Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake

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METHODS

Male AKR/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at ~ 4 wk of age. All mice were housed individually in hanging stainless steel cages with an automatic watering system in a room maintained at 26 ± 2°C with a 12:12-h light-dark cycle. Initially, the mice were fed ad libitum Purina Mouse Chow (diet no. 5015;Ralston Purina) and then were switched to a high-fat diet (diet no. D12290, %fat 45 kcal; Research Diets) 10 days before being randomly assigned to treatment diets. This period of adaptation to the high-fat diet was used in both studies described here to minimize the effect of high-fat diet-induced hyperphagia in the treatment groups at study initiation.

Two studies were conducted. In the dose-response study, 60 6-wk-old mice were randomly assigned to five treatment groups (n = 12/group) for feeding varied doses of CLA (0% control and 0.25, 0.50, 0.75, and 1.0% CLA by weight) in a high-fat diet (%fat 45 kcal). CLA was obtained from Nu Chek Prep (Elysia, MN), and the reported isomer content was as follows: 39.1% cis9;trans11 CLA and trans9;cis11 CLA; 40.7% trans10;cis12 CLA and 1.8% cis9;cis11 CLA; 1.3% cis10;cis12 CLA; 1.9% trans9;trans11 and trans10;trans12 CLA; 1.1% cis9;cis12 linoleic acid; and 14.1% remainder. The 45-kcal fat diets were formulated by Research Diets (New Brunswick, NJ), and the CLA-containing diets were prepared by replacing corn oil with CLA (Table 1). Mice were provided ad libitum access to a preweighed portion of the defined diets in pellet form placed on the bottom of each cage, with fresh preweighed diet given at each weighing. The animals and remaining diet were weighed three times each week (Monday, Wednesday, and Friday). Food intake was corrected for spillage, and all measurements were to the nearest 0.1 g. Measurements were recorded 7 days before the start of experimental diet feeding and continued throughout the 39 days of CLA feeding.

At the end of the study, after a 3-h fast, the mice were killed by cervical dislocation and decapitated. Truncal blood was collected, and plasma was frozen for later measurement of insulin and leptin by RIA (Linco Research, St. Louis, MO). Plasma glucose was measured in the control and 1.0% CLA groups by a colorimetric hexokinase glucose assay (Sigma Diagnostics, St. Louis, MO). Carcasses were dissected, and the following organs were removed and weighed: liver, spleen, left and right kidney, left and right testis, and heart. Similarly, the left and right inguinal, left and right epididymal, left and right retroperitoneal, and the mesenteric adipose depots were removed and weighed to the nearest 0.0001 g. The eviscerated carcass, which included all removed tissues except the left epididymal adipose depot, left testis, liver, spleen, and heart, was weighed and then frozen in liquid nitrogen for later body composition analysis.

The liver and spleen of control and 1% CLA groups were sectioned and fixed in 10% Formalin or glutaraldehyde for histopathological examination by light microscopy and trans-
mission electron microscopy. Histopathological analysis was performed by two separate laboratories [the Pathology Department at the Louisiana State University Veterinary School of Medicine (Baton Rouge, LA) and the Experimental Pathology Laboratories (Herndon, VA)].

The frozen eviscerated carcasses were homogenized in 3 vol of distilled water using a Polytron (PRO300D PRO; Scientific). Homogenates were dried to constant weight in a 70°C oven and then finely ground and mixed thoroughly using a mortar and pestle. The final product was analyzed in triplicate for lipid, protein, and ash content. Lipid was analyzed using a Soxtec System HT 1043 Extraction unit using 2:1 chloroform-methanol, protein with a Perkin-Elmer Series II Nitrogen Analyzer 2410, and ash by a CEM MAS 7000 Microwave Muffle Furnace. Water content of the carcass was calculated by subtracting the dried carcass weight from the original weight of the eviscerated carcass.

An adiposity index was calculated by dividing the summed weight of the seven excised adipose depots by the weight of the eviscerated carcass (which includes all organs but without the gastrointestinal tract and the 7 adipose depots). The adiposity index has been shown to be highly correlated with lipid content assayed chemically ($r = 0.97$; see Ref. 26).

In the time-course study, 80 6-wk-old mice were used. Due to the large number of animals, the study was divided into two cohorts of 40 (20 control, 20 treatment) with a staggered start of 4 wk. A total of 40 mice were used as controls and were fed an unadulterated high-fat diet, whereas 40 treatment animals were fed the high-fat diet containing 1% CLA. The 1% dose used in the previous study was chosen for its effect on body composition and lack of effect on energy intake. The diets were formulated by the same company with the same ingredient mixture as that used in the dose-response study.

Measurements of food intake and body weight were taken two times per week (Tuesday and Friday) using the same protocol as described in the above study.

Eight animals from the control group and eight from the treatment group were killed after 2, 4, 6, 8, and 12 wk of CLA

**Table 1. Diet composition**

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<tr>
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<th>g</th>
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<tbody>
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<tr>
<td>Corn oil</td>
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<tr>
<td>Sucrose</td>
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</tr>
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<td>Maltodextrin 10</td>
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<td>Corn starch</td>
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<td>Vitamin mix</td>
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</tr>
<tr>
<td>Choline bitartrate</td>
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<td>0</td>
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<tr>
<td>Total</td>
<td>204.8</td>
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Diet was formulated by Research Diets (New Brunswick, NJ). Diet composition was as follows: fat, 45 kcal; protein, 20 kcal; carbohydrate, 35 kcal. Dietary fat content was designed such that the fatty acid composition was approximately 1 part saturated and 3 parts unsaturated. For kcal values, energy content was calculated using 4 kcal/g for protein and carbohydrate and 9 kcal/g for fat. Mineral and vitamin mix content conformed to the minimum requirements as defined by the American Institute of Nutrition (AIN) 76A standard. In those diets containing conjugated linoleic acid (CLA), the CLA was substituted for corn oil on a weight basis.

**Fig. 1.** Body weight of 5 groups of mice fed high-fat diets containing increasing amounts of conjugated linoleic acid (CLA). Mice were weighed 3 times each week. Each point represents the mean ± SE for 12 animals.

**Fig. 2.** Cumulative food intake of 5 groups of mice fed high-fat diets containing increasing amounts of CLA. Food intake was weighed 3 times each week. Each point represents the mean ± SE for 12 animals. Energy intake was significantly increased relative to controls in the 0.25% CLA treatment.

**Fig. 3.** Weights of 4 adipose depots (inguinal, epididymal, retroperitoneal, and mesenteric) in 5 groups of mice fed high-fat diets containing increasing amounts of CLA. Each bar represents the mean ± SE of 12 animals. *$P < 0.05$ and **$P < 0.01$, difference from the control group. For all depots except the mesenteric depot the data are presented as average of left and right depot weights.
Dose-response study. There was a significant main effect of CLA on body weight (P < 0.05), an effect of CLA over time (P < 0.01), and a significant interaction between the two (P < 0.01; Fig. 1). When compared with the controls, body weight was significantly lower at day 18 in the 0.75% CLA group and day 21 in the 1.0% CLA group (P < 0.05) and remained lower throughout the remainder of the study. Energy intake was not significantly different from control except in the 0.25% CLA group, which was slightly increased relative to the control diet and other CLA doses (P < 0.05; Fig. 2).

At the end of treatment, there was a dose-dependent effect of CLA on all adipose depots relative to the controls (Fig. 3). The adipose depot most sensitive to the effects of CLA was the retroperitoneal, with a significant reduction in depot weight observed at 0.50, 0.75, and 1.0% CLA (P < 0.01 for all comparisons). The inguinal, epididymal, and mesenteric adipose depots showed a significant difference at the 0.75 and 1.0% doses (P < 0.05).

Body composition data and calculated adiposity index supported the adipose depot weight data for the effect of CLA to reduce body fat (Table 2). When data were expressed as percent of eviscerated carcass, body lipid content was significantly reduced at the 0.50, 0.75, (P < 0.05), and 1.0% (P < 0.01) CLA dose. Carcass lipid content (g) showed a significant decrease at the 0.75 and 1.0% CLA doses and was nearly significant at the 0.5% CLA dose (P < 0.053). All comparisons were made relative to the controls. Correspondingly, reductions in the adiposity index were observed for the 0.75 and 1.0% CLA doses relative to controls (P < 0.01). There was a trend for increased percent body protein content with higher doses of CLA, which was significant at the 1.0% dose (P < 0.01) compared with controls. Carcass ash content was not affected by CLA treatment.

Mice fed CLA tended to have enlarged spleens and livers, but this effect was not significant except at the highest dose of CLA (P < 0.01 for both organs; Table 2). CLA treatment had no effect on the weight of the kidneys, testes, or heart (Table 2). Liver samples were
analyzed by two veterinary pathologists in two separate laboratories. One laboratory reported minimal to moderate cytoplasmic vacuolization typical of lipid accumulation, which was higher \((P < 0.01)\) in the 1\% CLA group compared with the control group (Table 3). The other laboratory reported that histopathological examination of hepatic tissue showed mild to moderate cytoplasmic vacuolization consistent with the presence of intracellular glycogen. In addition, larger, clear, round vacuoles consistent with the presence of intracellular lipid deposits were present. However, there were no morphological features identified by light and transmission electron microscopy that distinguished the liver samples from the 1.0\% CLA treatment and the controls. Spleens were only mildly affected by lymphoid hyperplasia (lipid deposit), and no difference in the degree of severity was evident between the 1.0\% CLA and control groups.

Plasma leptin levels tended to decrease with the higher CLA doses, but there were no significant differences \((P > 0.05)\). Plasma insulin showed increased levels with higher doses of CLA \((P < 0.05)\), becoming significant from control in the 1.0\% group. Plasma insulin levels in the control group \((4.76 \pm 2.02 \text{ ng/ml})\) were similar to that observed previously in AKR/J mice \((3-4 \text{ ng/ml} \text{; see Ref. 5})\). Plasma glucose levels were not significantly affected by CLA treatment \((145 \pm 9 \text{ for controls and 153} \pm 11 \text{ mg/dl for 1\% CLA})\).

Time-course study. Body weight was significantly reduced by day 22 of CLA feeding compared with controls and remained lower throughout the 12 wk of treatment \((P < 0.005)\). However, as the study progressed, the difference between control and CLA treatment was diminished, particularly by 12 wk (Fig. 5). Cumulative energy intake was not affected by 12 wk of 1\% CLA treatment (Fig. 6).

As found in the dose-response study, the adipose depot most sensitive to CLA treatment was the retroperitoneal, with significant reductions in weight at all time points \((P < 0.005; \text{Fig. 7A})\). The inguinal and epididymal fat depots were significantly lower than control at week 4 and through week 12 of the study (Fig. 7, B and C). Mesenteric adipose depot weights were reduced by CLA treatment, but the effects of CLA on this depot were not apparent until week 6 (Fig. 7D).

Liver weights were significantly higher in the 1\% CLA group compared with controls at each time point \((P < 0.01; \text{Fig. 8})\). CLA treatment had a tendency to increase the weight of the spleen (Fig. 8), with a
significant difference observed at the 8-wk time point (P < 0.01) but not at the 12-wk time point (P < 0.09). Histopathological examination of control and 1% CLA livers and spleens at the 12-wk time point confirmed the results in the dose-ranging study. Livers showed mild (grade 2) to moderate (grade 3) centrilobular fatty change, which was higher (P < 0.05) in livers from the CLA group (2.75 ± 0.27) compared with controls (1.67 ± 0.31). Splenic changes consisting of lymphoid hyperplasia were similar in the control and CLA groups.

Body composition analysis of the control and CLA-treated animals at the 12-wk time point showed that CLA had a significant effect on protein and lipid content (P < 0.0001). Whether expressed in grams or percent of eviscerated carcass, 1.0% CLA treatment significantly decreased carcass lipid content compared with controls (P < 0.0005; Table 4). In contrast, carcass protein was significantly increased in the 1.0% CLA group (P < 0.0005). There was also an indication that ash may be increased by CLA treatment when expressed as percent of eviscerated carcass (P < 0.09; Table 4).

Plasma insulin levels showed a tendency to increase in the CLA treatment over time, becoming significantly different from controls at the 8-wk time point (P < 0.05; Fig. 9A). Interestingly, levels of plasma leptin in the CLA-treated animals were significantly reduced at the 6-wk time point (P < 0.001) but were not different from control values at the 8- and 12-wk time points. Plasma glucose levels were not significantly different between CLA-treated and control animals at the 12-wk time point (207 ± 13 in controls vs. 223 ± 9 in CLA group).

DISCUSSION

The major findings of the current studies are that CLA feeding causes a reduction in body fat accumulation, as well as an increase in body protein accumulation in male mice (AKR/J) at relatively low doses (0.5–1.0% CLA) and in as short a time as 2 wk at the 1% CLA dose. This confirms another study of the effects of 0.5% CLA in ICR female mice (18) and the findings of our previous study in male mice (24). In contrast to our previous report, there was no consistent suppression of food intake with the 1% CLA diet. We believe that the discrepancy regarding food intake between studies was due to differences in the experimental design. In the previous study, the novel high-fat diet was started at the same time as the CLA treatment. The interaction of the novel diet and CLA diet simultaneously could have led to a conditioned aversion (6) to the new diet if there were mild behavioral or central nervous system effects of the CLA. In the studies described in this report, an association between the novel taste of the diet and any central nervous system effects of CLA treatment were avoided by staging the presentations. The potential ability of CLA to condition a taste aversion should be explored further but was outside the scope of the present studies.

However, CLA may have an effect on food intake. The intake of the 0.25% CLA group was actually higher than the control group, there was a suggestion (P < 0.09) that the intake of the 0.75% CLA group was lower than controls (Fig. 2), and the cumulative intake of the
CLA group during the time-course study appeared to be lower at 8 wk \((P, 0.08)\). In a 28-day study examining the effects of 0.5% CLA in female mice, food intake was significantly lower at two time points but was not significantly different at the end of the study (18). Although not reported in that study, it appears from the daily food intake reported in Fig. 2 that cumulative food intake would be lower over the 28-day study. However, in the current study, the cumulative intake of the 1% CLA group was nearly identical to that of the control group in both the dose-response study and by the end of the time-course study. We conclude that, although CLA may have a slight and transient effect on energy intake, the effect on body fat accumulation at the 0.5, 0.75, and 1.0% CLA doses was not due to a suppression of food intake. The significant increase of food intake by the 0.25% CLA dose is interesting and deserves further study.

There are several possible mechanisms for the reduced body fat accumulation in response to CLA feeding. We previously demonstrated that CLA feeding causes an increase in energy expenditure and an increase in fat oxidation (24). That CLA increases fat oxidation is also supported by increased carnitine palmitoyltransferase activity in fat pad, muscle, and liver of mice fed CLA (18). Another possibility is a reduction in triglyceride uptake and storage in adipose depots, since CLA has been shown to inhibit lipoprotein lipase activity in 3T3-L1 adipocytes (18).

As we previously reported, the response of fat pads from different sites to CLA feeding was varied, ranging from a 40% reduction in the retroperitoneal and mesenteric fat pads to a 22% decrease in the epididymal fat pad. The fat pads also responded rapidly to CLA treatment, with significant decreases observed in the retroperitoneal fat pad 2 wk after the start of CLA treatment, the inguinal and epididymal sites at 4 wk, and the mesenteric fat pad at 6 wk. Although the majority of the CLA effect was observed by 4–6 wk, the decrease in fat pads relative to controls was not a transient effect, as the differences were observed throughout the 12-wk time-course study. The time-course study further suggests that CLA treatment reduces the accumulation of carcass lipid but does not prevent it when mice are fed a high-fat diet.

The feeding of CLA in this study was associated with a paradoxical increase in circulating insulin levels, suggesting that the animals were becoming insulin resistant over time. This was observed by 6 wk of feeding 1% CLA, and this hyperinsulinemia became even more pronounced by 12 wk of treatment (Figs. 4

<table>
<thead>
<tr>
<th>Table 4. Body composition of mice in time-course study</th>
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<td>Body composition, %</td>
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<tr>
<td>Fat</td>
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<tr>
<td>Protein</td>
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<td>Ash</td>
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<tr>
<td>Water</td>
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<td>Adiposity index</td>
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Data are presented as means ± SE. Body composition data after 12 wk of CLA treatment are presented as % of eviscerated carcass as well as in grams. Adiposity index is calculated by dividing the sum of the fat pad weights by the weight of the eviscerated carcass minus the weight of the fat pads. *P < 0.0005, significant difference from control.
and 9). The results are paradoxical, since a reduction in adipose mass, which was achieved by CLA treatment, is usually associated with improved insulin sensitivity and decreased plasma insulin levels (13). In addition, CLA has been shown to improve glucose tolerance and insulin sensitivity in the Zucker obese fatty rat, which has an early-onset massive obesity (8). Data from a reporter assay suggested that CLA may be acting as a weak PPARγ agonist, much like troglitazone, which is an insulin-sensitizing agent (8). It should also be noted that the mouse strain used in the current studies (AKR/J) has been shown to have higher insulin levels and respond to a high-fat diet with higher insulin levels than SWR/J mice (5, 25). Therefore, we will need to verify the effect of CLA on insulin levels in other strains.

The apparent paradox in the study reported here might be explained by the milder degree of obesity in the dietary obese mouse model compared with the Zucker fatty rat and the reported effects of CLA to stimulate lipolysis in adipocytes (18). If CLA induces a mild chronic lipolytic state such that fatty acids are more readily available to skeletal muscle, this could lead to a mild insulin resistance. Elevations of fatty acids are known to lead to impaired insulin-stimulated glucose disposal, perhaps through the glucose fatty acid cycle (19). Theoretically, the greater availability of fatty acids for oxidation inhibits glucose oxidation, which may be due to a reduced glucose uptake in muscle (7) and in the whole body (22). Fatty acids may also prime the β-cell for glucose-stimulated insulin secretion (3, 16, 21). When palmitate is added to the perfusion media in isolated pancreata, glucose-stimulated insulin secretion is restored in the presence of an inhibitor of malonyl-CoA synthesis (3). This effect of fatty acids on insulin release is dependent on chain length and degree of unsaturation (15); however, the effects of CLA on islet function have not been tested.

Therefore, the moderate hyperinsulinemia induced by CLA could be attributable to effects on both glucose utilization, resulting in mild peripheral insulin resistance, and CLA, having direct stimulatory effects on islet insulin release. The reason for the apparent improvement in insulin sensitivity in Zucker fatty rats could be due to the relative balance of effects in this model of severe obesity. Zucker fatty rats already have high levels of insulin and free fatty acids (14) due to decreased fatty acid oxidation (1, 12, 23). In addition, these animals exhibit an increased fatty acid esterification, leading to increased lipid deposition in pancreatic islets that may negatively alter β-cell function (11). Therefore, CLA may decrease fatty acid levels by increasing fatty acid oxidation as well as influencing PPARγ activation, resulting in improved insulin sensitivity. Clearly, the effect of CLA in various models of obesity must be tested to provide further insights into the mechanisms by which CLA influences lipid and carbohydrate metabolism.

The mechanism by which CLA increases total protein content is unknown. This response could be due to differential insulin resistance among tissues (7). If adipocytes become insulin resistant with CLA treatment this could partially explain the paradoxical increased fatty acid oxidation with increased insulin levels. If at the same time, skeletal muscle remains sensitive to insulin, the increased insulin levels could lead to increased protein synthesis and deposition. Another possibility is that CLA somehow causes an increase in growth hormone. This later possibility could also play a role in the increased fatty acid oxidation and increased insulin levels.

In summary, feeding mice CLA at 1% or lower concentrations as a dietary admixture reduces body fat accumulation and increases body protein accumulation. These effects of CLA on body composition occur fairly rapidly; some can be observed as early as 2 wk after treatment and persist. The increased liver weights observed in our previous and current study appear to be due to lipid accumulation. This could be due to the increased delivery of fatty acids to the liver in response to CLA feeding. That CLA feeding in the current studies causes an increase in serum insulin even as body fat stores are reduced is an interesting observation that requires further study.

This study was supported by the National Cattlemen’s Beef Association and Kraft Foods, Inc.

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Received 31 August 1998; accepted in final form 12 January 1999.
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