Comparison of the Effects of Triacylglycerol-CLA and Free Fatty Acid-CLA on Hepatic Lipid Metabolism in OLETF Obese Rats

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Abstract: The effects of conjugated linoleic acids with triacylglycerol form (TAG-CLA) and free fatty acid form (FA-CLA) on lipid mass and lipid metabolizing enzymes in the liver, and on serum metabolites was compared in Otsuka Long-Evans Tokushima fatty (OLETF) rats, which have high levels of hepatic and serum triacylglycerol (TAG) and higher serum insulin, glucose, leptin and tumor necrosis factor-alpha (TNF-α). Obese rats fed either forms of CLA significantly lowered the concentrations of TAG in the liver and serum to a similar extent, compared to the control dietary group. It was further demonstrated that both forms of CLA markedly enhanced mitochondrial carnitine palmitoyltransferase (CPT) activity, but reduced microsomal phosphatidate phosphohydrolase (PAP) activity in the liver. This suggests that the CLA-induced reduction in liver lipid mass may be attributable to increase in fatty acid β-oxidation. Both forms of CLA also lowered serum leptin levels. TAG-CLA had no significant effects on serum insulin and glucose levels, whereas FA-CLA raised the serum glucose level without affecting insulin levels. TNF-α concentrations were comparable between the groups. These results suggest that TAG-CLA and FA-CLA reduce TAG levels in the liver and serum, but have different effects on fasting serum glucose levels in OLETF obese rats.

Key words: conjugated linoleic acid (CLA), liver, lipid metabolism, carnitine palmitoyltransferase, triacylglycerol, obesity, OLETF rat

1 Introduction

CLA is a group of polyunsaturated fatty acids found in beef, lamb, and dairy products that exist as positional- and stereo-isomers of linoleic acid (9cis,12cis octadecadienoic acid) (1, 2). Numerous health benefits have recently been attributed to CLA in experimental animals and in human subjects, including protection against obesity, arteriosclerosis and several cancers (3-10).

CLA is naturally present as a component of TAG in foods (1), but most of the studies were conducted with the free fatty acid form (6-8), because of problems with the cost and/or availability of CLA preparations. Hence, it is necessary to study whether TAG-CLA and FA-CLA have similar or different effects on lipid metabolism to evaluate the relevance of later form in biological research. CLA influences lipid metabolism in adipose tissue (6-8). Earlier, we showed that feeding obese rats with both forms of CLA preparation reduces
abdominal fat mass, concomitant with the enhanced CPT activities in white adipose tissue, brown adipose tissues and muscle in OLETF rats (9, 10). We observed enhanced energy expenditure after CLA feeding in obese rats.

The liver is the main organ in which lipid and lipoprotein metabolism occurs. Some studies suggest that dietary CLA increases hepatic lipid contents in mice, and concomitant decreases body fat mass (6-8), although it is not so clear whether these results can extrapolate to rats and humans. Hence, we investigated the effects of CLA on hepatic lipid content and lipid metabolism in an obese model animal, OLETF rats, which are known to be prone to non-insulin dependent diabetes mellitus (NIDDM), and to show hypertriglyceridemia and hyperinsulinemia (11-13). The effects of the two CLA forms on serum concentrations of lipids, glucose, insulin, leptin and TNF-α were also compared.

2 Materials and Methods

2.1 Animal Experiment and Diet Composition

Both male OLETF and Long-Evans Tokushima Otsuka (LETO; wild lean type) rats of 5-wk old (B.W. 135-140 g) were supplied from Tokushima Research Institute of Otsuka Pharmaceutical Co. Ltd., (Tokushima, Japan). Rats were housed individually in metal cages in a temperature-controlled room under 12 h light/dark cycle. After one week adaptation period, the rats were divided into groups and given the following diets (Table 1): control diet (both LETO and OLETF control): 6.5% safflower oil, 1% TAG-CLA diet: 1% TAG-CLA+5.5% safflower oil, 1% FA-CLA diet: 1% FA-CLA+5.5% safflower oil. Both form of CLAs and safflower oil were supplied by Rinoru Oil Mills Co., Ltd. (Nagoya, Japan). The mass of total 18:2 in each diet was almost identical and the CLAs provided as TAG-form and FA-form contained same isomers (33.2% c-9, t-11/t-9, c-11-CLA, 34.2% t-10, c-12 and other CLA isomers and fatty acids) (Table 2). After the experimental period for 4 weeks being over, the rats were sacrificed after 10 h fasting and blood was taken from venacava. Serum was separated by centrifuging blood at 3000 rpm for 15 min. Livers were excised and stored at −80°C. The animal experiment was conducted according to the guideline provided by the ethical committee of experimental animal care at Saga University.

2.2 Preparation of Liver Subcellular Fractions

Liver homogenates and subcellular fractions were prepared as described previously (14). A piece of liver was homogenized in 4 volume of ice-cold 0.25 M sucrose homogenate solution containing 1 mM EDTA, 0.2 mM dithiothreitol and 10 mM Tris-HCl (pH 7.4) buffer. After centrifuging to sediment nuclei, cell debris, and the mitochondrial fraction at 20,000 xg for 20 min at 4°C, the resulting supernatant was recentrifuged at 105,000 xg for 45 min at 4°C to sediment microsomes, and the remaining supernatant being used as the cytosolic fraction. Microsomal pellet was resuspended with a small volume of the homogenate buffer solution. The fractions were stored at −80°C. Protein was assayed by the method of Lowry et al. (15) using bovine serum albumin as a standard.

2.3 Lipid Analysis

Lipids were extracted and purified by the method of Folch et al. (16). The hepatic concentrations of TAG

<table>
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<th>Ingredients</th>
<th>Control</th>
<th>TAG-CLA</th>
<th>FA-CLA</th>
</tr>
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<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>α-Corn starch</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
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<tr>
<td>DL-Methionine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
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<tr>
<td>Vitamin mixture</td>
<td>1.0</td>
<td>1.0</td>
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</tr>
<tr>
<td>Mineral mixture</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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</tr>
<tr>
<td>Safflower oil</td>
<td>6.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>CLA</td>
<td>—</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Sucrose to make 100</td>
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<table>
<thead>
<tr>
<th>Ingredients</th>
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<th>TAG-CLA</th>
<th>FA-CLA</th>
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<tbody>
<tr>
<td>16 : 0</td>
<td>6.7</td>
<td>7.3</td>
<td>6.6</td>
</tr>
<tr>
<td>18 : 0</td>
<td>2.5</td>
<td>2.4</td>
<td>2.4</td>
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<tr>
<td>18 : 1</td>
<td>15.9</td>
<td>15.2</td>
<td>18.1</td>
</tr>
<tr>
<td>18 : 2 (LA)</td>
<td>73.0</td>
<td>1.4</td>
<td>0.72</td>
</tr>
<tr>
<td>18 : 2 (CLA)*</td>
<td>—</td>
<td>73.2</td>
<td>71.6</td>
</tr>
<tr>
<td>Others</td>
<td>1.9</td>
<td>0.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The CLA provided as TAG form or FA form contained same isomers: 33.2% of 9c, 11t-CLA; 34.2% of 10t, 12c-CLA; and other isomers including 9c, 11c/10c, 12c-CLA; and 9t, 11t/10t, 12t-CLA.
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and cholesterol were measured by the method of Fletcher (17), and Sperry and Webb (18), respectively. The hepatic phospholipid was quantified by phosphorus content as reported previously (14). The total cholesterol, HDL-cholesterol, TAG and phospholipid in serum were measured enzymatically with commercial kits supplied by Wako Pure Chemical Ind. (Osaka, Japan) following the procedure supplied.

2.4 Assays of Enzyme Activity

The activities of CPT and PAP, and protein concentration were measured as described in the previous study (14).

CPT (EC 2.3.1.23) activity was assayed by measuring the CoA-SH formation. The reaction mixture contained 116mM Tris HCL (PH=8.0), 2.50 mM EDTA neutralized to PH 8.0 with Tris, 2.50 mM L-carnitine, 0.50 mM DTNB, 75mM palmitoyl-CoA and 0.2% Triton-X 100. The whole solution was equilibrated at 25°C. The reaction was initiated by addition of enzyme source and the absorbance was monitored for 2-4 min. The L-carnitine independent rate was determined in a second cuvette by only omitting L-carnitine. The difference between with and without L-carnitine gave the L-carnitine dependent rate for formation of CoA-SH. The assay was conducted in freeze-thawed homogenate.

PAP (EC 3.1.3.4) activity was assayed by the method of Possmayer and Walton with slight modification (19). The reaction mixtures contained 0.05 M Tris-HCl (pH 7.0), 1 mM L-α-phosphatidate and 1 mM phosphatidylcholine liposomes suspended in 1.55 M sodium chloride, in the presence of 1.25 mM magnesium sulfate, and 50 to 100 mg of liver enzyme protein in a final assay volume of 0.2 ml. The mixture was incubated for 15 min at 37°C and reaction was terminated by the addition of 0.8 ml of a solution containing 0.13% sodium dodecyl sulfate, 1.25% ascorbic acid, 0.32% ammonium molybdate-4H₂O and 0.75 N H₂SO₄ and the liber- ated inorganic phosphate was measured. The phosphomolybdate color was developed at 45°C for 20 min and the absorbance was measured at 820 nm. Non-enzymat- ic phosphate release was determined by inactivating the enzymes by boiling for 1 min without substrate. The enzyme activity expressed as nanomole in one minute per mg protein.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured as previously described (20). The reaction solution contained 0.32 M Tris-HCl (pH 7.6), 50 mM MgCl₂, 66 mM glucose-6-phosphate, 24 mM NADP⁺, 1unit 6-phosphogluconate dehydro- genase. The reaction solution was preincubated at 27°C, and the reaction was initiated by adding of cytosol (250 mg protein) in a final assay volume of 3.0 ml at 27°C for 2 min. The absorbance was determined at 340 nm. G6PDH activity was expressed as nanomole of enzyme produces NADPH/min/mg protein.

Malic enzyme (ME; EC 1.1.1.40) activity was determined as previously described (21). The reaction solution contained 0.4 M triethanolamine (pH 7.4), 30 mM malic acid, 0.12 M MnCl₂ and 3.4 mM NADP⁺. The reaction solution was preincubated at 27°C, and the reaction was initiated by adding of cytosol (250 mg protein) in a final assay volume of 3.0 ml at 27°C for 2 min. The absorbance was determined at 340 nm. Malic enzyme activity was expressed as nanomole of enzyme produces NADPH/min/mg protein.

2.5 Assays of Serum Concentrations of Insulin, Glucose, Leptin and TNF-α

Serum insulin concentration was determined with a radioimmunoassay kit (Linco, St. Charles, MO) standardized against rat insulin. Serum leptin concentration was determined with a radioimmunoassay kit (Linco, St. Charles, MO) for specifically determining rat leptin. The glucose in serum was measured enzymatically with commercial kits supplied by Wako Pure Chemical Ind. (Osaka, Japan) following the procedure supplied. Serum TNF-α concentration was determined with a rat TNF-α ELISA kit supplied by Wako Pure Chemical Ind. (Osaka, Japan) following the procedure supplied.

2.6 Statistical Analyses

Each value is presented as means ± SE. Data were analyzed by one-way ANOVA, all differences being inspected by Duncan’s new multiple-range test (22). A difference was considered significant at p<0.05.

3 Results

3.1 Lipid Concentrations in the Liver

Weight gains were slightly less (5% vs. control) in both CLA groups in comparison to the OLETF controls (data not shown). Feeding efficacy was comparable between groups. Relative liver weight (OLETF 4.27 ± 0.14 vs. LETO 3.66 ± 0.08 g/100g BW) and liver TAG content (OLETF 26.1 ± 1.8 vs. LETO 18.5 ± 1.7

mg/g liver) were markedly higher in OLETF controls than in LETO rats (Fig. 1). Administration of TAG-CLA significantly reduced hepatic TAG content compared to the OLETF controls (Fig. 1). The same level of reduction in TAG was observed in the FA-CLA group. No significant effects were seen, however, with either CLA diet on hepatic cholesterol or phospholipid content.

3.2 Serum Concentrations of Lipids

Serum TAG level was higher in the OLETF rats compared to the LETO rats. Treatment with both forms of CLAs lowered TAG concentration compared to the corresponding controls (Fig. 2). There were no significant differences in serum cholesterol and phospholipid levels between the OLETF groups.

3.3 Serum Concentrations of Glucose, Insulin, Leptin and TNF-α

Serum concentrations of glucose, insulin, leptin and TNF-α were higher in the OLETF controls in comparison to LETO controls, as shown in Fig. 3. Dietary TAG-CLA had no significant influence on serum concentrations of glucose and insulin, although the latter tended to be lower than in the corresponding controls. FA-CLA raised the serum glucose level, although serum insulin levels were comparable to the controls. Serum leptin concentration was lower in both CLA groups in comparison to controls. TNF-α levels were comparable between these groups.

3.4 Activities of Enzymes Involved in Lipid Metabolism

Hepatic CPT activity is shown in Fig. 4. CPT activity was lower in OLETF rats than in LETO rats, suggesting decreased fatty acid oxidation in these obese rats. A similar and significant enhancement of CPT activity (approximately 2-fold) was observed in both CLA groups compared to controls. There were no significant differences in microsomal PAP activity between the groups. ME and G6PDH activities were comparable between the OLETF groups, although administration of CLA tended to increase those activities.
The present study aimed to compare the effects of TAG-CLA and FA-CLA on liver lipid metabolism and serum metabolites in OLETF obese rats, which are known to be prone to NIDDM and have hypertriglyceridemia at an early stage and hyperinsulinemia in adulthood (11). As reported previously, serum concentrations of lipids, glucose, insulin, leptin and TNF-α toward to be higher in OLETF rats in comparison to LETO controls (Fig. 2, 3).

Earlier, we found that administration of CLA to male OLETF rats caused a reduction in abdominal fat tissue mass, which it was associated with enhancement of CPT activity in adipose tissue and muscle (9, 10), and increased energy expenditure (23). The liver is the central organ regulating lipid and lipoprotein metabolism, but little is known about the effect of CLA on liver lipid metabolism. Hepatic TAG metabolism is regulated by fatty acid synthesis and oxidation and lipoprotein secretion. Results of the present study revealed that TAG-CLA and FA-CLA reduce hepatic TAG mass significantly (Fig. 1), although hepatic concentrations of cholesterol and phospholipid were comparable between groups. CLA therefore has the ability to reduce both hepatic and serum TAG levels. The findings are in contrast with the earlier reports in which CLA accumulated TAG in mouse liver (24). The reasons for these differences may be species-specific. In this context, no evidence of hepatic injury or fatty liver has been reported with the supplementation of CLA in humans (25, 26).

In the present study, enhancement of hepatic CPT activity was seen by the treatment of both forms of CLA (Fig. 4). As CPT is a key enzyme necessary for fatty acid oxidation (13), these results suggest stimulation of fatty acid oxidation in the liver. In contrast, there were no significant changes in the activities of hepatic PAP, ME and G6PDH, indicating that CLA did not affect the synthesis of fatty acid and TAG in the liver. Some studies reported that CLA inhibited the hepatic activity of stearoyl-CoA desaturase (27, 28), a key enzyme in the desaturation of stearic acid to oleic acid. The present results suggest that CLA may directly affect fatty acid oxidation rather enhancing esterification process. Increased beta-oxidation may ultimately prevent the storage of TAG in the liver and adipose tissues. There are several possible mechanisms by which CLA may induce increased CPT activity. One proposed
mechanism is that CLA modulates lipid metabolism, in part by a mechanism dependent on the activation of the group of nuclear transcripional factors, peroxisome proliferator-activated receptors (PPARs) (29). In the liver, PPARα is thought to be a critical transcriptional factor for lipid metabolism, because several genes coding for enzymes involved with β-oxidation contain a functional peroxisome proliferator-responsive element in their enhancer regions (30). Several isomers of CLA are in fact high affinity ligands and activators of PPARα (31), and PPARα is thought to play a role in the ability of CLA to modulate lipid metabolism (32, 33). Recently, studies in PPARα-null mouse suggested, however, that PPARα may not be critical for lipid metabolism, because PPARα null mice fed a diet containing CLA had similar responses in comparison to the wild type, including CLA-dependent reduction of adiposity and induction of some PPAR-responsive genes in the liver (34). It is therefore likely that CLA also modulates lipid metabolism via PPARα-independent mechanisms.

Earlier, we showed that 10t,12c-CLA isomer reduced apoprotein B100 secretion in cultured hepatoma HepG2 cells (35). This is supported by the observation that serum TAG is lowered significantly by CLA in this study. We also observed that dietary CLA inhibits liver MTP activity (Wang et al., in press). These data suggest that CLA reduces TAG mass and inhibits MTP activity in the liver, which results in lower serum TAG concentrations.

It has been reported that the onset of diabetes was delayed by CLA in the Zucker diabetes fatty rat model (36). A recent study demonstrated that CLA enhanced glucose uptake in muscle in Zucker diabetes fatty rats (37). We were unable, however, to detect any influence of TAG-CLA on serum fasting glucose level, but TAG-CLA showed a tendency to reduced insulin levels in the present study, as shown in Fig. 3. In addition, FA-CLA modestly increased serum glucose levels. Hence, it is likely that different forms of CLA have different effects on fasting glucose in OLETF rats. It was reported that CLA reduces fasting insulin levels in diabetic animals, but enhances fasting insulin concentration in mice, pigs and humans (24, 38, 39). TNF-α is associated with insulin resistance and abdominal obesity in humans (40). In the present study, TAG-CLA had no significant effect on serum TNF-α levels, although FA-CLA tended to increase TNF-α levels. Further work is needed to explore the effects of the forms of CLA forms on these metabolic parameters.

In conclusion, both TAG-CLA and FA-CLA reduced hepatic and serum TAG concentrations in OLETF obese rats to similar degrees, which was associated with enhanced CTP activity. This suggests that TAG-CLA and FA-CLA have same ability to reduce liver and serum TAG levels, but the effects on serum glucose levels were different: TAG-CLA had no effect but FA-CLA raised serum glucose levels.

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References

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