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# Conjugated Linoleic Acid-Induced Fatty Liver Can Be Attenuated by Combination with Docosahexaenoic Acid in C57BL/6N Mice

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We investigated the effect of dietary combination of conjugated linoleic acid (CLA) and docosahexaenoic acid (DHA) to attenuate CLA-induced fatty liver in C57BL/6N mice. Mice were fed semisynthetic diets that contained either 6% high linoleic safflower oil (HL-SAF), 4% HL-SAF + 2% CLA, or 3.5% HL-SAF + 2% CLA + 0.5% DHA for 4 weeks. This 4 week feeding of CLA showed hepatic lipid accumulation concomitant with the decrease in adipose tissue weight in mice. However, 0.5% supplementation of DHA to the CLA diet could alleviate fatty liver without decreasing the antiobesity effect of CLA. The CLA diet promoted fatty acid synthesis in the liver, but DHA supplementation significantly attenuated the increase in enzyme activity induced by CLA. On the other hand, serum adipocytokines, leptin and adiponectin, were drastically decreased by CLA feeding, and DHA supplementation did not affect those levels. These results show that DHA supplementation to the CLA diet can attenuate CLA-induced fatty liver through the reduction of hepatic fatty acid synthesis without affecting adipocytokine production in C57BL/6N mice.

**KEYWORDS: Conjugated linoleic acid; docosahexaenoic acid; fatty liver; C57BL/6N mice**

## INTRODUCTION

Conjugated linoleic acid (CLA) is a group of polyunsaturated fatty acids found in beef, lamb, and dairy products that exist as positional and stereoisomers of linoleic acid (9-*cis*,12-*cis* octadecadienoic acid) (*1*, *2*). Numerous health benefits have recently been attributed to CLA in experimental animals and in human subjects, including protection against obesity, teriosclerosis, cancer, diabetes, and hypertension (*3*−*10*). In contrast to many favorable findings, feeding a mixture of CLA and the 10-*trans*,12-*cis*-CLA isomer with a low-fat diet induced lipodystrophy characterized by an increase in hepatic lipid contents concomitant with the decrease in body fat mass in mice (*11*, *12*). It has been suggested that lipodystrophy may occur in mice because they are too sensitive to the CLA-induced reduction in body fat (*13*−*16*). Although CLA-induced hepatic steatosis has been found only in mice and increasing the amount of fat in a CLA-supplemented diet substantially reduces the lipodystrophy effect (*11*, *17*), we expected that the simultaneous supplementation of docosahexaenoic acid (DHA), whose lipid lowering effect has been reported (*18*−*20*), would attenuate fatty liver in CLA-fed mice.

In the present study, we investigated the effect of a dietary combination of CLA and DHA on adipose tissue weight, liver weight, hepatic lipid metabolism, and serum adipocytokine levels in C57BL/6N mice.

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## MATERIALS AND METHODS

**Animals and Diets.** Male C57BL/6N mice aged 6 weeks were purchased from Japan Clea (Osaka, Japan) and housed individually in an air-conditioned room (24 °C) with a 12 h light/dark cycle. After a 1 week adaptation period, the mice were assigned to three groups (six mice each).

The basal diets were prepared according to recommendations of the AIN-93G. Dietary fats were composed of 6% high linoleic safflower oil (HL-SAF) in the control diet, a mixture of 4% HL-SAF + 2% CLA (triglyceride form) in the CLA diet, and a mixture of 3.5% HL-SAF + 2% CLA + 0.5% DHA (ethyl ester form) in the CLA + DHA diet. The composition of the semisynthetic diets and their fatty acid contents are given in **Table 1**. The mice received the diets ad libitum using Rodent CAFE (KBT Oriental Co. Ltd., Saga, Japan) for 4 weeks. At the end of the feeding period, mice were sacrificed by exsanguination of the heart after a 9 h starvation period. White adipose tissue (WAT) and livers were excised immediately, and serum was separated from the blood. All aspects of the experiment were conducted according to the guidelines provided by the Ethical Committee of Experimental Animal Care at Saga University.

**Analysis of Hepatic Lipids and Serum Parameters.** Liver lipids were extracted according to the method of Folch et al. (*21*), and the concentrations of triglyceride and cholesterol were measured by the methods of Fletcher (*22*) and Sperry and Webb (*23*), respectively. Triacylglycerol, cholesterol, and phospholipids in serum were measured using enzyme assay kits from Wako Pure Chemicals (Tokyo, Japan). Serum insulin, adiponectin, and leptin levels were measured using commercial mouse enzyme-linked immunosorbent assay kits (Shibayagi Co. Ltd., Gunma, Japan; Otsuka Pharmaceutical Co. Ltd., Tokyo; and Morinaga Co. Ltd., Yokohama, respectively).

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**Table 1.** Composition of Experimental Diets

| | weight % | | |
|---|---|---|---|
| | control | CLA | CLA + DHA |
| casein | 20 | 20 | 20 |
| dextrinized cornstarch | 13.2 | 13.2 | 13.2 |
| sucrose | 10 | 10 | 10 |
| cellulose | 5 | 5 | 5 |
| mineral mixture (AIN-93G) | 3.5 | 3.5 | 3.5 |
| vitamin mixture (AIN-93) | 1 | 1 | 1 |
| L-cystein | 0.3 | 0.3 | 0.3 |
| choline bitartrate | 0.25 | 0.25 | 0.25 |
| safflower oil[a] | 6 | 4 | 3.5 |
| CLA[b] | | 2 | 2 |
| DHA[c] | | | 0.5 |
| *tert*-butylhydroquinone | 0.0014 | 0.0014 | 0.0014 |
| cornstarch | 40.7486 | 40.7486 | 40.7486 |

[a] Contained 73.0% linoleic acid. [b] Contained 35.6% 9-*c*,11-*t*-CLA and 36.9% 10-*t*,12-*c*-CLA. [c] Contained 94.8% DHA.

**Preparation of Liver Subcellular Fractions.** A piece of liver was homogenized with a polytron homogenizer in six volumes of a 0.25 M sucrose solution that contained 1 mM EDTA in a 10 mM Tris-HCl buffer (pH 7.4). After the nuclei fraction was precipitated, the supernatant was centrifuged at 10000*g* for 10 min at 4 °C to obtain mitochondria. The resulting supernatant was recentrifuged at 125000*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. (*24*), with bovine serum albumin used as the standard.

**Assays of Hepatic Enzyme Activity.** The enzyme activities of carnitine palmitoyltransferase (CPT) (*25*), peroxisomal $\beta$-oxidation (*26*), fatty acid synthase (FAS) (*27*), glucose 6-phosphate dehydrogenase (G6PDH) (*28*), and malic enzyme (*29*) were determined as described elsewhere.

**Analysis of mRNA Expression.** Total RNA was extracted from 100 mg of liver, using the TRIZOL Reagent (Invitrogen, Tokyo, Japan). A TaqMan Universal PCR Master Mix (Applied Biosystems, Tokyo, Japan); Assays-on-Demand, Gene Expression Products [Mm00662319_m1 for FAS, Mm00486279_m1 for acyl-coenzyme A (CoA):cholesterol acyltransferase-1 (ACAT-1), and Hs99999901_s1 for 18S RNA, Applied Biosystems, Tokyo, Japan) and TaqMan MGB Gene Expression Kits for 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (forward primer, 5′-AGTGATTGTGTCAGTATTATTGTG-GAAG-3′; reverse primer, 5′-GGTACTGGCTGAAAAGTCACAA-GAG-3′; and TaqMan MGB probe, 5′-FAM-TTGCTGTTGTATG-TAAAGT-MGB-3′) were used for the quantitative real-time reverse transcribed polymerase chain reaction (RT-PCR) analysis of FAS, ACAT-1, 18S RNA, and HMG-CoA reductase expression in the liver. 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 18S RNA expression.

**Statistical Analyses.** All values are expressed as means ± standard error. Statistical analysis was carried out with Super ANOVA (Abacus Concepts, Berkeley, CA). The significance of differences among groups was analyzed with Tukey−Kramer, and differences were considered to be significant at $p < 0.05$.

## RESULTS

**Effects of Dietary Fatty Acids on Growth Parameters. Table 2** summarizes the growth parameters of C57BL/6N mice after 4 weeks of feeding on the different diets. Although there was no significant difference in final body weight or food intake among groups, the CLA diet significantly increased the liver weight of mice, as has been reported elsewhere (*11*, *12*, *17*). However, the supplementation of DHA to the CLA diet alleviated, but not significantly, the CLA-induced hepatomegaly in mice. Weights of west subcutaneous and abdominal, such as perirenal, epididymal, and omental, WATs were significantly decreased by the CLA diet, and the supplementation of DHA to the diet did not alter the antiobesity effect of CLA.

**Table 2.** Growth Parameters of C57BL/6N Mice after 4 Weeks of Feeding[a]

| | control | CLA | CLA + DHA |
|---|---|---|---|
| final body weight (g) | 21.9 ± 0.6 | 21.9 ± 0.5 | 22.3 ± 0.3 |
| food intake (g) | 69.8 ± 2.2 | 69.8 ± 2.1 | 69.1 ± 2.2 |
| liver weight (g/100 g BW) | 4.02 ± 0.12 a | 5.70 ± 0.33 b | 5.01 ± 0.35 b |
| | adipose tissue weight (g/100 g BW) | | |
| total | 4.23 ± 0.43 a | 1.36 ± 0.09 b | 1.62 ± 0.05 b |
| abdominal | 2.95 ± 0.29 a | 1.12 ± 0.08 b | 1.31 ± 0.04 b |
| epididymal | 1.46 ± 0.17 a | 0.242 ± 0.031 b | 0.270 ± 0.023 b |
| perirenal | 0.506 ± 0.070 a | 0.160 ± 0.016 b | 0.170 ± 0.026 b |
| omental | 0.981 ± 0.065 a | 0.722 ± 0.065 b | 0.866 ± 0.039 ab |
| subcutaneous | 1.28 ± 0.14 a | 0.236 ± 0.010 b | 0.316 ± 0.018 b |

[a] Values are expressed as means ± standard errors for six mice. Different superscript letters show significant differences at $p < 0.05$.

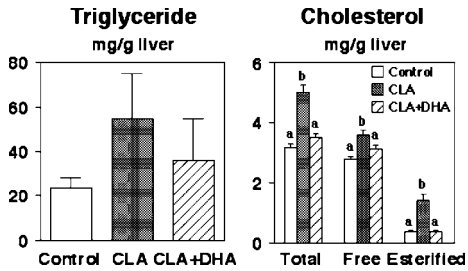


**Figure 1.** Effect of dietary fatty acids on the concentrations of hepatic lipids. Mice were fed semisynthetic diets that contained either 6% HL-SAF, 4% HL-SAF + 2% CLA, or 3.5% HL-SAF + 2% CLA + 0.5% DHA for 4 weeks. Values are expressed as means ± standard errors for six mice. See the Materials and Methods for the composition of diets. Different letters show significant differences at $p < 0.05$.

**Table 3.** Serum Parameters of C57BL/6N Mice after 4 Weeks of Feeding[a]

| | control | CLA | CLA + DHA |
|---|---|---|---|
| triglyceride (mg/dL) | 49.8 ± 7.3 a | 21.6 ± 0.9 b | 24.9 ± 2.5 b |
| cholesterol (mg/dL) | 110 ± 4 a | 151 ± 6 b | 106 ± 5 a |
| insulin (pg/mL) | 737 ± 187 | 966 ± 196 | 952 ± 140 |
| adiponectin ($\mu$g/mL) | 28.7 ± 1.1 a | 3.85 ± 0.214 b | 4.83 ± 0.12 b |
| leptin (pg/mL) | 1520 ± 425 a | 168 ± 82 b | 173 ± 76 b |

[a] Values are expressed as means ± standard errors for six mice. Different superscript letters show significant differences at $p < 0.05$.

**Effects of Dietary Fatty Acids on Hepatic Lipid Levels. Figure 1** shows hepatic triglyceride and cholesterol levels of C57BL/6N mice after 4 weeks of feeding of the diets. Although the difference was not significant, the hepatic triglyceride levels in mice fed a CLA diet were 2.2-fold that in the mice fed a control diet. However, supplementation of DHA to the CLA diet resulted in a 34% attenuation of triglyceride accumulation in the liver. In addition, hepatic cholesterol accumulation was induced by CLA feeding, and it was significantly attenuated by the CLA + DHA diet.

**Effects of Dietary Fatty Acids on Serum Parameters. Table 3** summarizes serum parameters of C57BL/6N mice after 4 weeks of feeding of the diets. Serum triglyceride levels were significantly decreased by a CLA diet, and this was not changed

**Table 4.** Activities of Hepatic Enzymes-related to Lipid Metabolism in C57BL/6N Mice[a]

| | nmo/min/mg protein | | |
|---|---|---|---|
| | control | CLA | CLA + DHA |
| FAS | 7.91 ± 0.96 a | 18.1 ± 1.2 b | 8.73 ± 0.53 a |
| malic enzyme | 108 ± 13 a | 733 ± 94 b | 217 ± 11 a |
| G6PDH | 11.8 ± 1.2 a | 11.8 ± 1.4 a | 7.29 ± 0.53 b |
| CPT | 7.38 ± 0.42 a | 10.2 ± 0.2 b | 7.96 ± 0.42 a |
| peroxisomal β-oxidation | 12.2 ± 1.4 a | 18.1 ± 0.8 b | 19.7 ± 0.8 b |

[a] Values are expressed as means ± standard errors for six mice. Different superscript letters show significant differences at $p < 0.05$.

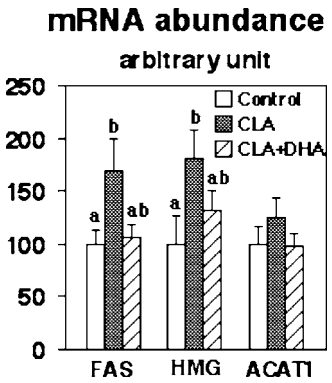


**Figure 2.** Effect of dietary fatty acids on the expression of mRNAs related to lipid metabolism in the livers of C57BL/6N mice. Mice were fed semisynthetic diets that contained either 6% HL-SAF, 4% HL-SAF + 2% CLA, or 3.5% HL-SAF + 2% CLA + 0.5% DHA for 4 weeks. Values are expressed as means ± standard errors for six mice. See the Materials and Methods for the composition of diets. Different letters show significant differences at $p < 0.05$.

by the CLA + DHA diet. On the other hand, serum cholesterol levels were significantly increased by the CLA diet. DHA supplementation, however, markedly alleviated CLA-induced hypercholesterolemia in mice. Serum insulin levels were 30% increased by a CLA diet, and this was not changed by the CLA + DHA diet. Serum levels of the adipocytokines, adiponectin and leptin, were drastically decreased by the CLA diet, as previously reported (*11*, *17*), and DHA supplementation did not alter those levels.

**Effects of Dietary Fatty Acids on Activities of Hepatic Enzymes Related to Lipid Metabolism. Table 4** summarizes hepatic enzyme activities of C57BL/6N mice after 4 weeks of feeding of the diets. The CLA diet markedly increased the activity of hepatic lipogenic enzymes such as FAS and malic enzyme. However, DHA supplementation significantly decreased those activities and that of G6PDH, as compared with a CLA diet. Mitochondrial β-oxidation, whose rate-limiting enzyme is CPT, and perosixomal β-oxidation were both significantly increased by a CLA diet. The CLA + DHA diet, however, decreased CPT activity as compared with a CLA diet.

**Effects of Dietary Fatty Acids on the Expression of mRNAs Related to Hepatic Lipid Metabolism. Figure 2** shows hepatic mRNA abundances of C57BL/6N mice after 4 weeks of feeding of the diets. Consistent with enzyme activity, mRNA levels of FAS were increased (69%) by a CLA diet, but the increase was attenuated (37%) by DHA supplementation. HMG-CoA reductase, a rate-limiting enzyme of cholesterol synthesis, and ACAT-1, a rate-limiting enzyme of cholesterol esterification, relate hepatic cholesterol storage. mRNA levels of those enzymes were also increased by a CLA diet during the onset of fatty liver, but DHA supplementation attenuated this.

## DISCUSSION

A CLA that drastically decreases the amount of adipose tissue has been reported to cause the induction of lipodystrophy, such as hepatic steatosis and hyperinsulinemia, in mice (*11*, *12*). Although increasing the amount of fat in a CLA-supplemented diet substantially reduces the lipodystrophy effect (*17*), we hypothesized that the simultaneous supplementation of other food components would also attenuate this. In fact, recent reports have shown that the coadministration of γ-linolenic acid or fish oil can prevent CLA-induced hepatic steatosis and hyperinsulinemia in mice (*30*, *31*). DHA is a long-chain highly unsaturated fatty acid that is abundant in fish oil and is a precursor of several eicosanoids. Additionally, it has been suggested that DHA has a lipid-lowering effect through the suppression of lipogenic genes expression in the liver of rodents (*32*−*34*). Therefore, the present study was undertaken to investigate the effects of the simultaneous supplementation with DHA during the onset of CLA-induced fatty liver in mice.

Consistent with previous reports, CLA reduced WAT weights but caused hepatomegaly accompanied by an accumulation of lipids in the liver of mice (*11*, *12*). Takahashi et al. previously demonstrated that CLA increases the activity and mRNA levels of hepatic lipogenic enzymes; they suggested that enhanced lipogenesis is a principal mechanism of the CLA-induced fatty liver in mice (*35*). In the present study, suppression of the activity and mRNA expression of FAS induced decreases in liver weight and hepatic triglyceride contents in mice fed a CLA + DHA diet. The gene expression of FAS is regulated by sterol regulatory element binding protein-1 (SREBP-1), a lipogenic transcriptional factor. The mature nuclear form of SREBP is produced from the membrane-bound precursor by proteolytic cleavage (*36*). Thus, the nuclear content of mature SREBP is under the regulation of gene expression and proteolytic cleavage of the precursor. Previous studies have shown that DHA has a suppressive effect on SREBP-1 mRNA expression (*32*), which suggests that this fatty acid can decrease fatty acid synthesis through the transcriptional suppression of SREBP-1 signaling. Therefore, the alleviation of CLA-induced fatty liver by DHA supplementation might be attributable to the transcriptional suppression of fatty acid synthesis. On the other hand, the effect of DHA supplementation on hepatic fatty acid oxidation observed in the present study was controversial. Both CLA and DHA have been reported to have the ability to enhance fatty acid β-oxidation through the activation of peroxisome proliferator-activated receptor-α, a lipolytic transcriptional factor (*37*−*39*). Although the reasons for the inconsistent decrease in CPT activity by DHA supplementation should be addressed by the future study, these results suggest that alterations in fatty acid β-oxidation were not responsible for the attenuation of fatty liver development in mice fed the CLA + DHA diet. In addition, the results showed that CLA feeding induced the accumulation of cholesterol in the liver and serum. A CLA diet increased mRNA levels of HMG-CoA reductase and ACAT-1, as compared with a control diet, which suggested that CLA enhanced cholesterol synthesis and storage in the liver of mice. DHA supplementation, however, suppressed those enhancements and also reduced hepatic cholesterol contents. As a consequence, serum cholesterol levels were also lowered by the CLA + DHA diet, as compared with the CLA diet.

Serum levels of adiponectin and leptin were drastically decreased in CLA-fed mice as previously reported (*11*, *17*).

Adiponectin and leptin are both abundantly secreted from adipose tissue and called adipocytokine (*40*−*42*). Both adipocytokines have several physiological functions, including the regulation of insulin sensitivity in humans and animals. Therefore, it has been reported that the deficiency of adipocytokine secretion induced by a paucity of adipose tissue would be the cause of lipodystrophy, which is characterized by a severe insulin resistance, and leads to hyperinsulinemia and hepatic steatosis (*43*−*46*). In fact, drastic decreases in adipose tissue by CLA induced lipodystrophy in mice, but continuous leptin infusions reversed hyperinsulinemia (*11*). Thus, we hypothesized that the DHA-mediated alleviation of CLA-induced fatty liver observed in this study was accompanied by a parallel increase in adipocytokine levels. However, a CLA + DHA diet did not affect adipocytokine levels, as compared with those seen with a CLA diet. Additionally, serum insulin levels were not changed by DHA supplementation to the CLA diet. Ide previously demonstrated that different amounts (1.5, 3, and 6%) of fish oil added to CLA-containing diets dose-dependently downregulated lipogenic parameters and decreased hepatic triglyceride levels in mice (*32*). In that study, however, CLA-induced hypoleptinemia, hypoadionectinemia, and hyperinsulinemia were alleviated only by the high dose (6%) fish oil supplementation. These results suggested that, in this study, the alleviation of CLA-induced fatty liver by DHA supplementation was attributable to the suppressive effect on the hepatic lipogenesis rather than a regulation of adipocytokine production and insulin sensitivity in mice.

In conclusion, our results show that DHA supplementation to the CLA diet can attenuate CLA-induced fatty liver through a reduction of hepatic fatty acid synthesis without affecting adipocytokine productions in C57BL/6N mice.

## ABBREVIATIONS USED

ACAT-1, acyl-CoA:cholesterol acyltransferase-1; CLA, conjutated linoleic acid; CPT, carnitine palmitoyltransferase; DHA, docosahexaenoic acid; FAS, fatty acid synthase; G6PDH, glucose 6-phosphate dehydrogenase; HL-SAF, high linoleic safflower oil; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; SREBP-1, sterol regulatory element binding protein-1.

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