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Note

Regulation of Lipid Metabolism by Palmitoleate and Eicosapentaenoic Acid (EPA) in Mice Fed a High-Fat Diet

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We investigated whether oral administration of palmitoleate ameliorates disorders of lipid metabolism to clarify the effects of one of the components of fish oil. Lipid levels in the liver and plasma were significantly decreased by palmitoleate and by EPA administration. These results suggest that palmitoleate, in addition to EPA, plays a role in the regulation of lipid metabolism by fish oil.

Key words: palmitoleate; EPA; fish oil; lipid metabolism; high-fat diet

With regard to lipid functions and metabolism, attention has recently focused not only on the quantity of lipids but also on the quality. It is thought that fatty acid composition is the main factor determining the quality of lipids as a nutrient. The importance of fatty acid composition in tissues has been shown in fatty acid elongase 6 (Elovl6) and stearoyl-CoA desaturase 1 (SCD1) knockout mice.[1–3] Recently, Cao *et al.* reported that *de novo* synthesized palmitoleate can suppress lipogenesis in the liver and enhance insulin action in skeletal muscle.[4] Hence, palmitoleate has been classified as a lipokine, and its effects on lipid and glucose metabolism have been investigated extensively.

On the other hand, fish oil improves lipid metabolism. It is thought that the main components of fish oil inducing these effects are docosahexaenoic acid (DHA; C22:6) and eicosapentaenoic acid (EPA; C20:5), which are known as n-3 polyunsaturated fatty acids (PUFA). However, recent studies have found that the effects of fish oil on metabolic disorders are not limited to those of DHA and EPA.[5–7] A relatively high amount of palmitoleate (C16:1) is present in fish oil compared with other oils and foods. Thus, palmitoleate as well as n-3 polyunsaturated fatty acids might play roles in improving lipid metabolism by fish oil. The role of orally administered palmitoleate has been studied using palmitoleate-rich foods, such as macadamia nuts. It has been reported that intake of macadamia nuts decreased triglyceride (TG) and total cholesterol (TC) levels in the plasma in humans and hamsters,[8–10] but macadamia nuts contain not only palmitoleate but other fatty acids, and the role of orally administered palmitoleate has not been clarified. In this study, we investigated whether orally administered palmitoleate at relatively low levels improves lipid metabolism in mice fed a high-fat diet in comparison with EPA, which is known to be present in fish oil and improves lipid metabolism.

C57BL/6J female mice were obtained from CLEA Japan, Inc. (Tokyo, Japan) at 6 weeks of age. The mice were maintained at a constant temperature of $23 \pm 3\,^{\circ}C$ and a humidity of $55 \pm 10\%$ under a fixed artificial light cycle (12 h light and 12 h dark). All procedures were approved by the Institutional Animal Care and Use Committee of Josai University.

The experimental diets were a lard diet (Lard) and a fish oil diet (FO). The fatty acid composition of the fish oil was measured by gas chromatography, and the resulting profiles are shown in Table 1. The compositions of the diets were modified so that the daily intake of dietary components, except for fat and carbohydrates, was nearly identical to that in AIN-93G.[11,12] Each experimental diet was designed to contain 39% carbohydrates, 40% fat, and 21% protein on a calorie basis. For each kilogram of food, the experimental diets contained 188.0 g of oil, 231.2 g of casein, 77.9 g of sucrose, 389.5 g of β-starch, 11.6 g of a vitamin mixture (AIN-93-VX, with added choline bitartrate), 40.5 g of a mineral mixture (AIN-93G-MX), 57.8 g of cellulose powder, 3.5 g of L-cystine, and 0.038 g *t*-butylhydroquinone. The fish oil was a gift from NOF corporation (Tokyo, Japan). L-Cystine and *t*-butylhydroquinone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The other materials were from Oriental Yeast (Tokyo, Japan).

The mice consumed FO or Lard for 10 wk. Thereafter, the mice fed the lard diet were divided into three groups (Lard, Lard + EPA, and Lard + C16:1) and were housed for 2 wk. Twenty milligrams of EPA and C16:1 mixed with 10 mg of melted lard was orally administered by microspatula once a day to the Lard + EPA and the Lard + C16:1 group respectively. The same amount of lard (30 mg) was administered orally to the Lard group. The FO group was fed the same diet for 2 wk. The dose of palmitoleate was adjusted to a

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calculated amount of fatty acid according to the daily food intake in the FO group. The dose of EPA was determined to the same amount of palmitoleate. EPA and palmitoleate were purchased from Larodan Fine Chemicals (Malmö, Sweden) and MP Biomedicals, LLC (Solon, OH, USA) respectively.

TG and TC levels in the plasma were measured with colorimetric slides using the analyzer (FUJI DRI-CHEM 3500, FUJIFILM corporation, Tokyo, Japan). NEFA levels were measured using NEFA C-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Total lipid extracts of the liver were obtained by the method described by Bligh and Dyer.[13] Then, total lipid levels were measured, and TG, TC NEFA, and PL levels were measured from total lipids using Triglyceride E-test Wako, Cholesterol E-test Wako, NEFA C-test Wako and Phospholipid C-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Ceramide and diacylglycerol (DAG) were analyzed by thin-layer chromatography (TLC). After TLC, the plate was sprayed with 10% copper sulfate in 8% phosphoric acid solution, and this was heated at 150 °C for 30 min. After that, spots of ceramide and DAG were analyzed using Image Master 1D Elite ver.3.00 (Amersham Biosciences, Uppsala, Sweden).

Statistical comparisons of the two groups were made by Student's *t*-test. The other statistical comparisons of the groups were made by one-way ANOVA, and each group was compared with the others by Fisher's PLSD test (protected least significant difference test) (Statview 5.0; SAS Institute Inc., Cary, NC, USA). p values of less than 0.05 were considered to indicate statistical significance. Values are means ± SE.

There was no significant difference in energy intake at 6 wk and body weight at 10 wk between the FO and the Lard groups (Table 2). At 10 wk, TG and TC levels in the plasma were significantly higher in the Lard group than those in the FO group ($p < 0.01$, Table 2). We therefore confirmed that lipid metabolism of the Lard group became worse than those of the FO group.

Energy intake at 11 wk, final body weight, and TG levels in the plasma at 12 wk were not significantly different among the 4 groups (Table 3). In contrast, TC levels in the plasma in the Lard group were the highest, those in the FO group were the lowest, and those in the Lard + EPA and the Lard + C16:1 groups were at intermediate levels between the Lard and the FO groups ($p < 0.01$, Table 3). NEFA levels in the plasma showed no significant differences among the 4 groups (Table 3).

Table 1. Profiles of Dietary Oils

	Fatty acids (%)	Fish oil (FO)	Lard (Lard)
14:0	(Myristic acid)	3.0	1.6
16:0	(Palmitic acid)	18.2	23.0
16:1	(Palmitoleic acid)	4.2	2.3
18:0	(Stearic acid)	4.9	13.0
18:1 n-9	(Oleaic acid)	18.8	40.0
18:2 n-6	(Linoleic acid)	1.3	8.9
18:3 n-3	(α-Linolenic acid)	0.3	0.5
20:0	(Arachidic acid)	—	0.2
20:4 n-6	(Arachidonic acid)	2.0	0.1
20:5 n-3	(EPA; eicosapentaenoic acid)	6.8	—
22:0	(Behenic acid)	—	—
22:6 n-3	(DHA; docosahexaenoic acid)	22.8	—
S:M:P		10:10:14	38:42:10
n-6/n-3 ratio		0.1	18.0

Fatty acids in fish oil were measured by gaschromatography, and those in lard were based on the food composition table in Japan.

Table 2. Energy Intake, Body Weight, and TG, TC Levels in the Plasma during the Dietary Habitation Period (About 10 wk)

		FO	Lard
Energy intake	(kcal/mouse/d)	9.7 ± 0.4	9.4 ± 0.5
Initial body weight	(g)	18.4 ± 0.4	18.2 ± 0.2
At 10 wk body weight	(g)	24.1 ± 0.3	24.1 ± 0.3
Plasma TG	(mg/dL)	54.8 ± 3.1	73.1 ± 2.8**
Plasma TC	(mg/dL)	44.6 ± 0.9	102.4 ± 3.5**

Energy intake was calculated using the amount of food intake for 3 d at 6 wk. Energy intake was calculated based on the daily food intake, and the values from 3 d were averaged. The standard errors for energy intake were calculated from the variations in the daily intake of each group, not from those of individual mice. Blood samples of mice fed each diet for 10 wk were collected from the retro-orbital plexus after a 4-h fast. Data points represent the mean ± SE for 5 mice in the FO group and 14 mice in the Lard group. ** $p < 0.01$ for the Lard group compared to the FO group.

Table 3. Energy Intake, Final Body Weight and Lipid Parameters in the Plasma and the Liver during the Experimental Period (10–12 wk)

		FO	Lard	Lard + EPA	Lard + C16:1
Energy intake	(kcal/mouse/d)	11.5 ± 0.2	10.1 ± 0.4	10.1 ± 0.8	10.5 ± 0.3
Final body weight	(g)	24.4 ± 0.3	24.5 ± 0.3	25.3 ± 0.3	24.9 ± 0.4
Plasma					
TG	(mg/dL)	28.80 ± 4.84	39.50 ± 4.87	35.00 ± 1.79	31.80 ± 2.69
TC	(mg/dL)	38.80 ± 3.76[a]	89.50 ± 3.80[b]	60.20 ± 4.28[c]	62.20 ± 2.85[c]
NEFA	(mEq/L)	0.48 ± 0.06	0.45 ± 0.04	0.42 ± 0.02	0.42 ± 0.01
Liver					
Total lipid	(mg/g liver)	55.23 ± 2.99[a]	76.66 ± 6.18[b]	51.55 ± 5.79[a]	58.80 ± 3.08[a]
TG	(mg/g liver)	27.97 ± 3.60	37.49 ± 5.94	34.98 ± 3.68	33.07 ± 2.43
TC	(mg/g liver)	4.11 ± 0.19[a]	5.91 ± 0.09[b]	6.18 ± 0.10[b]	5.71 ± 0.32[b]
NEFA	(mg/g liver)	1.64 ± 0.06[a]	2.17 ± 0.23[b]	2.16 ± 0.08[b]	1.96 ± 0.13[ab]
PL	(mg/g liver)	14.18 ± 0.43	12.52 ± 0.90	12.58 ± 0.74	11.84 ± 0.60
Ceramide (pooled sample)	(mg/g liver)	1.02	0.54	0.46	0.55
DAG (pooled sample)	(mg/g liver)	0.21	1.71	0.70	2.15

Energy intake was calculated using amount of food intake for 3 d at 11 wk. Mice fed one of the four experimental diets for 12 wk were killed after a 4-h fast, and then blood samples were collected from the postcaval vein. Total lipid, TG, TC, NEFA, and PL levels in the liver were measured. Each data point represents the mean ± SE for 4–5 mice. [a,b,c] Values not sharing the same superscript are significantly different at $p < 0.05$ by Fisher's PLSD test. Ceramide and DAG levels in the mixed liver section from each of the 4 groups were measured using TLC.

Total lipid levels in the liver were significantly higher in the Lard group than those in the FO group ($p < 0.05$, Table 3), and interestingly, these levels in both the Lard + EPA and the Lard + C16:1 groups were significantly decreased to almost the same levels as the FO groups ($p < 0.05$, Table 3). TG levels in the liver in the Lard group showed an increase over those in the FO group ($p = 0.1$, Table 3), but these levels in the Lard + EPA and the Lard + C16:1 groups were not significantly different from those in the Lard group. Similarly, although TC and NEFA levels in the liver of the Lard group were significantly higher than those of the FO group ($p < 0.05$, Table 3), TC and NEFA levels in the Lard + EPA and the Lard + C16:1 groups were not significantly different from those in the Lard group. PL levels in the liver did not show any significant difference. Thus, this study indicates that palmitoleate and EPA reduced other lipid class levels, besides TG, TC, NEFA, and PL in the liver. In this study, more than 50% of the lipids in the liver were TG. In previous studies, TG levels of in the livers of lard-fed mice increased or unchanged compared with those in fish oil-fed mice.[6,7,14–17] Furthermore, several recent studies have found that accumulations of TG and fatty acid metabolites such as fatty acyl-CoA, DAG, ceramide, and glycosphingolipid are associated with hepatic steatosis and insulin resistance.[18–20] As for DAG, it has been reported that DAG levels were higher in subjects with nonalcoholic fatty liver disease (NAFLD), including nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH).[21,22] In our preliminary experiments, DAG levels in the Lard group were higher than those in the FO group. Although EPA administration decreased DAG levels in the liver, palmitoleate administration did not show any such effect. These results suggest that n-3 fatty acid such as EPA and DHA, but not palmitoleate, decrease the levels of DAG in the liver. It is speculated that the mechanisms causing decreases in the lipids in the liver are different between EPA and palmitoleate. In addition, it has been reported that selective knockdown lysophosphatidylglycerol acyltransferase 1 gene expression in the liver, which enzyme synthesizes DAG from monoacylglycerol and fatty acyl-CoA, caused an increase in hepatic TG and TC and played a significant role in hepatic lipids synthesis and secretion.[23] Thus, although palmitoleate might reduce monoacylglycerol or acyl-CoA in the liver, further study is needed.

In this study, we found that increased TC levels in the plasma and total lipid levels in the liver in the Lard group were significantly decreased by orally administered palmitoleate. In addition, the effect of palmitoleate in reducing total lipid levels in the liver was equal to that of EPA. This is surprising, because palmitoleate in our study was administered at relatively low levels and the experimental period was short (2 wk). Moreover, we used pure palmitoleate, while previous studies used macadamia nuts containing complex lipids other than palmitoleate. In human studies using a macadamia nut diet, the amount of calculated palmitoleate per kg of body weight was less than that in our experiment.[8,9] In animal study, 165 mg/d/hamster (about 26 mg/d/mouse) of palmitoleate as diet containing macadamia oil was fed,[10] and the dose of palmitoleate per g of body weight was almost the same as that in our experiment. The experimental period of intake in the previous studies; 6 wk, 30 d and 5 wk[8–10] were longer than that in our study, 2 wk. Thus, our study indicates the effects of orally administered palmitoleate on lipid metabolism even at relative low levels and over a short period of administration. Therefore, our study suggests that palmitoleate, in addition to EPA, is a key player in the regulation of lipid metabolism by fish oil.

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