Highly purified eicosapentaenoic acid prevents the progression of hepatic steatosis by repressing monounsaturated fatty acid synthesis in high-fat/high-sucrose diet-fed mice

Satoshi Kajikawa, Tsuyoshi Harada *, Akiko Kawashima, Kazunori Imada, Kiyoshi Mizuguchi

Development Research, Pharmaceutical Research Center, Mochida Pharmaceutical Company Limited, 722 Uenohara, Jimba, Gotemba, Shizuoka 412-8524, Japan

ABSTRACT

Eicosapentaenoic acid (EPA) is a member of the family of n-3 polyunsaturated fatty acids (PUFAs) that are clinically used to treat hypertriglyceridemia. The triglyceride (TG) lowering effect is likely due to an alteration in lipid metabolism in the liver, but details have not been fully elucidated. To assess the effects of EPA on hepatic TG metabolism, mice were fed a high-fat and high-sucrose diet (HFHSD) for 2 weeks and were given highly purified EPA ethyl ester (EPA-E) daily by gavage. The HFHSD diet increased the hepatic TG content and the composition of monounsaturated fatty acids (MUFAs). EPA significantly suppressed the hepatic TG content that was increased by the HFHSD diet. EPA also altered the composition of fatty acids by lowering the MUFAs C16:1 and C18:1 and increasing n-3 PUFAs, including EPA and docosahexaenoic acid (DHA). Linear regression analysis revealed that hepatic TG content was significantly correlated with the ratios of C16:1/C16:0, C18:1/C18:0, and MUFA/n-3 PUFA, but was not correlated with the n-6/n-3 PUFA ratio. EPA also decreased the hepatic mRNA expression and nuclear protein level of sterol regulatory element binding protein-1c (SREBP-1c). This was reflected in the levels of lipogenic genes, such as acetyl-CoA carboxylase (ACC), fatty acid synthase, stearoyl-CoA desaturase 1 (SCD1), and glycerol-3-phosphate acyltransferase (GPAT), which are regulated by SREBP-1c. In conclusion, oral administration of EPA-E ameliorates hepatic fat accumulation by suppressing TG synthesis enzymes regulated by SREBP-1c and decreases hepatic MUFAs accumulation by SCD1.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasingly recognized cause of liver-related morbidity and mortality, which is frequently associated with metabolic syndromes such as obesity, insulin resistance (IR), and hyperlipidemia [1–3]. IR is believed to be a central mechanism in the progression of NAFLD [4], because the resulting hyperinsulinemia increases glucose levels in the blood and free fatty acids in adipose tissue, which then causes excessive accumulation of triglyceride (TG) in the liver [2]. Hyperlipidemia, especially hypertriglyceridemia, also increases lipoproteins and free fatty acids taken into liver resulting enhanced hepatic synthesis of TG [5,6]. Reduction of hepatic TG accumulation may prevent the progression from steatosis to NAFLD, because an aberrant accumulation of TG in the liver is an underlying cause of these diseases [7].

It is well established that TG is the most substantial fat component in the fatty liver. A molecule of TG is composed of a backbone of glycerol with three fatty acids bound to it. Palmitic acid (C16:0), palmitoleic acid (C16:1), and oleic acid (C18:1) are the predominant fatty acids in the liver in patients diagnosed with NAFLD [8]. It has been reported that increased plasma concentrations of monounsaturated fatty acids (MUFAs), mainly C16:1 and C18:1, were observed along with progression of hepatic damage [9]. High proportions of C16:0 in serum and adipose tissue have been consistently related to IR and arteriosclerosis [10,11]. Changes in hepatic fatty acid composition and content may be important in the pathogenesis of NAFLD.

The effects of n-3 long-chain polyunsaturated fatty acids (PUFAs) on coronary heart disease, obesity, dyslipidemia, and IR have been extensively investigated [12–14]. n-3 long-chain
2. Materials and methods

2.1. Drugs

EPA-E (98% purity; Mochida Pharmaceutical, Tokyo, Japan) or polyeneporphosphatidylcholine (PC, Taiyo Pharmaceutical Industry, Nagoya, Japan) was suspended in 5% arabic gum (Wako Pure Chemical Industries, Osaka, Japan) solution using a homogenizer (Pryocoton NS-56S; Microtec, Funabashi, Japan), and was administered orally. Pioglitazone hydrochloride (PIO, ChemPacific, Baltimore, MD, USA) was homogenized in 5% arabic gum solution using a Teflon homogenizer.

2.2. Animals

Male C57BL/6J mice (8 weeks of age) were purchased from Charles River Laboratories Japan (Yokohama, Japan). Mice were housed individually in a room under controlled temperature (21–25 °C), humidity (40–70%), and 12-h light/dark cycle with free access to sterilized water and a standard pellet diet (STD) containing no fish products (F1; Funabashi Farms, Funabashi). All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals at the Pharmaceutical Research Center of Mochida Pharmaceuticals.

2.3. Experimental protocols

After a 1-week acclimation, mice were randomly divided into five groups of eight mice each. They were fed STD (per weight basis; 4.4% crude fat, 61.4% carbohydrate, 22.3% crude protein, and 2.7% crude fiber) or a high-fat and high-sucrose diet (HFHSD) (TD.88137; Harlan Teklad, Madison, WI, USA) (per weight basis; 21% anhydrous milkfat, 34.1% sucrose, 15% corn starch, 19.5% casein, 0.15% cholesterol, and 5% cellulose) in pellet form for 2 weeks. The fatty acid composition of each of the two diets is shown in Table 1. The detailed combination of treatment in each group was as follows: group 1, STD-fed (control I); group 2, HFHSD-fed (control II); groups 3–5, HFHSD-fed and administered with EPA-E, PIO, or PC (1, 0.03, or 1 mg/g body weight, respectively) by gavage daily for 2 weeks. Groups 1 and 2 received 5% arabic gum solution as vehicle in the same way. Heparinized blood was collected from the inferior vena cava under anesthesia, and the liver was removed just after euthanasia. For measurement of hepatic TG content a portion of liver was immediately homogenized in saline using a Teflon homogenizer, and subjected to the assays described below. Another portion of the liver was fixed with 10% buffered formalin for histology, and the other was frozen in liquid nitrogen for analysis of fatty acids. To assess the dose-related effects of EPA-E (0.1, 0.3 and 1 mg/g), another set of mice (five groups of eight mice) was treated as above. Six out of eight mice were randomly assigned for analysis of nuclear SREBP-1 level before the experiment. A portion of liver was homogenized as mentioned above, and subjected to analyses of hepatic TG content and nuclear SREBP-1 level described below. For lipogenic gene analysis in the liver, an additional portion was immersed in RNAlater (Ambion, Austin, TX, USA) and stored in liquid nitrogen until RNA isolation.

2.4. Histology

Lipid accumulation was assessed by oil red-O staining of 4–5 μm frozen sections of fixed liver specimen. Briefly, OCT-embedded cryosections were pre-treated with 60% isopropanol, stained with 0.18% oil-red-O in 60% isopropanol for 15–30 min, and then washed with 60% isopropanol. Sections were counterstained with Mayer’s hematoxylin and mounted with aqueous solution.

### Table 1: Fatty acid composition of STD and HFHSD diets.

| Fatty acid composition of STD and HFHSD diets. | STD | HFHSD |
| % (w/w) of total fatty acids | | |
| Capric acid | C6:0 | ND | 1.2 |
| Caprylic acid | C8:0 | ND | 1.0 |
| Capric acid | C10:0 | ND | 2.5 |
| Lauric acid | C12:0 | ND | 3.2 |
| Myristic acid | C14:0 | ND | 2.2 |
| Pentadecanoic acid | C15:0 | ND | 1.3 |
| Palmitic acid | C16:0 | 17.4 | 31.1 |
| Heptadecanoic acid | C17:0 | 0.1 | 0.9 |
| Stearic acid | C18:0 | 5.6 | 12.0 |
| Arachidic acid | C20:0 | 0.3 | 0.2 |
| Behenic acid | C22:0 | 0.3 | ND |
| Lignoceric acid | C24:0 | 0.2 | ND |
| Total SFAs | 24.1 | 64.7 |
| Myristoleic acid | C14:1n-5 | ND | 0.8 |
| Palmitoleic acid | C16:1n-7 | 0.1 | 1.8 |
| Oleic acid | C18:1n-9 | 21.6 | 18.6 |
| Eicosenoic acid | C20:1n-9 | 0.5 | ND |
| Docosenoic acid | C22:1n-11, n-9 | 0.4 | ND |
| Total MUFAs | 22.6 | 26.1 |
| Linoleic acid | C18:2n-6 | 48.7 | 1.3 |
| α-Linolenic acid | C18:3n-3 | 3.9 | 0.8 |
| Eicosapentaenoic acid | C20:5n-3 | ND | 0.1 |
| Docosapentaenoic acid | C22:5n-3 | ND | 0.1 |
| Docosahexaenoic acid | C22:6n-3 | ND | ND |
| Total PUFAs | 52.6 | 3.5 |

ND: Not detected.
2.5. Measurement of hepatic TG content

The liver was homogenized in saline with a Teflon homogenizer, and lipid was extracted according to the method of Folch et al. [35]. The TG concentration was determined using commercial kits (Triglyceride E-test Wako, Wako Pure Chemical Industries).

2.6. Assay for nuclear SREBP-1 level in the liver

The SREBP-1 level in the liver was determined as described by Sheng et al. [36]. Briefly, liver was suspended in buffer that contained 10 mM HEPES, 25 mM KCl, 1 mM Na2EDTA, 2 M sucrose, 10% (v/v) glycerol (pH 7.9) supplemented with 50 μg/mL ALLN, 5 μg/mL pepstatin A, 10 μg/mL leupeptin, 1 mM Pefabloc, 2 μg/mL aprotinin, 0.15 mM spermine, and 2 mM spermidine, followed by high-speed centrifugation at 24,000 rpm for 70 min at 4 °C. The pellets were resuspended in buffer that contained 10 mM HEPES, 100 mM KCl, 2 mM MgCl2, 1 mM Na2EDTA, 10% (v/v) glycerol (pH 7.9) supplemented with 50 μg/mL ALLN, 5 μg/mL pepstatin A, 10 μg/mL leupeptin, 1 mM Pefabloc, 2 μg/mL aprotinin, and 1 mM dithiothreitol, followed by addition of 0.5 M NaCl. After high-speed centrifugation at 85,000 rpm for 45 min at 4 °C, the supernatant containing the nuclear fraction was removed. Protein concentrations were determined by a colorimetric method using the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Aliquots (5.3 μg of protein) were mixed with Tris–SDS sample-treating buffer, boiled for 5 min, loaded onto a 10% SDS–polyacrylamide gel, and electrophoresed at a constant current of 20 mA. Proteins were then transferred from the gel to a polyvinylidene difluoride membrane and blocked in 5% (w/v) ECL blocking agent (GE Healthcare, Buckinghamshire, UK) in Tris-buffered saline supplemented with 0.1% Tween 20. SREBP-1 was detected by immunoblotting with an anti-SREBP-1 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Enhanced fluorescence (ECL plus Western Blotting Detection System, GE Healthcare) of HRP-labeled anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA, USA) was visualized with an image analyzer (Typhoon 9410, GE Healthcare, UK). Quantitative densitometric analyses were performed on digitized images of immunoblots using ImageQuant TL v2003.02 software (GE Healthcare).

2.7. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the liver in RNAAlater was isolated with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) and an RNeasy mini kit (Qiagen, Hilden, Germany) as described by the manufacturer's instructions. Total RNA (5 μg) from each sample was reverse transcribed into cDNA using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Hepatic mRNA levels of SREBP-1a, SREBP-1c, acetyl-CoA carboxylase α (ACCα), FAS, SCD1, and GPAT were evaluated using quantitative real-time RT-PCR with acidic ribosomal phosphoprotein P0 (36B4) as internal control. Quantitative real-time RT-PCR and analysis were carried out using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) with the SYBR green PCR master mix (Applied Biosystems). The amounts of initial template cDNA for the target gene were quantified by applying the threshold cycle to the standard curve. The final result for each sample was normalized to the respective 36B4 value. Sequences of all primers are shown in Table 2.

2.8. Fatty acid profiles

The total liver lipid extracts obtained by the method of Folch et al. [35] were hydrolyzed and converted to fatty acid methyl esters (FAMEs). Briefly, total lipids were hydrolyzed with 9:1 (v/v) 0.5 mol/L HCl CH3CN/water for 45 min at 100 °C. Samples were then mixed with chloroform/water, and centrifuged to obtain the organic layer. After drying under a nitrogen atmosphere, the lipids were converted to FAMEs with 0.4 mol/L CH3OK and 14% BF3/methanol at 100 °C for 6 min. FAMES were mixed with water, hexane, and silica gel and then centrifuged. The supernatant obtained was used for fatty acid analysis.

For fatty acid analysis, FAMES were examined by gas–liquid chromatography (GC–17A, Shimadzu, Kyoto, Japan) using an apparatus equipped with a flame ionization detector. FAMES were separated on an Omegawax 250 column (0.25 mm diameter, 0.25 μm coating thickness, Supelco, Bellefonte, PA, USA) using helium carrier. Fatty acid composition is expressed as wt% of total fatty acids or tissue concentration (μg/g liver).

Table 2

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
<th>Genbank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCα</td>
<td>TGGCTCTGGGTGTGATCCGA</td>
<td>TCGGTCAGGTCTATCCCAT</td>
<td>64</td>
<td>NM_133360</td>
</tr>
<tr>
<td>FAS</td>
<td>CTCGAGCTCGTACGTGAAGAG</td>
<td>GCCAGTCTTTACACCACTTGT</td>
<td>68</td>
<td>NM_007988</td>
</tr>
<tr>
<td>SCD1</td>
<td>AGCTCAGCTCCTACCTCTCTCT</td>
<td>CAGCCAGCTTGTGATCCATCT</td>
<td>71</td>
<td>NM_009127</td>
</tr>
<tr>
<td>GPAT</td>
<td>TCATCAGTACGTGACATTCACA</td>
<td>GCAAAGCCAGCAGTCACTC</td>
<td>67</td>
<td>NM_008149</td>
</tr>
<tr>
<td>SREBP-1a</td>
<td>CAGGAGTGAGGAGGAAGTG</td>
<td>GCTGGAAGATGGGTGATCT</td>
<td>67</td>
<td>NM_011480</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>CCGCGCGCCAGGCGCT</td>
<td>TGGATCATTGCTCATTCAT</td>
<td>73</td>
<td>NM_011480</td>
</tr>
<tr>
<td>36B4</td>
<td>CCTGAAGTGTCGACATCACA</td>
<td>GCCGTTGACCTATGATGATG</td>
<td>141</td>
<td>NM_007475</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Control I</th>
<th>Control II</th>
<th>EPA-E</th>
<th>PIO</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26.7 ± 0.2</td>
<td>28.3 ± 0.2***</td>
<td>29.0 ± 0.2</td>
<td>29.0 ± 0.5</td>
<td>28.6 ± 0.4</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.39 ± 0.02</td>
<td>1.40 ± 0.03</td>
<td>1.33 ± 0.03</td>
<td>1.68 ± 0.06*</td>
<td>1.33 ± 0.02</td>
</tr>
<tr>
<td>Relative liver weight (%)</td>
<td>5.19 ± 0.05</td>
<td>4.92 ± 0.08*</td>
<td>4.39 ± 0.09</td>
<td>5.80 ± 0.15***</td>
<td>4.64 ± 0.05</td>
</tr>
</tbody>
</table>

Mice were fed STD or HFHSD for 2 weeks. EPA-E (1 mg/g/day), PIO (0.03 mg/g/day), or PC (1 mg/g/day) was administered by gavage for 2 weeks in HFHSD-fed mice. Values are expressed as the mean ± SE for eight mice in each group. *P<0.05, **P<0.001 versus control I (Student’s t-test), *P<0.05, ***P<0.001 versus control II (Dunnett or Steel test).
2.9. Statistical analysis

Values were expressed as the mean±SE. Results were tested for homogeneity of variance using the F-test (between two groups) or the Bartlett’s test (among three groups or more). For evaluation of differences between two groups, the Student’s t-test was performed if the variance was homogenous, and the Aspin–Welch t-test was done if the variance was heterogeneous. For evaluation of differences among three groups or more, the Dunnett test was performed if the variance was homogenous, and the Steel test was done if the variance was heterogeneous. Correlation was determined by simple linear regression analysis. For all analyses, SAS statistical programs were used (Version 9.1.3, SAS Institute, Japan, Tokyo). Differences with \( P < 0.05 \) were considered statistically significant.

3. Results

3.1. Body weight, liver weight, and relative liver weight

The body weight of mice fed the HFHSD for 2 weeks was significantly higher than that of mice fed the STD, whereas the liver weight was similar in the two groups (Table 3).

Oral administration of EPA-E (1 mg/g/day) or PC (1 mg/g/day) daily for 2 weeks did not affect body weight, liver weight, or relative liver weight in HFHSD-fed mice. In contrast, PIO (0.03 mg/g/day) significantly increased the liver weight and relative liver weight. The food consumption did not differ significantly among the groups of HFHSD-fed mice (data not shown).

3.2. Suppression of hepatic TG accumulation by EPA-E administration

Oil red-O staining revealed fat accumulation in the liver of HFHSD-fed mice (Fig. 1A and B). In contrast, administration of EPA-E (1 mg/g/day) prevented HFHSD-induced hepatic fat accumulation (Fig. 1C).

To clarify the effect of HFHSD feeding on the fat accumulation in liver, hepatic TG content was measured. HFHSD feeding to mice caused a 6.5-fold increase in hepatic TG content as compared to STD-fed mice (Fig. 2A and B).

Administration of EPA-E (0.1, 0.3, or 1 mg/g/day) for 2 weeks in HFHSD-fed mice significantly reduced hepatic TG content (Fig. 2A and B), which was consistent with the histological changes observed. On the other hand, the hepatic TG content in mice treated with PIO (0.03 mg/g/day) was 2.0-fold higher than that in control II mice. No reduction was observed in hepatic TG content in mice treated with PC (1 mg/g/day) (Fig. 2B).

3.3. Down-regulation of hepatic SREBP-1 level and lipogenic gene expressions by EPA-E administration

Immunoblot analysis of liver nuclear extracts showed that mice treated with EPA-E for 2 weeks had 42% lower SREBP-1 mature protein than control II mice (Fig. 3). To clarify the mechanism by which EPA down-regulates mature SREBP-1 protein, the mRNA levels of SREBP-1 were examined by quantitative real-time RT-PCR. Hepatic expression of SREBP-1c was suppressed by EPA-E treatment, although the SREBP-1a mRNA level was comparable to that of control II mice treated with EPA-E (Fig. 4A and B).

EPA is reported to decrease the expression of fatty acid synthesis-related genes regulated by SREBP-1, such as FAS and SCD1, in mice fed a high-carbohydrate, fat free-diet, and in genetically obese mice [17,37]. Consistent with this, EPA-E treatment attenuated the increase in mRNA levels of ACCa, FAS, SCD1, and GPAT in HFHSD-fed mice (Fig. 4C–F). These findings
confirm that EPA prevents hepatic TG accumulation by inhibiting gene expression of lipogenic enzymes in HFHSD-fed mice.

3.4. Effects of EPA-E administration on fatty acid composition in liver total lipids

The composition of fatty acids in the total liver lipids was analyzed by gas–liquid chromatography. Marked increases in C16:1n-7, C18:1n-9, and C20:5n-3 in the liver of HFHSD-fed mice compared to STD-fed mice were seen, along with a significant and marked decrease in C18:0 and C22:6n-3 (Table 4). There were no significant changes in C16:0. In comparison to STD-control values, total fatty acids and total MUFAs in the liver of HFHSD-fed mice were 64% and 129% higher, respectively. Total SFAs, total n-6 PUFAs, and total PUFAs were 9%, 65%, and 50% lower, respectively. There was no significant change in total n-3 PUFAs.

Administration of EPA-E (1 mg/g/day) increased total SFAs, mainly C18:0, whereas the level of C16:0 was comparable to that seen in control II mice. EPA-E treatment significantly reduced total MUFAs, especially C16:1n-7, C18:1n-9, and conversely increased total PUFAs, total n-3 PUFAs, C20:5n-3 (EPA), and C22:6n-3 (DHA). In contrast to EPA-E, PIO treatment (0.03 mg/g/day) reduced total SFAs and C16:0, while C18:0 was comparable to that of control II mice. PIO treatment increased C16:1n-7, but maintained levels of n-3 PUFAs. PC treatment (1 mg/g/day) increased total n-6 PUFAs, whereas no significant change was observed in the levels of SFAs, MUFAs, and n-3 PUFAs, in both total and each major component.

3.5. The relationship between fatty acid concentration and hepatic TG content

Correlations between hepatic TG content and hepatic concentrations of major fatty acid components (C16:0, C18:0, C16:1n-7, C18:1n-9, C18:2n-6, C20:4n-6, C20:5n-3, or C22:6n-3) were evaluated by simple linear regression analysis. Significant correlations were observed between hepatic TG content and C16:0 ($R^2 = 0.3579$; $P < 0.0001$), C16:1n-7 ($R^2 = 0.5329$; $P < 0.0001$), and C18:1n-9 ($R^2 = 0.3325$; $P < 0.0001$). Correlations were not observed between hepatic TG content and C16:0 ($R^2 = 0.0977$), C18:2n-6 ($R^2 = 0.1393$), C20:4n-6 ($R^2 = 0.0645$), C20:5n-3 ($R^2 = 0.0547$), and C22:6n-3 ($R^2 = 0.0883$) (Fig. 5).

In addition, there was no significant correlation between hepatic TG content and the n-6/n-3 PUFA ratio ($R^2 = 0.0555$) (Fig. 6A). On the other hand, significant correlation was observed between hepatic TG content and the MUFA/n-3 PUFA ratio ($R^2 = 0.621$; $P < 0.0001$) (Fig. 6B). The ratio of MUFAs to the SFAs, C16:1/C16:0 and C18:1/C18:0, is proportional to the hepatic TG content ($R^2 = 0.8443$; $P < 0.0001$, and $R^2 = 0.5147$; $P < 0.0001$, respectively) (Fig. 6C and D).
In a large scale, prospective, randomized clinical trial, highly purified EPA-E reduced the risk of major coronary events through a LDL cholesterol-independent mechanism in Japanese hypercholesterolemia patients treated with concurrent administration of statins [38]. The alternation of lipid metabolism by EPA is presumed to be involved in the LDL cholesterol-independent mechanism, however, precise action of EPA is poorly understood. To investigate the effect of EPA on lipid metabolism and its mechanisms, we here examined the pharmacological effect of EPA-E on hepatic fat accumulation in HFHSD-fed mice, and the relationship between hepatic TG accumulation and hepatic fatty acid composition.

This study demonstrates that EPA is capable of decreasing hepatic TG content, which is accompanied by inhibition of the activation of SREBP-1. Several reports have indicated that dietary feeding of fish oil reduces the mature proteolytically active form of SREBP-1c, but not the protein level of precursor SREBP-1c [17,37]. Nakatani et al. [39] showed that 10 energy% of fish oil feeding reduces the SREBP-1 proteolytic cascade but not the precursor SREBP-1 protein level, while 30 energy% decreases the mRNA level of SREBP-1 in mouse liver. Although oral administration of 1 mg EPA-E/g body weight/day as was used in this study provided a lower dose of n-3 PUFA than the diet impregnated with fish oil, it reduced the levels of hepatic SREBP-1c mRNA and mature SREBP-1 protein in the nuclear fraction. These data indicate that highly purified EPA-E is an efficient suppressor of SREBP-1 cascade in the liver.

EPA can be metabolized to DHA endogenously through the activity of elongase, Δ6-desaturase, and peroxisomal β-oxidation, but both fatty acids are considered equally effective suppressors of SREBP-1 [40]. The hepatic expression of Δ6-desaturase is reduced by dietary fat, including PUFAs [41], and Sekiya et al. [17] observed that the hepatic content of DHA in mice that ingested a 5% EPA diet was not increased. Conversely, elevation of the hepatic content of DHA was observed in this study when a low dose of EPA-E (1 mg/g/day) was administrated to the mice. The reasons for

![Diagram](image-url)
this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of liver lipids into plasma, (3) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced desaturation of hepatic expression of this difference are not clear, but it is possible that the oral administration of EPA-E is of too low dose to suppress the hepatic expression of A6-desaturase. Alternatively, there may be differences between the ability of EPA and DHA to regulate this enzyme. Further studies are required to clarify differences in the metabolism of EPA and DHA in the liver.

The thiazolidinedione (TZD) drug pioglitazone is a selective ligand of the PPARγ pathway, which acts as an insulin sensitizer. It has been used in the treatment for patients with type 2 diabetes and other insulin-resistant conditions [42]. In line with our data, it has been reported that pioglitazone treatment increases liver weight, which is accompanied by hepatic hypertrophy as seen by histology and morphometry in the KK-Av mice [43]. In addition, rosiglitazone, another TZD derivative, enhanced hepatic steatosis in ob/ob mice [44]. TZD may increase hepatic steatosis by interacting with multiple pathways, including (1) reduction of the mobilization of liver lipids into plasma, (2) enhanced “de novo” synthesis of fatty acids, and (3) induction of hepatic expression of PPARα responsive genes involved in free fatty acid uptake [44,45]. On the other hand, pioglitazone prevents hepatic steatosis and fibrosis in rat liver induced by a choline-deficient diet [46]. In a clinical trial, the treatment of NASH patients with pioglitazone improved this metabolic and histological disorder, and reduced the content of liver fat [47].

The decreased hepatic MUFA of TG in SCDF-deficient mice cannot be reversed with dietary MUFA supplementation, suggesting that de novo synthesis of MUFA is essential for hepatic TG synthesis [25,48]. The key enzyme in the biosynthesis of MUFA is SCOT, which catalyzes the introduction of the first cis-double bond in the Δ9 position in palmitoyl (C16:0)- and stearoyl (C18:0)-CoA, which are then converted into palmitoleoyl (C16:1)- and oleoyl (C18:1)-CoA, respectively. The desaturation index, C16:1/C16:0 and/or C18:1/C18:0, is used to estimate SCOT activity [49,50]. In our study, both the hepatic C16:1/C16:0 and C18:1/C18:0 ratios were correlated with hepatic TG accumulation, especially the C16:1/C16:0 ratio, whereas the HHFD diet used in this study contained a high proportion of C16:0. Attie et al. [49] reported that, in healthy and familial combined hyperlipidemia patients, the desaturation index of plasma C18:1/C18:0 ratio correlates with plasma TG concentration, while the C16:1/C16:0 ratio does not. In hypertiglyceridemic men, the C16:1/C16:0 ratio is increased in plasma, while the C18:1/C18:0 ratio is not [51]. Although the hepatic TG content was correlated with the SCOT activity index with C16:1/C16:0 and C18:1/C18:0 ratios in this study, it will be necessary to investigate the relationship between fatty acid composition in plasma and hepatic TG to determine differences among various diseases.

Plasma concentrations and the hepatic content of palmitoleic acid (C16:1) and oleic acid (C18:1) are increased in steatosis, NAFLD, and non-alcoholic steatohepatitis (NASH) subjects, but the EPA and DHA levels are decreased [52–54]. It has been reported that total fat, n-6 PUFA composition, and the n-6/n-3 PUFA ratio in the liver are higher in patients with hepatic steatosis and NAFLD, and that the n-6/n-3 PUFA ratio is significantly correlated with
severity of steatosis [8,53]. In the mouse model for diet-induced steatosis, a relationship between hepatic TG content and the n-6/n-3 PUFA ratio was not observed, probably because n-6 PUFA content was not increased. Araya et al. [8] suggested that a higher n-6 and lower n-3 PUFA composition in liver is accompanied by an enhancement of (1) the n-6/n-3 PUFA ratio in liver and adipose tissue, (2) the C18:1(n-9) trans level in adipose tissue, and (3) the hepatic lipid peroxidation index. These conditions may favor lipid synthesis over oxidation and secretion, thereby leading to steatosis. On the other hand, we have demonstrated that the composition of hepatic MUFAs was increased, and the MUFA/n-3 PUFA ratio was related to the hepatic TG content. This index may be a biomarker for the diagnosis of steatosis and NAFLD. In agreement with this idea, several reports indicate the importance of

Fig. 5. The relationship between hepatic fatty acid concentration and hepatic TG content. Mice were fed STD or HFHSD for 2 weeks, and EPA-E (1 mg/g/day), PIO (0.03 mg/g/day), or PC (1 mg/g/day) was administered by gavage for 2 weeks in HFHSD-fed mice (n = 8 for each group). Total hepatic lipids were analyzed by gas–liquid chromatography. Correlations between hepatic TG content and the fatty acid concentrations of C16:0 (A), C18:0 (B), C16:1n-7 (C), C18:1n-9 (D), C18:2n-6 (E), C20:4n-6 (F), C20:5n-3 (G), and C22:6n-3 (H) were evaluated by simple linear regression analysis.
of increased MUFA and decreased n-3 PUFA in these disorders [8,52,53]. However, further studies are required to confirm the efficacy and reliability of this index in animals and clinical studies. In conclusion, this study demonstrates that oral administration of highly purified EPA-E ameliorated hepatic fat accumulation through the suppression of SREBP-1 and the key lipogenic enzymes regulated by SREBP-1, which include FAS, GPAT, and SCD1, and also increased the hepatic contents of n-3 PUFAs. Furthermore, highly purified EPA-E, an anti-hyperlipidemia drug, also has a therapeutic potential for NAFLD.

Acknowledgement

We would like to thank Ms. Chiaki Masaki for her skillful assistance.

References


