly decreased liver weight, whereas CLA did not have a significant effect.

Effects of CLA and leptin on serum analytes and insulin tolerance

After 2 weeks of diets and treatments, CLA significantly increased fasting glucose levels in the absence of leptin (**Table 2**). Increased fasting glucose levels were maintained, but not increased, by CLA after 4 weeks of diets and treatments. In the presence of leptin, CLA did not significantly increase fasting glucose levels at 2 or 4 weeks. Leptin also prevented an increase in fasting glucose in mice fed the CON diet by 4 weeks. In the absence of leptin, CLA significantly increased fasting insulin levels compared with CON, whereas leptin significantly reduced fasting insulin levels in both diet groups (Table 2).

After 4 weeks of diets and treatments, an ITT was conducted to assess the response to insulin. In the absence of leptin, glucose levels in mice fed CLA did not decrease at any time point after the administration of insulin, indicating unresponsiveness to insulin (**Fig. 2A**). In the presence of leptin, glucose levels in mice fed CLA decreased significantly after insulin injection, as they did in mice fed the CON diet. In Fig. 2B, the net areas contained within the ITT curves were quantified. As implied by the positive area, in the absence of leptin, mice fed CLA were unresponsive to insulin, which was significantly different from the other groups. There were no significant differences among the other groups, and the net areas were negative, indicating insulin responsiveness. A second estimation of insulin resistance by HOMA showed that in the absence of leptin, CLA worsened insulin resistance. Leptin decreased insulin resistance in both diet groups (Fig. 2C). Together, the ITT and HOMA data show that in the absence of leptin, CLA worsens insulin resistance, but it does not do so in the presence of leptin.

Effects of CLA and leptin on adipokines

Serum adiponectin levels were reduced significantly by CLA in both the absence and presence of leptin (Table 2). Leptin had no effect on serum adiponectin levels in either diet group. Serum resistin, however, was significantly decreased by leptin and CLA in the absence and presence of leptin. Similar to serum adiponectin, CLA, in the absence and presence of leptin, significantly reduced epididymal white adipose tissue (WAT) adiponectin mRNA expression compared with the respective CON diet groups (**Fig. 3A**). Unlike serum levels, however, leptin significantly increased WAT adiponectin mRNA expression in CON-fed mice. Both leptin and CLA significantly decreased WAT resistin mRNA expression; however, the reduction induced by CLA, regardless of leptin treatment, was significantly greater than with leptin alone (Fig. 3B).

Effects of CLA and leptin on hepatic lipid metabolism

CLA had no effect on hepatic triglyceride concentrations in the absence of leptin (**Fig. 4A**). However, in the presence of leptin, CLA increased hepatic TG. Leptin significantly reduced hepatic triglyceride levels in both diet groups; however, leptin only partially reduced hepatic triglycerides in mice fed CLA compared with CON. Hepatic mRNA expression of markers of lipogenesis [sterol-regulatory element binding protein-1 (SREBP-1) and FAS] and lipid transport (FAT/CD36) was not altered by CLA (Fig. 4B) but was reduced by leptin in both diet

TABLE 1. Effects of CLA and leptin on body and tissue weights

	Diet ± Leptin				Factors (P)		
Variable	CON−	CLA−	CON+	CLA+	Diet	Trt	Int
Final body weight (g)	42.20 ± 0.97c	28.98 ± 0.97a	34.79 ± 0.97b	28.41 ± 0.97a	<0.001	<0.001	0.001
Change in body weight (g)	9.74 ± 0.56c	−3.54 ± 0.56a	2.39 ± 0.56b	−4.30 ± 0.56a	<0.001	<0.001	<0.001
Epididymal adipose weight (g)	2.96 ± 0.14c	1.71 ± 0.14a	2.16 ± 0.14b	1.36 ± 0.14a	<0.001	<0.001	0.004
Liver weight (g)	2.76 ± 0.17b	3.18 ± 0.17b	1.29 ± 0.17a	1.56 ± 0.17a	0.057	<0.001	0.694

CLA, conjugated linoleic acid (and CLA diet); CON, control diet. Values represent least square means ± SEM, with significant differences ($P < 0.05$) within rows denoted by different letters. Factors include the main effects of diet and leptin treatment (Trt) and the interaction (Int) between diet and leptin treatment.

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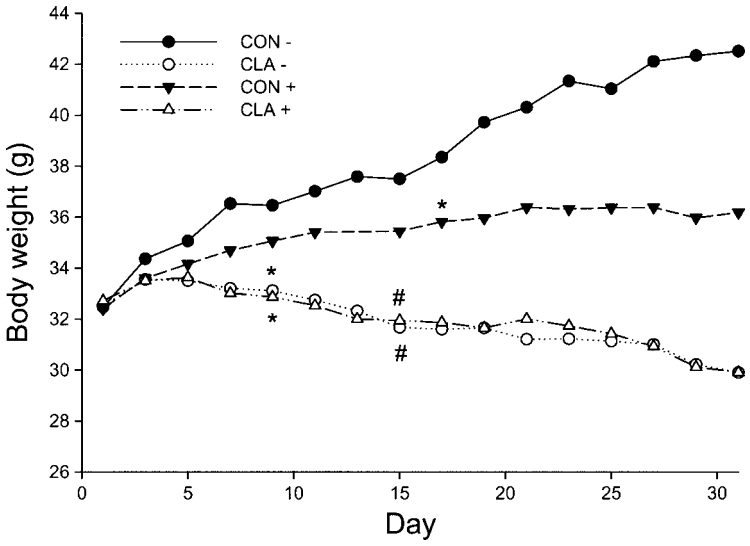

Fig. 1. Effects of conjugated linoleic acid (CLA) and leptin on body weights over time. Mice were fed either control (CON) or CLA-supplemented (CLA) diet and received either leptin (+) or vehicle (−) by intraperitoneal injection daily. Body weights were measured every other day. Values represent least square means ± SEM of eight mice per group. * $P < 0.05$ compared with CON−; # $P < 0.05$ compared with CON+. The symbols indicate the first day that a significant difference was observed; differences were maintained through the completion of the study.

groups. In the absence of leptin, CLA did not alter markers of fatty acid oxidation [peroxisome proliferator-activated receptor α (PPARα), carnitine palmitoyltransferase 1α, fatty acid binding protein 1, and acyl-CoA oxidase 1] compared with vehicle-treated mice fed the CON diet. These markers were increased by leptin in CON-fed mice. In the presence of leptin, CLA decreased these markers to levels of vehicle-treated mice in either diet group.

Effects of CLA and leptin on markers of inflammation and macrophage infiltration

Both CLA and leptin significantly reduced serum IL-6 compared with CON− mice (Table 2). Serum TNF-α did not significantly change with any diet or treatment. Contrary to the trend in serum IL-6, only leptin significantly decreased WAT IL-6 mRNA expression compared with vehicle-treated mice on either diet (**Fig. 5A**). Likewise, only leptin significantly decreased WAT TNF-α mRNA expression compared with vehicle-treated mice (Fig. 5B). CC chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1, a macrophage recruiter, was decreased significantly by leptin treatment regardless of diet but was not altered by CLA (Fig. 5C). In the absence of leptin, CLA significantly increased F4/80 mRNA expression, a macrophage-specific marker, nearly 2-fold compared with all other groups (Fig. 5D).

DISCUSSION

Adipokines, particularly leptin and adiponectin, produced by adipose tissue are recognized as key mediators in both insulin sensitivity and inflammation and provide an important link of communication among tissues. Rapid depletion of both adiponectin and leptin coincides with increased inflammation and subsequent insulin resistance and hepatic steatosis with CLA supplementation in mice (4, 16). In the present study, recombinant leptin was chronically administered to ob/ob mice to determine the effects of dietary CLA in the absence or presence of leptin on insulin resistance, hepatic steatosis, and inflammation. This study demonstrates that in the absence of leptin, CLA augments insulin resistance without evidence of increased hepatic steatosis or inflammation in serum or WAT. When coadministered with leptin, CLA fails to worsen insulin resistance but increases hepatic steatosis.

TABLE 2. Effects of CLA and leptin on fasted serum analytes

	Diet ± Leptin				Factors (P)		
Variable	CON−	CLA−	CON+	CLA+	Diet	Trt	Int
Fasting glucose (mg/dl)							
Baseline	115.12 ± 10.38	101.25 ± 10.38	117.00 ± 10.38	107.38 ± 10.38			
2 weeks[a]	130.25 ± 10.38a,b	161.59 ± 12.10b	106.25 ± 10.38a	102.50 ± 10.38a			
4 weeks[a]	122.87 ± 10.38b	153.63 ± 10.38c	89.50 ± 10.38a	98.38 ± 10.38a,b			
Insulin (pmol/l)	441.24 ± 49.45b	771.05 ± 58.51c	94.99 ± 46.25a	209.00 ± 46.25a	<0.001	<0.001	0.042
Adiponectin (μg/ml)	9.37 ± 0.57b	3.91 ± 0.57a	10.61 ± 0.57b	5.23 ± 0.57a	<0.001	0.502	0.405
Resistin (ng/ml)	22.13 ± 2.20b	14.67 ± 2.20a	9.17 ± 2.20a	13.91 ± 2.20a	0.547	0.009	0.017
Interleukin-6 (pg/ml)	25.89 ± 5.01b	1.22 ± 5.01a	5.87 ± 4.68a	4.41 ± 4.68a	0.012	0.094	0.024
Tumor necrosis factor-α (pg/ml)	2.01 ± 0.99	0.20 ± 0.99	1.18 ± 0.92	2.85 ± 0.92	0.940	0.347	0.079

Values represent least square means ± SEM, with significant differences ($P < 0.05$) within rows denoted by different letters. Factors include the main effects of diet and leptin treatment (Trt) and the interaction (Int) between diet and leptin treatment. Fasting glucose levels were analyzed by repeated-measures ANOVA; only differences within diet ± leptin group and within time are shown.

[a] There were no significant differences ($P < 0.05$) between 2 weeks and 4 weeks within any diet ± leptin group.

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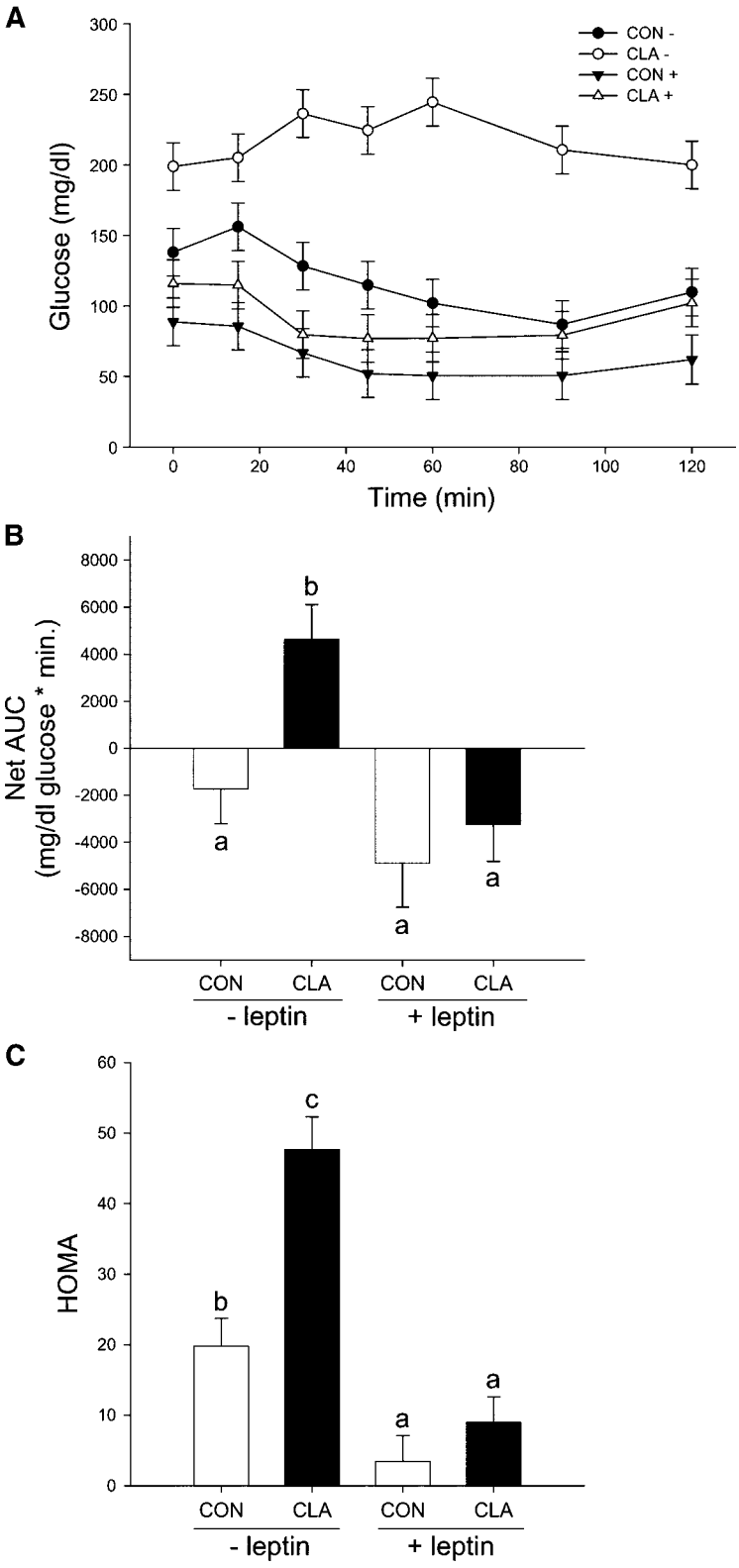

Fig. 2. Effects of CLA and leptin on insulin tolerance. A: Insulin tolerance test (ITT). After 4 weeks, mice received 1.5 U/kg insulin by intraperitoneal injection after an overnight fast (12 h). Blood glucose was measured at the intervals indicated over 2 h. Statistical differences at each time point were omitted for clarity. B: Net area contained within the curve (AUC) of the ITT. C: Homeostasis model assessment (HOMA) estimates of insulin resistance measured as follows: insulin (mU/l)/22.5$e^{-\ln \text{ glucose (mM)}}$. Mice were fed either CON or CLA diet and received either leptin (+) or vehicle (−) by intraperitoneal injection daily. Values represent least square means ± SEM of eight mice per group, with significant differences ($P < 0.05$) denoted by different letters.

CLA induces hyperinsulinemia and hepatic steatosis preceded by the depletion of adipokines, specifically leptin and adiponectin, in mice (4). Tsuboyama-Kasaoka et al. (2) reported that 12 days of leptin infusion decreased nonfasted plasma insulin and hepatic steatosis in mice that had been fed CLA for 8 months prior. However, the effect of leptin infusion on adiponectin concentrations was not reported, nor was it compared with a control-fed group. Leptin treatment has been shown to increase plasma adiponectin (24); therefore, although speculative, insulin concentrations and hepatic steatosis may have been attenuated by a leptin-induced increase in adiponectin.

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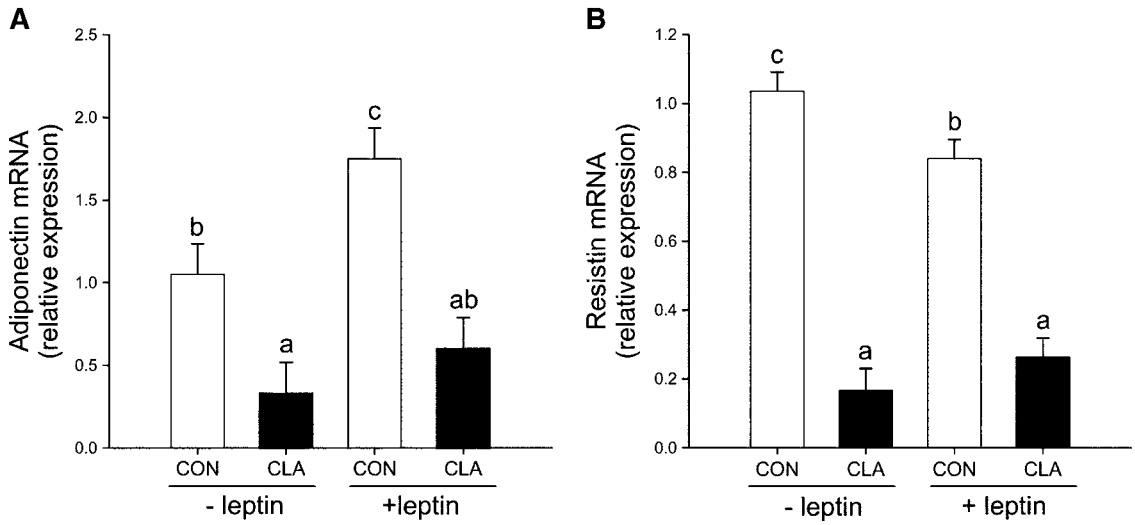

Fig. 3. Effects of CLA and leptin on epididymal white adipose tissue (WAT) gene expression of adipokines. A: Adiponectin. B: Resistin. mRNA expression is expressed as $2^{-\Delta\Delta Ct}$ relative to the 18S endogenous control and the CON− group. Mice were fed either CON or CLA diet and received either leptin (+) or vehicle (−) by intraperitoneal injection daily for 4 weeks. Values represent least square means ± SEM of six to eight mice per group, with significant differences ($P < 0.05$) denoted by different letters.

In the present study, induction of hyperinsulinemia and insulin resistance in ob/ob mice by CLA paralleled the depletion of adiponectin. In the presence of leptin, CLA supplementation did not worsen insulin resistance, but serum adiponectin levels were still reduced. Although CLA did not significantly increase serum insulin levels in the presence of leptin, insulin levels of these mice were double those of the CON-fed group treated with leptin. This finding, that CLA induces hyperinsulinemia without worsening insulin resistance in the presence of leptin, suggests that there may be direct, opposing effects of CLA and leptin on insulin secretion. Hyperinsulinemia induced by CLA has been shown to be associated with pancreatic β-cell hyperplasia and increased insulin secretion (4). Leptin, conversely, reduces insulin release from pancreatic β-cells (25).

The incomplete attenuation of CLA-induced hyperinsulinemia with leptin treatment also suggests that these effects of CLA may be dependent on adiponectin, or perhaps a basal level of both leptin and adiponectin, as adiponectin was suppressed by CLA regardless of leptin treatment. Our group reported previously that although CLA rapidly depleted adiponectin levels in mice, upon removal of CLA from the diet, adiponectin and leptin levels increased significantly and insulin sensitivity improved (5). Yamauchi et al. (26) reported that only a combination of adiponectin and leptin could correct insulin resistance in lipodystrophic mice: leptin or

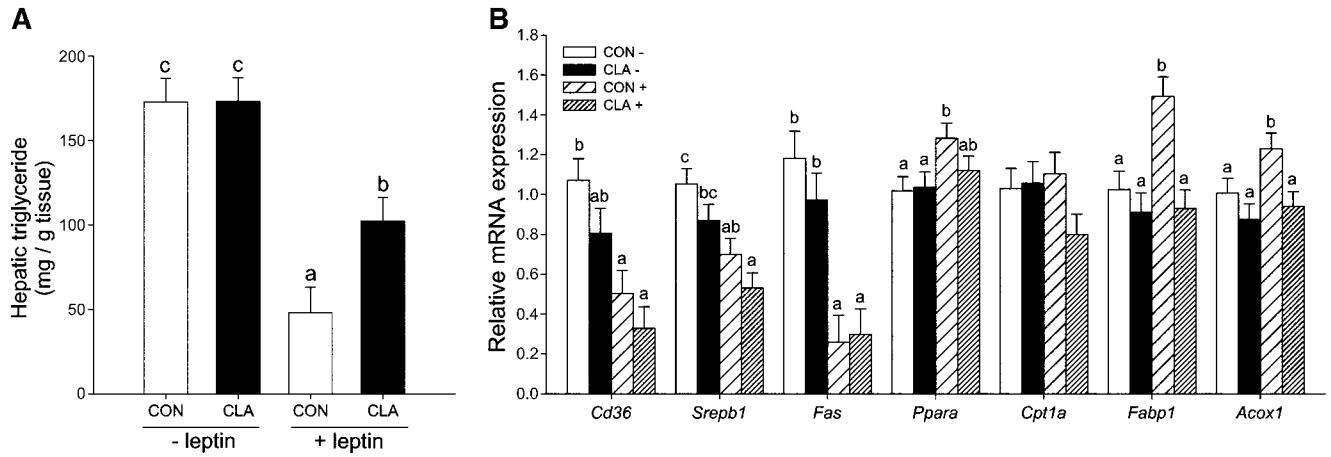

Fig. 4. Effects of CLA and leptin on hepatic lipid metabolism. A: Hepatic triglyceride levels. B: Hepatic mRNA expression of markers of lipid metabolism. mRNA expression is expressed as $2^{-\Delta\Delta Ct}$ relative to the 18S endogenous control and the CON− group. Mice were fed either CON or CLA diet and received either leptin (+) or vehicle (−) by intraperitoneal injection daily for 4 weeks. Values represent least square means ± SEM of six to eight mice per group, with significant differences ($P < 0.05$) denoted by different letters. *Acox1*, acyl-coenzyme A oxidase 1; *Cpt1α*, carnitine palmitoyltrasferase 1α; *Fabp1*, fatty acid binding protein 1; *Pparα*, peroxisome proliferator-activated receptor α; *Srebp1*, sterol-regulatory element binding protein 1.

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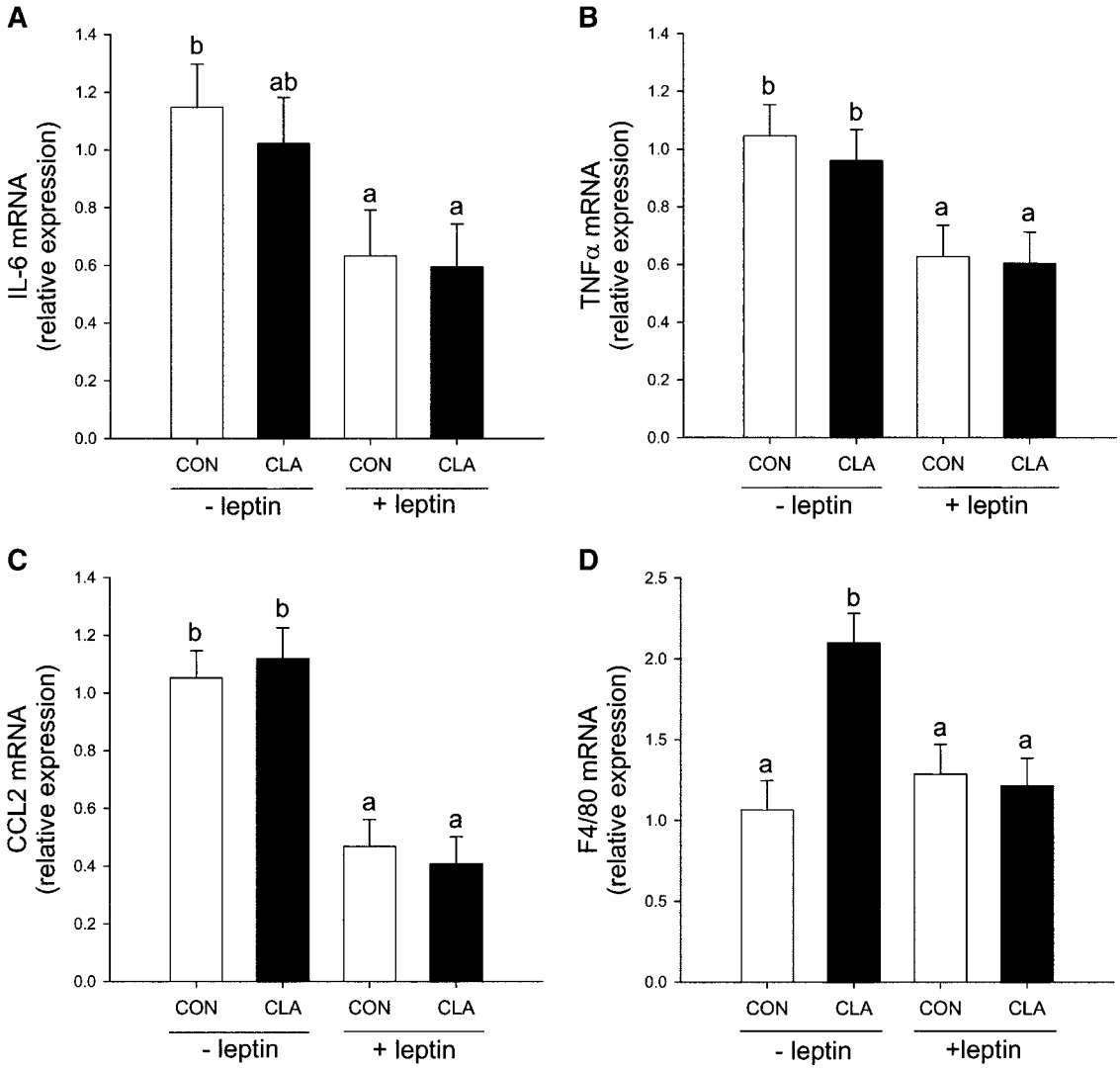

Fig. 5. Effects of CLA and leptin on WAT gene expression of markers of inflammation and macrophage infiltration. Interleukin-6 (IL-6; A), tumor necrosis factor-α (TNF-α; B), CC chemokine ligand 2 (CCL2; C), and F4/80 mRNA (D) expression was measured in epididymal adipose tissue. mRNA expression is expressed as $2^{-\Delta\Delta Ct}$ relative to the 18S endogenous control and the CON− group. Mice were fed either CON or CLA diet and received either leptin (+) or vehicle (−) by intraperitoneal injection daily for 4 weeks. Values represent least square means ± SEM of six to eight mice per group, with significant differences ($P < 0.05$) denoted by different letters.

adiponectin alone only partially reversed insulin resistance. Increased levels of resistin, another adipokine, have been associated with insulin resistance, but the data are contradictory (27–29). Here, in the absence of leptin, CLA decreased serum and WAT expression of resistin despite increased insulin resistance. In the presence of leptin, which also decreases resistin (30), and with improved insulin sensitivity, CLA still reduced WAT expression of resistin. These data support previous reports that CLA decreases resistin levels (16, 18, 31) and also support a disconnect between resistin levels and insulin resistance. These data also suggest that leptin status does not influence the effect of CLA on WAT resistin expression.

Hepatic steatosis and hepatomegaly generally develop with CLA-induced insulin resistance (2, 12, 32). In this study, we did not observe a significant increase in liver weight or hepatic steatosis by CLA in the absence of leptin in ob/ob mice. Although hepatic steatosis in nonalcoholic fatty liver disease is defined as an accumulation of lipid to 5–10% of the liver by weight (33), livers of both the CON and CLA groups in vehicle-treated mice were 17.3% triglycerides. Therefore, it may be possible that CLA did not increase hepatic lipid levels in livers of ob/ob mice because they were already grossly steatotic. When leptin was present, livers of CON-fed mice were 4.8% lipid and not steatotic, but CLA increased hepatic lipid concentration to 10.2%. These differences in hepatic triglyceride levels may be attributed to changes in insulin levels and adipokines. Insulin contributes to increased hepatic lipogenesis through the induction of SREBP-1 (34). Appropriately, hyperinsulinemia in vehicle-treated mice fed the CON diet was accompanied by hepatic steatosis; however, the increase in insulin by CLA did not contribute to greater levels of hepatic triglycerides or markers of lipogenesis (SREBP-1 or FAS). Roche et al. (11) also reported that the *trans*-10,*cis*-12-CLA isomer did not alter

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hepatic mRNA expression of SREBP-1c despite hyperinsulinemia in ob/ob mice, but hepatic triglyceride or adipokine levels were not reported.

In addition to insulin, both leptin and adiponectin contribute to hepatic lipid metabolism to modulate triglyceride levels. Adenovirus-induced hyperleptinemia reduced hepatic mRNA expression of SREBP-1c and the lipogenic enzymes FAS and acetyl-CoA carboxylase in lean ($^{+/+}$) ZDF rats (35). Leptin increases fatty acid oxidation by upregulating PPARα and its target genes (36). Adiponectin promotes fatty acid oxidation through the activation of AMPK (37) and the increased expression of PPARα and target genes involved in fatty acid oxidation (38). In this study, leptin lessened hepatic steatosis and liver mass in both diet groups. Interestingly, however, CLA only increased hepatic triglycerides in the presence of leptin. The decrease in hepatic triglycerides by leptin coincided with the reduction of markers associated with lipogenesis and increased markers of lipid oxidation. CLA, however, blunted the increase of markers of lipid oxidation by leptin. This lack of increase in these markers by leptin in CLA-fed mice may be attributed to the depletion of adiponectin, again suggesting that a basal level of both leptin and adiponectin may be critical to maintain energy homeostasis.

Chronic inflammation is a major factor in obesity-driven insulin resistance. Reports in both animal models of obesity and insulin resistance and in humans show strong relationships between the expression of TNF-α and IL-6, as well as macrophage infiltration into adipose tissue, with increased adiposity and insulin resistance (39–41). Despite these and previous reports of CLA-mediated increased inflammation (16, 42, 43), in this study, two prominent markers of inflammation, serum and WAT mRNA expression of TNF-α and IL-6, were not increased by CLA. Leptin reduced WAT expression of both TNF-α and IL-6 regardless of diet. The reduction in WAT inflammation by leptin may have contributed to the increased insulin sensitivity in CLA-fed mice. It also may have been a factor in the reductions of fasting insulin and glucose, as well as hepatic steatosis, in both CON- and CLA-fed mice. Similarly, CLA did not have an effect on WAT mRNA expression of CCL2, a macrophage recruiter, but increased WAT F4/80 mRNA expression, a macrophage-specific marker. Leptin reduced the expression of CCL2, which may have prevented the increase in F4/80 expression in WAT of mice fed CLA, but did not have an effect on the level of macrophage infiltration in WAT of CON-fed mice.

After 4 weeks, the induction of insulin resistance by CLA coincided with macrophage infiltration, but without increased levels of WAT IL-6 and TNF-α. A previous study showed that 3 and 7 days of treatment with CLA significantly increased WAT mRNA expression of markers of inflammation (IL-6 and TNF-α) and macrophage infiltration (F4/80, CD68, and monocyte chemoattractant protein-1). These changes occurred simultaneously with the depletion of adiponectin and leptin and an increase in insulin levels (16). Tsuboyama-Kasaoka et al. (2) reported that the maximum increase in TNF-α mRNA expression in WAT by CLA in C57BL/6J mice occurred at 4 days, but subsequently it declined. Wargent et al. (3) showed that after initially inducing impaired glucose tolerance and hyperinsulinemia, prolonged supplementation (10 weeks) with CLA actually improved glucose tolerance and insulinemia in ob/ob mice, regardless of reduced adiponectin concentrations. Markers of WAT inflammation were not reported in that study. This study demonstrates that after 4 weeks of CLA supplementation in ob/ob mice, the mice are hyperinsulinemic and insulin-resistant without overt evidence of increased inflammation compared with the control. Increased F4/80, but not CCL2, mRNA expression in the WAT of CLA-fed mice suggests that there was still evidence of macrophage infiltration in WAT, which is associated with inflammation (39), but that macrophage recruitment had perhaps subsided. Together, the results from these studies suggest that some events of CLA-mediated inflammation (such as changes in WAT TNF-α and IL-6 and macrophage infiltration) and the possible, subsequent effects on insulin resistance may be transient.

In summary, this study demonstrates that insulin resistance induced by CLA in ob/ob mice was prevented by leptin without an increase in adiponectin. However, the ability of CLA to increase hyperinsulinemia and hepatic steatosis in the presence of leptin suggests that these effects may be dependent on other factors, such as adiponectin, a basal level of both leptin and adiponectin, and/or other factors not yet identified. Furthermore, IL-6 and TNF-α, markers of inflammation frequently associated with obesity and insulin resistance, were not increased after 4 weeks of supplementation with CLA. Future studies are needed to determine the mechanistic roles of adiponectin and/or markers of inflammation in lipodystrophic-like insulin resistance and hepatic steatosis induced by CLA.

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