Dietary Conjugated Linoleic Acid Alleviates Nonalcoholic Fatty Liver Disease in Zucker (fa/fa) Rats

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ABSTRACT Nonalcoholic fatty liver disease (NAFLD) is the preferred term to describe the spectrum of liver damage ranging from hepatic steatosis to steatohepatitis, liver fibrosis, and cirrhosis, and it is emerging as the most common liver disease in industrialized countries. Thus, the discovery of food components that would ameliorate NAFLD is of interest. Conjugated linoleic acid (CLA), a mixture of positional and geometric isomers of linoleic acid, has attracted considerable attention because of its potentially beneficial biological effects both in vitro and in vivo. We tested whether dietary CLA protects Zucker (fa/fa) rats from hepatic injury. After 8 wk of feeding, hepatomegaly, hepatic triglyceride (TG) accumulation, and elevated hepatic injury markers in plasma were markedly alleviated in CLA-fed Zucker rats compared with linoleic acid–fed (control) rats. These effects were attributed in part to the enhanced hepatic activities of carnitine palmitoyltransferase, a key enzyme for fatty acid β-oxidation, and microsomal TG transfer protein, an important factor for lipoprotein secretion due to the CLA diet. We previously reported that the severe hyperinsulinemia in control Zucker rats was attenuated in CLA-fed rats due to an enhanced level of plasma adiponectin, which improves insulin sensitivity. In the present study, the adiponectin concentration was increased and the mRNA expression of tumor necrosis factor-α, an inflammatory cytokine, was markedly suppressed in the liver of CLA-fed Zucker rats. We speculate that the enhanced level of liver adiponectin may prevent the development and progression of NAFLD in CLA-fed Zucker rats. J. Nutr. 135: 9–13, 2005.

KEY WORDS: • conjugated linoleic acid • nonalcoholic fatty liver disease • adiponectin • tumor necrosis factor-α • Zucker (fa/fa) rats

Nonalcoholic fatty liver disease (NAFLD) is emerging as the most common liver disease in industrialized countries (1–3). NAFLD is the preferred term to describe the spectrum of liver damage, ranging from hepatic steatosis to steatohepatitis, liver fibrosis, and cirrhosis. Most liver-related morbidity and mortality are associated with the development of cirrhosis. Cirrhosis is most likely to occur in individuals who have progressed from hepatic steatosis to steatohepatitis. Although the processes through which steatohepatitis evolves from hepatic steatosis are not fully understood, it is necessary to develop effective therapies for the treatment of NAFLD. Several experimental models of NAFLD exist including the Zucker (fa/fa) rat (4–6). Zucker rats develop a syndrome with multiple metabolic and hormonal disorders that shares many features with human obesity. Zucker rats have hyperphagia, because they have a missense mutation on the leptin receptor gene; they become obese and develop hyperinsulinemia, diabetes, hypertension, and NAFLD. Therefore, Zucker rats are also a good model for Syndrome X, in which multiple risks cluster in an individual.

Using functional food components is one of the strategies to reduce the risk of NAFLD. Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. It is found in meat and dairy products, such as beef, milk, and processed cheese (7,8). CLA has attracted considerable attention because of its potentially beneficial biological effects in inhibiting carcinogenesis, attenuating atherosclerosis, alleviating diabetes, and reducing body fat in animal models and humans (9–13). Recently, we also reported that CLA and its isomer (10trans,12cis-CLA) prevent the development of obesity-related hypertension in obese animals (14,15). In contrast to many favorable findings, feeding a CLA mixture and the 10trans,12cis-CLA isomer with a low-fat diet induced lipodystrophy (characterized by an increase in hepatic lipid concentration concomitant with a decrease in body fat mass) in mice (16,17). However, these effects have been found only in mice, and have not been reported in other species, including humans (18–20). It was suggested that lipodystrophy occurs in mice because they are so sensitive to CLA-induced body fat reduction. It should also be noted that increasing the amount of fat
in a CLA-supplemented diet substantially reduces lipodystrophy in mice (21). Because NAFLD is often linked to obesity and diabetes in humans, we evaluated the effect of dietary CLA on the development of NAFLD in obese, diabetic rats.

**MATERIALS AND METHODS**

**Animals and diets.** All aspects of the experiment were conducted according to the guidelines provided by the ethical committee of experimental animal care at Saga University. Male Zucker rats, 6 wk old, were purchased from Japan SLC. The rats were housed individually in metal cages in a temperature-controlled room (24°C) under a 12-h light:dark cycle. After a 1-wk adaptation period, the rats were assigned to 2 groups (n = 6) and fed 1 of 2 diets: a purified diet supplemented with 5% corn oil plus 1% high-oleic safflower oil (control group), or a purified diet supplemented with 5% corn oil and 1% CLA (CLA group). All sample oils were provided by Riorin Oil Mills. The composition of the semisynthetic diets and their fatty acid contents are given in Table 1. The rats consumed the diets for 8 wk.

**Measurement of lipid levels and hepatic injury marker activities in plasma.** At the end of the feeding period, the rats were killed by aortic exsanguination under diethyl ether anesthesia. EDTA plasma was prepared from blood by centrifugation at 1750 × g for 15 min. Liver and abdominal white adipose tissue were excised immediately. The plasma triglyceride (TG) and cholesterol levels were measured using commercial enzyme assay kits (Triglyceride E-test and Cholesterol E-test; Wako Pure Chemicals). Activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) in plasma were measured using commercial enzyme assay kits (Wako Pure Chemicals).

**Preparation of liver subcellular fractions.** A piece of liver was homogenized in 6 volumes of a 0.25 mol/L sucrose solution that contained 1 mmol/L EDTA in a 10 mmol/L tris-HCl buffer (pH 7.4). After precipitating the nuclei fraction, the supernatant was centrifuged at 10,000 × g for 10 min at 4°C, to obtain mitochondria. The resulting supernatant was recentrifuged at 125,000 × g for 60 min to precipitate microsomes, and the remaining supernatant was used as the cytosol fraction. The protein concentration was determined according to the method of Lowry et al. (23), with bovine serum albumin used as the standard.

**Assays of enzyme activity.** The activity of carnitine palmitoyltransferase (CPT) in the hepatic mitochondrial fraction was measured according to the method of Markwell et al. (24). The activity of the microsomal TG transfer protein (MTP) in the hepatic microsomal fraction was measured using a fluorometric MTP activity assay kit (Calbiochem). The assay was performed according to the manufacturer’s recommendation.

**Table 1**

<table>
<thead>
<tr>
<th>Composition of the experimental diets</th>
<th>Control</th>
<th>CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td>g/100 g</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral mixture&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mixture&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Safflower oil (high linoleic)</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CLA&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>to make 100</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Mineral and vitamin mixture, AIN-76 (22).<br><sup>2</sup> Contained different CLA isomers: 46.0% 9c,11t; 47.3% 10t,12c; 3.2% 9c,11c/10c,12c; and 0.4% 9t,11t/10t,12t.

**Measurement of triglyceride and adiponectin levels in the liver.** Liver lipids were extracted and purified according to the method of Folch et al. (25). The concentration of TG was measured according to the method of Fletcher (26). For the assay of adiponectin concentration in the liver, a piece of liver was homogenized in a 0.1 mol/L tris-HCl buffer (pH 7.6) that contained 1 mol/L NaCl, 2% fat-free bovine serum albumin, 2 mmol/L EDTA, 80 U/L aprotinin, and 0.02% NaN₃, at 15% wet tissue wt/vol. The homogenate was centrifuged at 13,500 × g for 30 min, and the supernatant was subjected to a commercial ELISA assay kit (Otsuka Pharmaceutical) for mouse/rat adiponectin (MW: 26,839.75).

**Analysis of mRNA expression.** Total RNA was extracted from 300 mg of liver, using a TRI ZOL Reagent (Invitrogen). A TaqMan Universal PCR Master Mix (Applied Biosystems); Assay-on-Demand, Gene Expression Products [Rn00562055_m1 for tumor necrosis factor-α (TNF-α), Rn00567070_m1 for insulin receptor (Ins-r), Hs99999901_s1 for 18S RNA, Applied Biosystems], and TaqMan MGB Gene Expression Kits for adiponectin receptor (ADP-r) 1 and 2 were used for the quantitative real-time RT-PCR analysis of TNF-α, Ins-r, 18S RNA, ADP-r1, and ADP-r2 expression in the liver. The details of the TaqMan Gene Expression Kits were as follows: ADP-r1 (forward primer, 5'-GGCTTTATGCTGCTCGGATT-3'; reverse primer, 5'-GATGAGCTGGAAACCATTAGTCAA-3'; and TaqMan MGB probe, 5'-FAM-AGGCGCTTICTTTCG-MGB-3'), ADP-r2 (forward primer, 5'-CTGCCACCATAGGGCAGATAG-3'; reverse primer, 5'-AAAGACGGCTCAGGGGAATAG-3'; and TaqMan MGB probe, 5'-FAM-CTATATACAOGGCTCG-MGB-3'). The amplification was performed with a real-time PCR system (ABI Prism 7000 Sequence Detection System; Applied Biosystems). Results were expressed as a relative value after normalization to the 18S RNA expression.

**Statistical analysis.** All values are expressed as means ± SEM, n = 6. Statistical analysis was carried out with Stat View J-4.5 (Abacus Concepts). The significance of differences between means for the 2 groups was determined by Student’s t test. Linear regression analysis was used to assess the relation between plasma adiponectin levels and enzyme activities. Differences were considered significant at P < 0.05.

**RESULTS**

The 2 groups of rats did not differ in final body weight (control, 394 ± 8; CLA, 392 ± 6 g), body weight gain (control, 193 ± 7; CLA, 190 ± 6 g/8 wk), food intake (control, 1052 ± 5; CLA, 1053 ± 18 g/8 wk), or food efficiency (control, 183 ± 0.7; CLA, 181.1 ± 0.5 g gain/100 g food intake). In contrast, the relative liver weight and TG concentration differed between rats fed the control and CLA diets (Table 2). After the 8-wk feeding period, control Zucker rats had severe NAFLD. The relative liver weight was 26% less in CLA-fed rats, and this was associated with a marked reduction (78%) in the TG accumulation in the liver. In contrast to the hepatic TG level, the plasma TG level in CLA-fed rats was 160% higher than that in control rats. However, the plasma total cholesterol level was lower in CLA-fed rats than in control rats (control, 6.93 ± 0.56; CLA, 5.74 ± 0.31 mmol/L; P < 0.05). Consistent with the alleviation of hepatomegaly and hepatic steatosis by the CLA diet, the activities of hepatic injury markers such as AST, ALT, LDH, and ALP were significantly decreased (by 56, 60, 58, and 45%, respectively) in the plasma of CLA-fed rats compared with controls (Table 2). These results indicate that CLA protects Zucker rats from the development of NAFLD.

To examine further the effect of the CLA diet on the liver, hepatic proteins related to lipid homeostasis were analyzed. Activities of CPT, a key enzyme of fatty acid β-oxidation, and MTP, an important factor for the assembly and secretion of lipoproteins, were significantly greater in control than in rats fed the CLA diet (Table 2). The activity of fatty acid synthase...
TABLE 2
Liver relative weight, TG concentration, and lipid metabolizing enzyme activities and hepatic injury marker enzyme activities in plasma of rats fed control or CLA diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CLA</th>
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<tbody>
<tr>
<td>Liver weight, g/100 g body weight</td>
<td>4.74 ± 0.36</td>
<td>3.50 ± 0.17*</td>
</tr>
<tr>
<td>TG concentrations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver, mmol/liver</td>
<td>7.92 ± 0.81</td>
<td>1.74 ± 0.19*</td>
</tr>
<tr>
<td>Plasma, mmol/L</td>
<td>2.08 ± 0.15</td>
<td>5.40 ± 0.31*</td>
</tr>
<tr>
<td>Plasma enzyme activity, IU/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>66.3 ± 12.7</td>
<td>23.6 ± 1.7*</td>
</tr>
<tr>
<td>ALT</td>
<td>48.3 ± 7.0</td>
<td>19.4 ± 1.5*</td>
</tr>
<tr>
<td>ALP</td>
<td>205 ± 19</td>
<td>113 ± 10*</td>
</tr>
<tr>
<td>LDH</td>
<td>502 ± 101</td>
<td>209 ± 46*</td>
</tr>
<tr>
<td>Hepatic lipid metabolism enzymes, nmol/(min · mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT</td>
<td>7.66 ± 0.35</td>
<td>9.09 ± 0.40*</td>
</tr>
<tr>
<td>MTP</td>
<td>1.65 ± 0.10</td>
<td>1.91 ± 0.05</td>
</tr>
</tbody>
</table>

* Values are means ± SEM, n = 6; † different from the control group, P < 0.05.

(FAS), a key enzyme of fatty acid synthesis, and the mRNA abundances of CPT, MTP, and FAS did not differ between the groups (data not shown). Thus, the alleviation of NAFLD by CLA is associated with the enhancement of the activities of CPT and MTP in the liver.

We previously reported that feeding the CLA diet for 8 wk to the same rats markedly reduced the plasma insulin level to 35% of control rats and increased the plasma adiponectin concentration (control, 239 ± 24; CLA, 378 ± 17 nmol/L; P < 0.05) (15). The plasma adiponectin concentration and hepatic enzyme activities in plasma of these rats were negatively correlated (vs. AST, r = -0.801, P < 0.05; vs. ALT, r = -0.846, P < 0.05; vs. ALP, r = -0.946, P < 0.05; vs. LDH, r = -0.846, P < 0.05, n = 12) (Fig. 1A and B).

In addition, the CLA diet significantly increased the level of adiponectin in the liver of Zucker rats (Table 3). Therefore, the levels of mRNAs that are related to the transport of adiponectin into the liver were measured by quantitative real-time RT-PCR (Table 3). Ins-r, Adp-r1, and Adp-r2 mRNA levels did not differ between the groups. However, the mRNA expression of TNF-α, a hepatic inflammatory cytokine, was markedly suppressed (by 86%) by the CLA diet. The suppression of TNF-α mRNA expression may contribute to the prevention of the development and progression of NAFLD in CLA-fed Zucker rats.

DISCUSSION

We investigated the effects of dietary CLA on the development and progression of NAFLD in obese, diabetic Zucker rats. The results indicated that dietary CLA protects against hepatic injury partly through the enhancement of adiponectin production in Zucker rats.

NAFLD is common in type 2 diabetic and obese patients. Although the mechanisms responsible for the TG accumulation in the liver are unclear, it was suggested that hepatic steatosis results from accelerated fatty acid mobilization from expanded visceral fat stores and their deposition in the liver as well as decreased hepatic fatty acid β-oxidation (1,2). In the present study, dietary CLA counteracted both the hepatomegaly and the hepatic steatosis that occur in control Zucker rats. The alleviation of hepatic steatosis in CLA-fed Zucker rats may be due in part to the enhancement of activities of CPT, a key enzyme of fatty acid β-oxidation, and MTP, an important factor for lipoprotein secretion, in the liver. The changes are consistent with another report showing that isolated perfused livers from rats fed CLA produced significantly more ketones than livers from rats fed linoleic acid (27). We also reported previously that dietary CLA enhanced fatty acid β-oxidation not only in the liver but also in other tissues in obese rats (28–30). The extent and the direction (increase or decrease) of changes in liver lipoprotein synthesis and secretion during the development of NAFLD have been controversial (1,2,31). However, the enhancement of MTP activity was consistent with the decrease in the hepatic TG level and the increase in the plasma TG level in CLA-fed Zucker rats. Hepatic steatosis links to progressive disorders such as steatohepatitis and cirrhosis because of the weakened resistance against viruses and other stresses, and those liver dysfunctions cause the decisive injury to the individual. Therefore, we consider that maintaining the normal function of the liver at the expense of the rise in the plasma TG level was a favorable action of CLA. In fact, plasma cholesterol and the activities of other markers of hepatic injury in plasma were markedly lower in CLA-fed Zucker rats than in control rats. Although we did not do a hepatic histological evaluation, these data suggest that dietary CLA prevented the development and progression of NAFLD to a certain extent.

Insulin resistance is the essential first pathologic step in the development of NAFLD (32–34). In fact, hepatic steatosis is now proposed as a feature of the insulin resistance syndrome.
along with type 2 diabetes mellitus, visceral obesity, hyperlipidemia, and hypertension (32–34). We previously reported that feeding CLA for 8 wk alleviated the severe hyperinsulinemia that occurs in control Zucker rats (15). The result is consistent with previous data showing that CLA acts as an insulin sensitizer and that it results in enhanced glucose tolerance as well as insulin-stimulated glucose transport activity and glycogen synthase activity in the skeletal muscle of Zucker rats (12,35,36). In addition, we reported that CLA acts as an adiponectin inducer and that it alleviates hyperinsulinemia and obesity-related hypertension in Zucker rats (15). Adiponectin is one of the most abundant secretory proteins from adipose tissue in rodents and humans (37–40). Because several reports indicated that adiponectin can enhance insulin action in vitro and in vivo (37–40), it is strongly suggested that adiponectin plays a protective role against insulin resistance. A recent study also indicated that adiponectin alleviates alcohol- and obesity-induced hepatomegaly, hepatic steatosis, and serum ALT abnormality in mice (41). In humans, it was reported that plasma adiponectin levels are associated with plasma concentrations of various liver function indices such as ALT, ALP, and γ-glutamyltransferase (41–43). These results suggest that adiponectin has a protective role against NAFLD. Very recently, 2 receptors for adiponectin were cloned and named Adp-r1 and Adp-r2 (44). The authors demonstrated that Adp-r1 is abundantly expressed in skeletal muscle, whereas Adp-r2 is found predominantly in liver. In the present study, the CLA diet significantly increased the level of adiponectin in the liver of Zucker rats. Therefore, we hypothesized that dietary CLA increases the hepatic adiponectin concentration by upregulating either or both adiponectin receptors. However, there were no differences in their expression between the 2 groups. Thus, we suggest that the transport of adiponectin into the liver was enhanced at the post-transcriptional regulation of its receptors in CLA-fed Zucker rats.

The pathogenesis of steatohepatitis, the more advanced form of NAFLD, has yet to be clearly defined, but the recent major theory is the “two-hit” hypothesis (45,46). The first “hit” is the TG accumulation within the liver. It was proposed that lipid-laden hepatocytes are more susceptible to a second “hit,” i.e., injury by oxidative stress and inflammatory cytokines, such as TNF-α. In addition, lipid peroxidation products trigger cytokine production within the liver, and this accelerates TNF-α-mediated liver injury. In the present study, the mRNA expression of TNF-α was markedly suppressed by the CLA diet, compared with the control diet, in the liver of Zucker rats. Both in vivo and in vitro studies demonstrated that adiponectin and TNF-α suppress each other’s production and also antagonize each other’s action in their target tissues (37). Thus, the increased hepatic adiponectin concentration may have prevented the expression of hepatic TNF-α mRNA and it may contribute to the prevention of the development and progression of NAFLD in CLA-fed Zucker rats.

LITERATURE CITED


