Slow Absorption of Conjugated Linoleic Acid in Rat Intestines, and Similar Absorption Rates of 9c,11t-Conjugated Linoleic Acid and 10r,12c-Conjugated Linoleic Acid

Tsuyoshi TSUZUKI1,4 and Ikuo IKEDA2

1Department of Food Management, School of Food, Agricultural and Environmental Sciences, Miyagi University, Sendai 982-0215, Japan
2Laboratory of Food and Biomolecular Science, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

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We have previously shown that the 9c,11t-conjugated linoleic acid (CLA) concentration was always significantly higher than the 10r,12c-CLA concentration following the administration of these compounds to mice and rats, and considered that structural differences between the conjugated double bonds in these isomers affected absorption in the small intestine. This study investigates the absorption of CLA in the rat intestine by a lipid absorption assay of lymph from the thoracic duct. In Study 1, we used safflower oil and a triacylglycerol form of CLA (CLA-TG), while in Study 2, we used 9c,11t-CLA and 10r,12c-CLA. The cumulative recovery of CLA was lower than that of linoleic acid until two hours after sample administration. There was no difference in the extent of lymphatic recovery of 9c,11t-CLA and 10r,12c-CLA after the administration of CLA-TG, 9c,11t-CLA, and 10r,12c-CLA to the rats, suggesting that geometrical and positional isomerism of the conjugated double bonds did not influence the absorption.

Key words: absorption; conjugated linoleic acid; conjugated fatty acid; linoleic acid; lymph

Conjugated fatty acids (CFAs) is a generic term for fatty acids with a conjugated double bond, as exemplified by conjugated linoleic acid (CLA).1 There are several CLA isomers as a result of positional and geometrical isomerism of the conjugated double bonds, the major naturally-occurring CLA isomer being referred to as 9c,11t-18:2.1 CLA was first reported to have an anticarcinogenic effect, and various physiological effects have also subsequently been demonstrated, including an antiatherosclerotic effect and a role in the regulation of lipid metabolism.1–5 These CLA activities are associated with the conjugated double bond system. CLA is found naturally, and is especially present in such ruminant fats as beef tallow and milk fat.1 However, the CLA level in these foodstuffs is around 1% (w/w), and this prevents natural fats containing CLA from being used as health-promoting foods. Therefore, at present, oils that include CLA are prepared by alkali-isomerization of such vegetable oils as safflower oil, and these products are marketed as health supplements.6,7

CFAs other than CLA exist in nature: the seed oils of certain plants contain CFAs such as α-eleostearic acid and punicic acid, and red and green algae contain CFAs such as conjugated EPA and conjugated DHA.8–12 We are particularly interested in CFAs and have previously shown them to have a stronger antitumor effect than unconjugated fatty acids both in vitro and in vivo.13–16 An interesting phenomenon was found for CLA that allowed its use as a contrast when the physiological functions of these CFAs were examined. When a diet containing equal amounts of 9c,11t-CLA and 10r,12c-CLA was administered to rats for four weeks, the 9c,11t-CLA concentrations in the plasma and liver were very much higher than the 10r,12c-CLA concentrations.17 In addition, when mice were administered equal quantities of 9c,11t-CLA and 10r,12c-CLA separately for four weeks, greater amounts of 9c,11t-CLA than of 10r,12c-CLA were found in the liver and tumor tissue.13 We therefore thought that 9c,11t-CLA might be more easily absorbed by the small intestine than 10r,12c-CLA.

Surprisingly, little is known about the intestinal absorption of CLA. However, the intestines are the first step of nutrient delivery to tissues and, as such, may modulate the bioavailability of ingested fatty acids and also, therefore, their biological effects. Sugano et al. have examined the intestinal transport of dietary CLA in
free fatty acid form into the lymph of rats.\\(^1\)\\(^8\) The analytical procedures used in that study, however, are likely to have resulted in an inaccurate evaluation of CLA isomers recovered from the lymph, as was evident by the unusually high level of the \(t\)-isomer found. Martin et al. have examined the intestinal transport of dietary CLA in triacylglycerol form into the lymph of rats.\\(^1\)\\(^9\) Unfortunately, in that experiment, lymph was collected over 0–6 h and 6–24 h periods after CLA administration, and we have previously shown that differences in the rates of absorption of fatty acids with different structures were apparent within six hours after administration.\\(^2\\) Therefore, CLA absorption characteristics have yet to be clearly determined \textit{in vivo}, and the absorption of 9c,11t-CLA and 10r,12c-CLA in the small intestine is particularly unclear. For example, the following issues remain unresolved: whether there is any difference between the rates of absorption of CLA and linoleic acid (LA, 9c,12c-18:2, with the same molecular weight as CLA) within 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 h after administration; whether there is any difference between the rates of absorption of 9c,11t-CLA and 10r,12c-CLA within 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 h after administration; whether there is any difference between the rates of absorption of 9c,11t-CLA and 10r,12c-CLA when administered separately or together. Hence, we investigated here the absorption of CLA in the rat intestine by a lipid absorption assay of lymph from the thoracic duct.

To clarify the absorption of CLA in the rat intestine, we performed two studies with samples in different states. In Study 1, we used two oils (safflower oil and CLA-TG): safflower oil contains a triacylglycerol (TG) form of LA and was used as the control oil (Table 1), while CLA-TG contains a TG form of CLA and was used as the test oil (Table 1). In Study 2, we used two test oils, 9c,11t-CLA and 10r,12c-CLA; the former contains a free form of 9c,11t-18:2, while the latter contains a free form of 10r,12c-18:2 (Table 1).

\textbf{Materials and Methods}

\textit{Materials.} Safflower oil was kindly presented by Nippon Oil and Fats Co. (Tokyo, Japan). CLA-TG (the triacylglycerol form of CLA prepared from high-linoleic safflower oil), 9c,11t-CLA (80% purity), and 10r,12c-CLA (80% purity) were obtained from Nissin OilliO Group (Tokyo, Japan). Trimethylsilyldiazomethane (10% in hexane, v/v) was purchased from GL Sciences (Tokyo, Japan), and sodium methoxide/methanol (1 mol/l) solutions were purchased from Wako Pure Chemical Industries (Osaka, Japan).

\textit{Fatty acid composition of the test oils.} In Study 1, safflower oil and CLA-TG were used as the test oils, and in Study 2, 9c,11t-CLA and 10r,12c-CLA were used. Safflower oil and CLA-TG, with a known amount of heptadecanoic acid methyl ester (17:0 ME, Sigma, St. Louis, MO, USA) as an internal standard, were methylated by adding sodium methoxide/methanol for 5 min at room temperature, as described previously.\\(^2\\)\\(^0\)\\(^2\\) Each of the two test oils was dissolved in a 1-mol/l sodium methoxide/methanol solution. After a 5-min incubation at room temperature, the reaction was stopped by adding a saturated NaCl solution, and the fatty acid methyl esters were extracted with n-hexane and subjected to gas chromatography (GC; GC 353B gas chromatograph, GL Sciences, Tokyo, Japan) An FID instrument and a Supelcowax-10 fused silica capillary column (60 m x 0.32 mm i.d., Supelco, Bellefonte, PA, USA) were used, with helium as the carrier gas. The injector and detector temperatures were 200°C and 250°C, respectively, and the column oven temperature was increased by 20°C/min from 50°C to 220°C and then held constant for 31.5 min. The peak components were identified by comparing their retention times with those of commercial fatty acid methyl esters (Funakoshi, Tokyo, Japan). 9c,11t-CLA and 10r,12c-CLA, with a known amount of heptadecanoic acid (17:0, Sigma, St. Louis, MO, USA) as an internal standard, were methylated by adding trimethylsilyldiazomethane for 30 min at room temperature, as described previously.\\(^2\\)\\(^1\)\\(^2\) Each of the two test lipids in 9 ml of MeOH/benzene (2:7, v/v) were added to 1.3 mmol/l trimethylsilyldiazomethane in n-hexane. After standing for 30 min at room temperature, the reaction mixture was dried under a stream of nitrogen gas, and the fatty acid methyl esters were extracted with n-hexane and subjected to GC under the conditions just described. The fatty acid compositions of the four test oils are shown in Table 1.

\textit{Rat thoracic duct cannulation.} This study was conducted in conformity with the policies and procedures detailed in the Animal Experiment Guidelines of Tohoku University. Operations on and maintenance of the rats and all other procedures were performed as described previously.\\(^2\\)\\(^0\) Nine-week-old male Sprague-Dawley rats were obtained from Japan SLC (Hamamatsu, Japan) and housed in stainless-steel wire-mesh cages in a room kept at 23 ± 1°C with a 12-h light:dark cycle. After acclimatizing with MF Standard Rodent Chow (Oriental Yeast Co.) and distilled water (free access) for 1 wk as previously described,\\(^2\\)\\(^0\\)\\(^2\\) a cannula (SV35, Dural Plastics, Tokyo, Japan) was inserted into the left thoracic channel to collect lymphatic fluid, and a catheter (SP-55, Dural Plastics) was also inserted into the stomach. After surgery, each rat was placed in a restraining cage in a warm recovery room. A physiological solution containing 139 mmol/l of glucose and 85 mmol/l of NaCl was continuously infused overnight at a rate of 3 ml/h through the stomach cannula. The same solution was also provided as drinking water. The next morning, after collecting the lymph for 2 h as a blank control, the rats were infused with 3 ml of an emulsion as a single bolus through the stomach catheter.

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Test emulsions containing 200 mg of each test oil (Study 1, safflower oil or CLA-TG; Study 2, 9c,11t-CLA or 10t,12c-CLA), 50 mg of fatty acid-free albumin, and 200 mg of sodium taurocholate were prepared by ultrasonication. After infusing an emulsion into the rats, infusion with the glucose/NaCl solution was continued. Lymph was collected in an EDTA-containing tube for analysis during the following periods after the test oil infusion: 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–8, and 8–24 h. After the lymph flow had been measured, the lymph was stored at −30 °C until needed for analysis.

### Measurement of fatty acids in lymph

Total lipids from the lymph were extracted by Folch’s procedure and then methylated (with a known amount of heptadecanoic acid as an internal standard) by adding trimethylsilyldiazomethane for 30 min at room temperature and sodium methoxide/methanol for 5 min at room temperature, as previously described. Total lipids in 9 ml of MeOH/benzene (2:7, v/v) were added to 1.3 mmol/l of trimethylsilyldiazomethane in n-hexane. After standing for 30 min at room temperature, the reaction mixture was dried under a stream of nitrogen gas, and the dried residue was dissolved in 1 mol/l of sodium methoxide/methanol solution. After a 5-min incubation at room temperature, the reaction was stopped by adding a saturated NaCl solution. The fatty acid methyl esters were extracted with n-hexane and subjected to GC under the same conditions as those just described (Fatty acid composition of the test oils). The peak components were identified by comparing their retention times with those of commercial fatty acid methyl esters.

### Statistical analysis

Each result is expressed as the mean ± SD. A statistical analysis was performed by using Student’s t test for comparisons between two groups, after Bartlett’s test had been used to check that variances were homogeneous. A difference is considered to be significant at P < 0.05.

### Results

#### Study 1

Study 1 was aimed at clarifying the absorption properties of CLA in the rat small intestine. Safflower oil (TG form) containing 75.6% linoleic acid (LA) and CLA-TG (TG form) containing 77.6% conjugated linoleic acid (CLA), an isomer of LA, were used as the test oils (Table 1). No CLA was detected in the safflower oil (Table 1). The proportion of conjugated fatty acids in the safflower oil was 0%, and in CLA-TG was 77.6% (Table 1). Lymph from the thoracic duct of the rats that had been infused with emulsions containing each oil had normal flow properties, confirming that the surgery and maintenance of the rats had been carried out appropriately. The lymph flow rates did not differ, and were 95 ± 12 and 107 ± 17 ml/24 h in the safflower oil and CLA-TG groups, respectively. There were no conjugated fatty acids in rat lymph immediately before administering the test oils, this being determined by a GC analysis. LA was present in the lymph collected from −2 h to 0 h. Consequently, the recovery of LA in the lymph 0–24 h after administration was corrected for the amount of LA in the lymph collected from −2 h to 0 h. With this correction, the lymphatic recovery of LA in the safflower oil-treated rats was 78% 8 h after administration and 95% after 24 h (Fig. 1A); therefore, almost all the administered LA had been recovered. Similarly, the lymphatic recovery of CLA in CLA-TG-treated rats was 82% 8 h after administration and 93% after 24 h (Fig. 1A). Hence, most of the administered LA and CLA had been absorbed within 24 h. The cumulative recovery of LA and CLA in the thoracic duct lymph at each time point was compared (Fig. 1A). The recovery of CLA in the rats infused with CLA-TG was less than that of LA in the rats infused with safflower oil for in only the 0–1 and 0–2 h time periods. The lymphatic recovery of LA and CLA was similar for all other periods.

### Table 1. Fatty Acid Composition of the Test Oils

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Safflower oil</td>
<td>CLA-TG</td>
</tr>
<tr>
<td>16:0</td>
<td>8.0</td>
<td>6.3</td>
</tr>
<tr>
<td>18:0</td>
<td>3.4</td>
<td>2.9</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>12.1</td>
<td>11.0</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>75.6</td>
<td>0.9</td>
</tr>
<tr>
<td>9c,11t-18:2</td>
<td>—</td>
<td>36.8</td>
</tr>
<tr>
<td>10t,12c-18:2</td>
<td>—</td>
<td>37.5</td>
</tr>
<tr>
<td>Other-CLA</td>
<td>—</td>
<td>3.3</td>
</tr>
<tr>
<td>Other</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Total CFA</td>
<td>0.0</td>
<td>77.6</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>9c,11t-CLA</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>10t,12c-CLA</td>
<td>11.3</td>
<td>11.9</td>
</tr>
<tr>
<td>9c,11t-CLA</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>10t,12c-CLA</td>
<td>80.3</td>
<td>4.4</td>
</tr>
<tr>
<td>10t,12c-CLA</td>
<td>5.0</td>
<td>81.2</td>
</tr>
<tr>
<td>Other-CLA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Other</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Total CFA</td>
<td>85.3</td>
<td>85.6</td>
</tr>
</tbody>
</table>

*Fatty acid methyl esters prepared from each test oil were analyzed by GC.

a—, not detected (below 0.1%).
calculated again for each collection period (Fig. 1B). There was a significant difference between recovery in the two groups for the 0–1, 3–4, 4–5, and 8–24 h periods (\( P < 0.05 \), Fig. 1B). The recovery of CLA was significantly less than that of LA in the 0–1 and 8–24 h periods (57% and 63%, respectively; \( P < 0.05 \) for each, Fig. 1B), but significantly greater in the 3–4 and 4–5 h periods (179% and 182%, respectively; \( P < 0.05 \), for each Fig. 1B). There was no significant difference between the two groups for any other period. These results show, therefore, that the rate of absorption of CLA was lower than that of LA immediately after administering a test oil, but that there was no difference 24 hours after the administration. Next, to compare the rates of absorption of 9c,11t-18:2 and 10t,12c-18:2, the lymphatic recovery of 9c,11t-18:2 and 10t,12c-18:2 in the CLA-TG-treated animals was determined (Fig. 2). The lymphatic recovery and secretion rate of each oil for each time period was almost the same, there being no significant difference between the two. These results show that the rates of absorption of 9c,11t-18:2 and 10t,12c-18:2 were similar when administered together.

**Study 2**

Study 2 was aimed at clarifying the absorption properties of 9c,11t-CLA and 10t,12c-CLA in the small intestine when administered separately. 9c,11t-CLA containing an 80.3% free fatty acid form of 9c,11t-18:2 and 10t,12c-CLA containing of an 81.2% free fatty acid form of 10t,12c-18:2 were used as the test oils (Table 1). The proportion of conjugated fatty acids in the test oils was 85.3% in 9c,11t-CLA and 85.6% in 10t,12c-CLA (Table 1). Lymph from the thoracic duct of rats that had been infused with emulsions containing...
each oil had normal flow properties, confirming that the surgery and maintenance of the rats had been carried out appropriately. The lymph flow rates did not differ, being 99 ± 13 and 104 ± 24 ml/24 h in the 9c,11r-CLA and 10r,12c-CLA groups, respectively. There were no conjugated fatty acids in the rat lymph immediately before administering a test oil, this being determined by a GC analysis. The lymphatic recovery of 9c,11r-18:2 in the 9c,11r-CLA-treated rats was 71% 8 h after administration and 95% after 24 h (Fig. 3A); therefore, almost all the administered 9c,11r-18:2 had been recovered. Similarly, the lymphatic recovery of 10r,12c-18:2 in the 10r,12c-CLA-treated rats was 70% 8 h after administration and 93% after 24 h (Fig. 3A). Therefore, most of the administered 9c,11r-CLA and 10r,12c-CLA had been absorbed within 24 h. The cumulative recovery of 9c,11r-18:2 and 10r,12c-18:2 was calculated again for each collection period (Fig. 3B), there being no significant difference between the two groups for any period. The results show that the rates of absorption of 9c,11r-18:2 and 10r,12c-18:2 were similar when 9c,11r-CLA and 10r,12c-CLA were administered separately.

Discussion

Our study was designed to evaluate the lymphatic recovery of CLA. The fatty acid content of the thoracic duct lymph was measured in rats administered with LA and CLA to examine the intestinal absorption properties of 9c,11r-CLA and 10r,12c-CLA.

In Study 1, we showed that most of the administered LA and CLA had been absorbed within 24 h (Fig. 1A). The cumulative recovery of CLA in the thoracic duct lymph from rats infused with CLA-TG was less than that of LA in the thoracic duct lymph of rats infused with safflower oil for the 0–1 and 0–2 h time periods only. It was examined at which time point there was a difference in the absorption rate. The recovery of CLA was significantly less than that of LA in the 0–1 and 8–24 h periods, and significantly greater than that of LA in the 3–4 and 4–5 h periods (Fig. 1B). Martin et al. have reported that CLA glycerol esters were less readily hydrolyzed by lipase than LA glycerol esters in an in vitro pancreatic lipase assay. It was therefore thought that the rate of absorption of CLA was lower than that of LA immediately after administering the test oils because of differences in their reactivity with pancreatic lipase. There was no difference in the extent of absorption of CLA and LA 24 h after the administration, and therefore the rate of delivery of CLA from the small intestine to the lymph might have been faster than that of LA. The lymphatic recovery of 9c,11r-CLA and 10r,12c-CLA was almost the same in the CLA-TG-treated animals (Fig. 2) and, moreover, almost the same when 9c,11r-CLA and 10r,12c-CLA were administered separately in Study 2 (Fig. 3). It is concluded that the rates of absorption of 9c,11r-CLA and 10r,12c-CLA were similar.

Previous studies by ourselves and others have shown that the 9c,11r-CLA concentration was always significantly higher than the 10r,12c-CLA concentration following the administration of these compounds to mice and rats. We initially thought that structural differences in the conjugated double bonds of these isomers affected absorption in the small intestine; however, in the current study, there was no difference in the lymphatic recovery of 9c,11r-CLA and 10r,12c-CLA after administering CLA-TG, 9c,11r-CLA, and 10r,12c-CLA to rats (Figs. 2 and 3), suggesting that geometrical and positional isomerism of the conjugated double bonds did not influence absorption. An alternative explanation is that 10r,12c-CLA was more easily metabolized than 9c,11r-CLA in rats, as it has been reported to activate the β-oxidation system more
strongly than 9c,11t-CLA and is more easily oxidized. We will attempt in the future to clarify why the 10c,12t-CLA concentration was less than that of 9c,11t-CLA in vivo.

Acknowledgments

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