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

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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 α (ACCα), 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-1 and decreases hepatic MUFAs accumulation by SCD1.

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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

PUFAs are decreased in the hepatic tissue of patients with NAFLD, and are negative regulators of hepatic lipogenesis and inflammatory response [15,16]. It has been reported that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) down-regulate hepatic TG accumulation by decreasing sterol regulatory element binding protein-1 (SREBP-1) transcriptional activity and inducing fatty acid catabolism by activating the peroxisome proliferator-activated receptors (PPARs)-mediated pathway [17–20]. SREBP-1c, which is the predominant isoform of SREBP-1 in the liver, controls the transcription and expression of lipogenic enzymes such as stearoyl-CoA desaturase 1 (SCD1), fatty acid synthase (FAS), and glycerol-3-phosphate acyltransferase (GPAT) [21,22]. SCD1 converts saturated fatty acids (SFAs) into MUFAs, and is the rate-limiting enzyme in the biosynthesis of C16:1 and C18:1 [23]. A deficiency in the SCD1 gene leads to resistance to diet-induced obesity, decreased hepatic TG accumulation, and increased insulin sensitivity and metabolism [24,25].

Evidence from in vitro and in vivo studies suggests differential effects of EPA and DHA [26,27]. Several studies have suggested that EPA may be primarily responsible for the hypotriglyceridemic effect of n-3 long-chain PUFAs [28–31]. Rambjor et al. [32] concluded that EPA is responsible for the TG-lowering effect of fish oils in humans. In contrast, a hypolipidemic effect of DHA was also shown in patients with hypercholesterolemia and combined hyperlipidemia [33,34]. However, EPA is the only member in the class of n-3 long-chain PUFAs that is used clinically as a single-agent treatment for hypertriglyceridemia.

In this study, we assessed the pharmacological effects of highly purified EPA ethyl ester (EPA-E) on fatty liver induced by a Western-style diet, and investigated the relationship between the hepatic fatty acids and TG accumulation.

2. Materials and methods

2.1. Drugs

EPA-E (98% purity; Mochida Pharmaceutical, Tokyo, Japan) or polyenephosphatidylcholine (PC, Taiyo Pharmaceutical Industry, Nagoya, Japan) was suspended in 5% arabic gum (Wako Pure Chemical Industries, Osaka, Japan) solution using a homogenizer (Physcotron 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;

Table 1
Fatty acid composition of STD and HFHSD diets.

		STD	HFHSD
		% (w/w) of total fatty acids	
Caproic 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	0.2	11.1
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.

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.

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 Na_2EDTA, 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 $MgCl_2$, 1 mM Na_2EDTA, 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). 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 RNAlater 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 CH_3CN/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 CH_3OK and 14% BF_3/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
Primer pairs used for quantitative real-time RT-PCR.

Genes	Forward primer	Reverse primer	Amplicon size (bp)	Genbank accession no.
ACCα	TGGTTCTTGGGTTGTGATCGA	TCGGTCAGCGTACATCTCCAT	64	NM_133360
FAS	CTGCAGCTGTCAGTGTGAAGAAG	GCAGCATTTTTACCAGGTTGGT	68	NM_007988
SCD1	AGCTCAGTCTCACTCCTTCCCTTA	CAGCCAGCCTCTTGACTATTCC	71	NM_009127
GPAT	TCATCCAGTATGGCATTCTCACA	GCAAGGCCAGGACTGACATC	67	NM_008149
SREBP-1a	CAGACACTGGCCGAGATGTG	GCTGGAGCATGTCTTCGATGT	67	NM_011480
SREBP-1c	CGGCGCGGAAGCTGT	TGCAATCCATGGCTCCGT	73	NM_011480
36B4	CCTGAAGTGCTCGACATCACA	GCGCTTGTACCCATTGATGA	141	NM_007475

Table 3
Body weight, liver weight, relative liver weight.

Group HFHSD	Control I –	Control II +	EPA-E +	PIO +	PC +
Body weight (g)	26.7 ± 0.2	28.3 ± 0.2[†††]	29.0 ± 0.2	29.0 ± 0.5	28.6 ± 0.4
Liver weight (g)	1.39 ± 0.02	1.40 ± 0.03	1.33 ± 0.03	1.68 ± 0.06[¶]	1.33 ± 0.02
Relative liver weight (%)	5.19 ± 0.05	4.92 ± 0.08[†]	4.59 ± 0.09	5.80 ± 0.15[¶¶¶]	4.64 ± 0.05

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$\pm$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
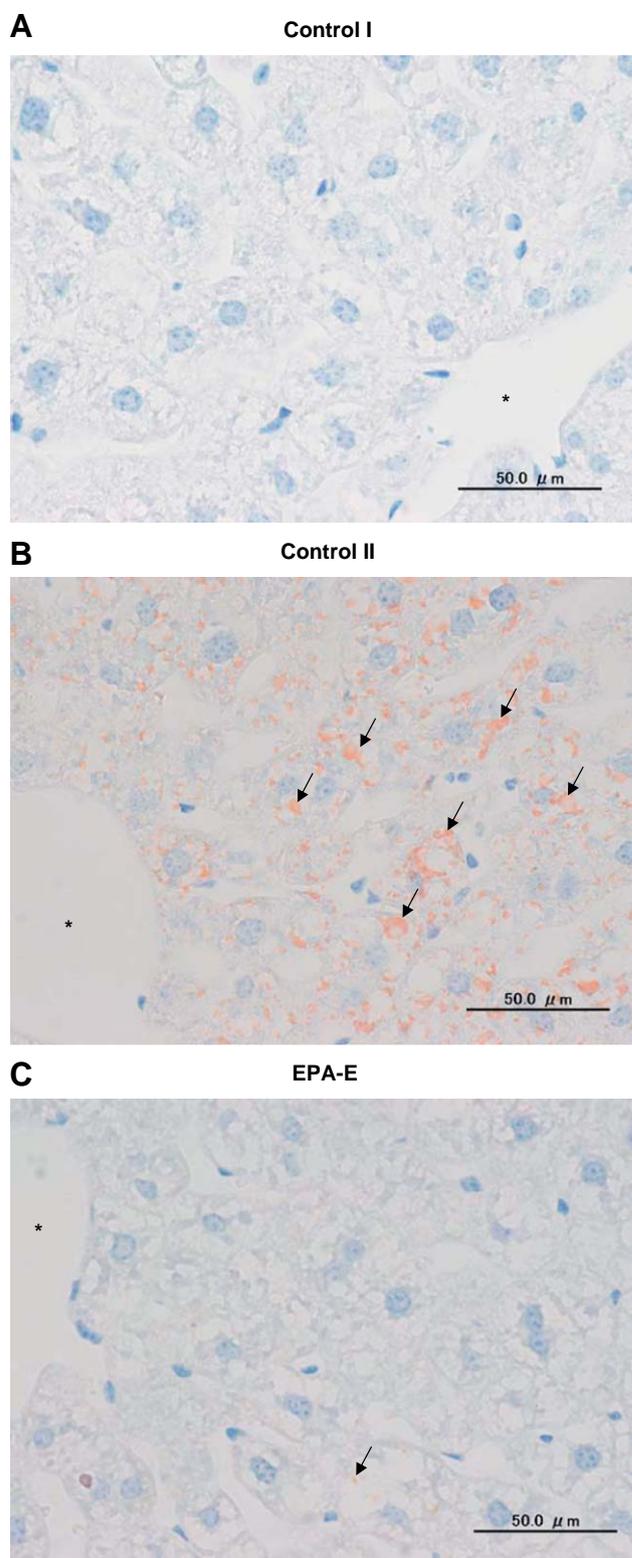

Fig. 1. Representative histological appearance of oil red-O staining of liver sections from mice fed STD (Control I) (A), HFHSD (Control II) (B), and HFHSD plus EPA-E (C) for 2 weeks (original magnification: $\times 400$). Arrows indicate fat accumulation. Asterisks indicate central vein.

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 ACCα, FAS, SCD1, and GPAT in HFHSD-fed mice (Fig. 4C–F). These findings
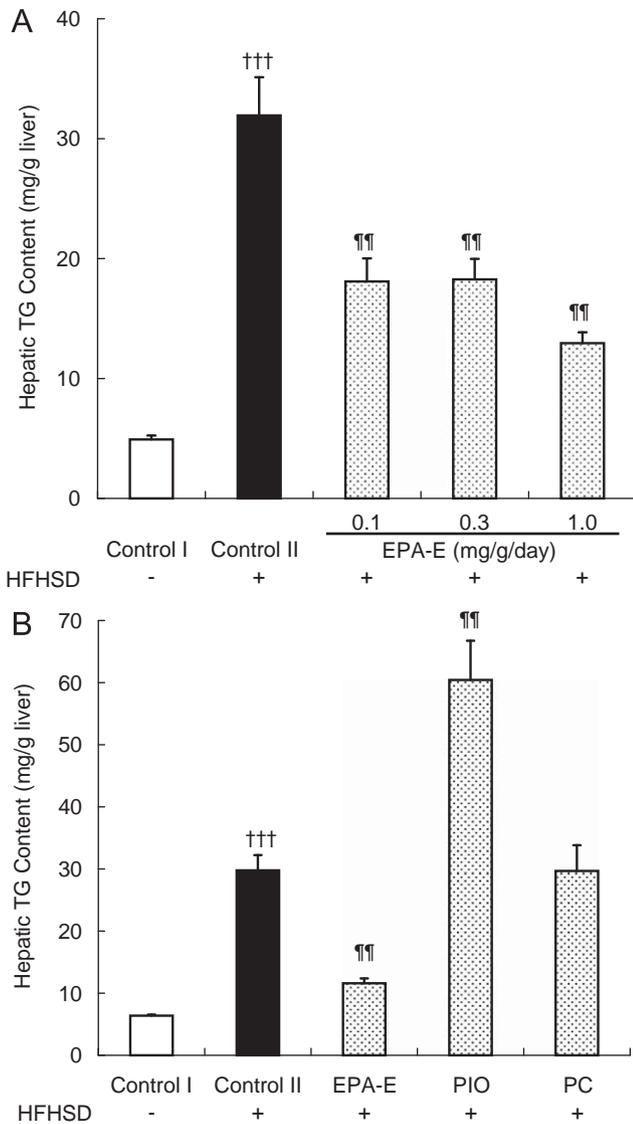
Fig. 2. Effects of EPA-E, PIO, and PC on hepatic TG content in HFHSD-fed mice: (A) mice were fed STD or HFHSD for 2 weeks, and EPA-E (0.1, 0.3, or 1 mg/g/day) was administered by gavage for 2 weeks in HFHSD-fed mice. (B) Mice were fed STD or HFHSD for 2 weeks. EPA-E (1 mg/g/day), PIO (0.03 mg/g/day), 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. $^{†††}P<0.001$ versus control I (Aspin–Welch t-test), $^{¶¶}P<0.01$ versus control II (Steel test).

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.
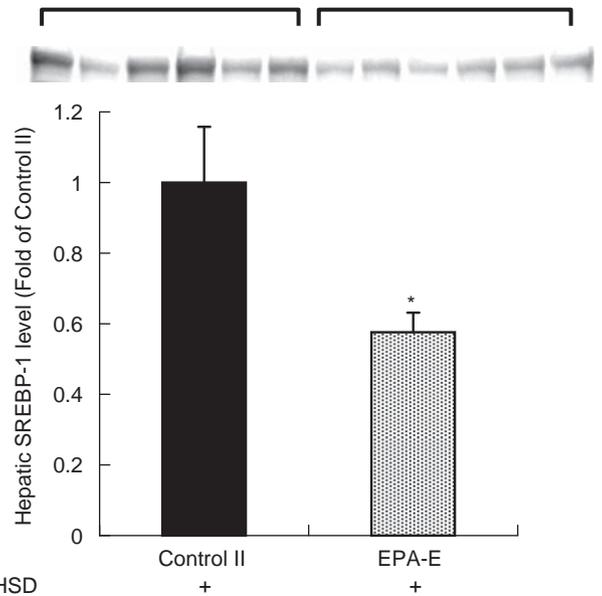

Fig. 3. Effect of treatment with EPA-E on hepatic nuclear SREBP-1 level in HFHSD-fed mice. Mice were fed HFHSD for 2 weeks with or without EPA-E administration (1 mg/g/day). Hepatic nuclear SREBP-1 level were determined by western blotting. The intensity of bands was analyzed by densitometry and expressed relative intensity to the mean control II level. Values are expressed as the mean±SE for six mice in each group. $^{*}P<0.05$ versus control II (Aspin–Welch t-test).

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, and 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 C18:0 ($R^2 = 0.3579$; $P<0.0001$), 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).
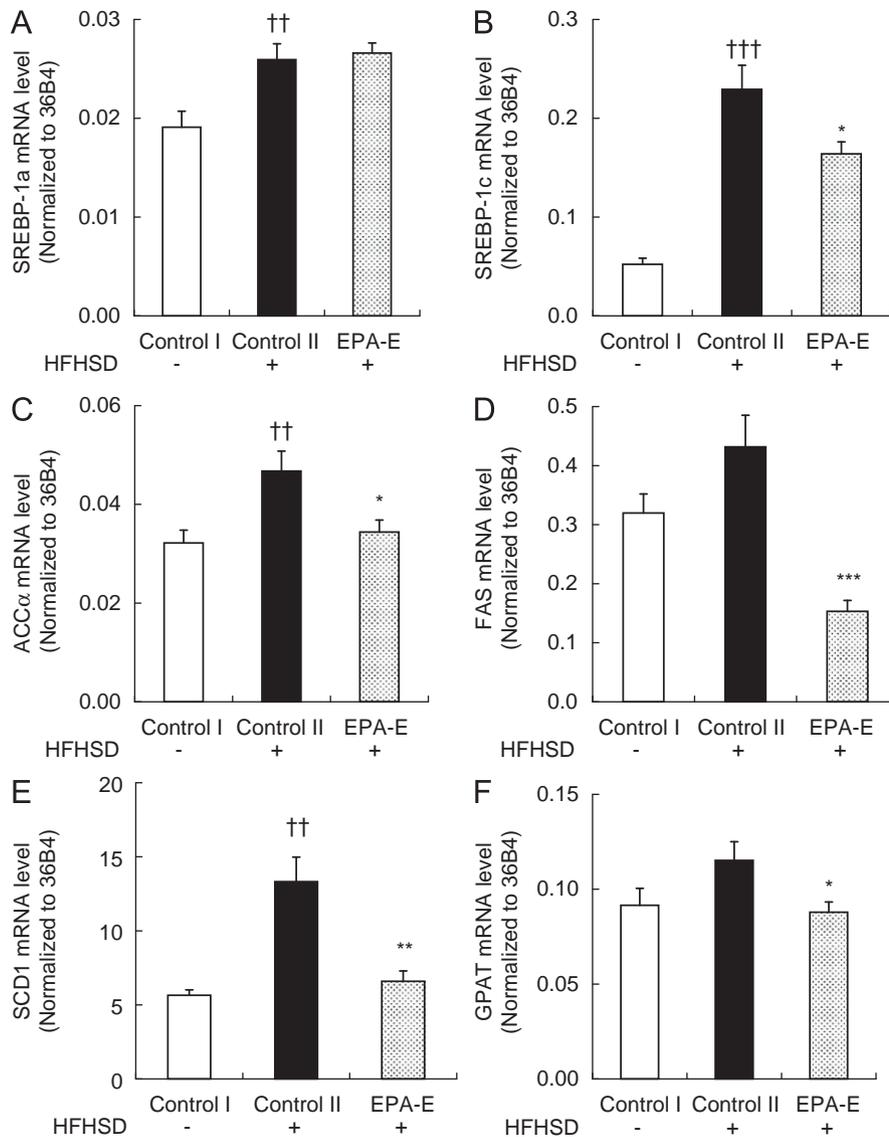
Fig. 4. Effects of EPA-E on the liver mRNA levels of SREBP-1a (A), SREBP-1c (B), ACCα (C), FAS (D), SCD1 (E), and GPAT (F) in HFHSD-fed mice. Mice were fed STD or HFHSD for 2 weeks, and EPA-E (1 mg/g/day) was administered by gavage for 2 weeks in HFHSD-fed mice. mRNA levels were measured using quantitative real-time RT-PCR and normalized to 36B4 levels. Values are expressed as the mean±SE for eight mice in each group. $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ versus control I (Student's or Aspin–Welch t-test), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control II (Student's or Aspin–Welch t-test).

4. Discussion

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

Table 4
Fatty acid composition in hepatic total lipids.

Fatty acids		Fatty acid composition (wt% of total fatty acid)				
	Group HFHSD	Control I −	Control II +	EPA-E +	PIO +	PC +
Lauric acid	C12:0	ND	0.06 ± 0.01	0.04 ± 0.01	0.03 ± 0.00¶¶	0.06 ± 0.00
Myristic acid	C14:0	0.25 ± 0.00	1.53 ± 0.08†††	1.07 ± 0.09¶	0.83 ± 0.03¶¶¶	1.41 ± 0.11
Palmitic acid	C16:0	27.44 ± 0.24	27.17 ± 0.30	26.79 ± 0.25	24.78 ± 0.22¶¶¶	27.09 ± 0.39
Stearic acid	C18:0	11.50 ± 0.12	7.03 ± 0.59†††	10.22 ± 0.51¶¶¶	7.15 ± 0.40	7.29 ± 0.63
Arachidic acid	C20:0	0.25 ± 0.00	0.25 ± 0.02	0.33 ± 0.01¶¶¶	0.19 ± 0.01¶¶	0.27 ± 0.01
Behenic acid	C22:0	0.44 ± 0.01	0.30 ± 0.03††	0.49 ± 0.03¶¶¶	0.28 ± 0.02	0.32 ± 0.03
Lignoceric acid	C24:0	0.39 ± 0.01	0.21 ± 0.02†††	0.30 ± 0.02¶	0.18 ± 0.01	0.18 ± 0.02
Total SFAs		40.26 ± 0.19	36.55 ± 0.52†††	39.24 ± 0.46¶¶¶	33.46 ± 0.42¶¶¶	36.60 ± 0.34
Palmitoleic acid	C16:1n-7	2.69 ± 0.11	7.92 ± 0.38†††	4.41 ± 0.37¶¶¶	9.87 ± 0.40¶¶	7.16 ± 0.53
Oleic acid	C18:1n-9	15.26 ± 0.29	34.12 ± 1.52†††	24.03 ± 0.94¶¶¶	34.43 ± 1.16	32.45 ± 1.47
Eicosenoic acid	C20:1n-9	0.36 ± 0.01	0.62 ± 0.04†††	0.34 ± 0.02¶¶¶	0.56 ± 0.03	0.60 ± 0.03
Erucic acid	C22:1n-9	0.05 ± 0.00	0.07 ± 0.01††	0.05 ± 0.01¶	0.06 ± 0.00	0.05 ± 0.00¶
Nervonic acid	C24:1n-9	0.38 ± 0.01	0.17 ± 0.02†††	0.17 ± 0.01	0.22 ± 0.02	0.17 ± 0.02
Total MUFAs		18.74 ± 0.38	42.91 ± 1.88†††	28.98 ± 1.32¶¶¶	45.14 ± 1.40	40.43 ± 1.99
Eicosatrienoic acid	C20:3n-9	0.26 ± 0.01	0.81 ± 0.10†††	0.10 ± 0.01¶¶	1.19 ± 0.11¶	0.56 ± 0.06
Linoleic acid	C18:2n-6	16.14 ± 0.25	5.42 ± 0.29†††	5.58 ± 0.26	5.19 ± 0.21	7.15 ± 0.37¶¶¶
γ-linolenic acid	C18:3n-6	0.38 ± 0.01	0.11 ± 0.01†††	0.06 ± 0.00¶¶¶	0.16 ± 0.01¶¶¶	0.13 ± 0.00¶
Eicosadienoic acid	C20:2n-6	0.29 ± 0.01	0.09 ± 0.00†††	0.08 ± 0.00	0.09 ± 0.00	0.15 ± 0.00¶¶¶
Dihomo-γ-linolenic acid	C20:3n-6	1.99 ± 0.04	1.00 ± 0.08†††	0.57 ± 0.02¶¶	1.38 ± 0.08¶	1.22 ± 0.10
Arachidonic acid	C20:4n-6	13.42 ± 0.17	4.64 ± 0.45†††	3.78 ± 0.20	5.70 ± 0.35	5.11 ± 0.50
Docosatetraenoic acid	C22:4n-6	0.38 ± 0.01	0.13 ± 0.01†††	0.09 ± 0.01¶¶	0.12 ± 0.01	0.14 ± 0.01
Total n-6 PUFAs		32.60 ± 0.36	11.38 ± 0.75†††	10.16 ± 0.42	12.64 ± 0.60	13.90 ± 0.95¶
Linolenic acid	C18:3n-3	0.25 ± 0.01	0.37 ± 0.02††	0.62 ± 0.03¶¶¶	0.21 ± 0.01¶¶¶	0.40 ± 0.03
Eicosapentaenoic acid	C20:5n-3	0.31 ± 0.01	1.57 ± 0.13†††	9.58 ± 0.49¶¶	1.73 ± 0.10	1.42 ± 0.11
Docosapentaenoic acid	C22:5n-3	0.44 ± 0.01	0.57 ± 0.03††	3.53 ± 0.15¶¶	0.56 ± 0.03	0.64 ± 0.02
Docosahexaenoic acid	C22:6n-3	7.13 ± 0.11	5.83 ± 0.53†	7.80 ± 0.39¶	5.07 ± 0.29	6.06 ± 0.63
Total n-3 PUFAs		8.13 ± 0.11	8.34 ± 0.68	21.53 ± 0.74¶¶¶	7.57 ± 0.41	8.52 ± 0.74
Total PUFAs		41.00 ± 0.45	20.54 ± 1.49†††	31.78 ± 0.96¶¶¶	21.41 ± 1.05	22.98 ± 1.72
Total fatty acids (μg/g liver)		23,314 ± 321	38,216 ± 3447††	27,028 ± 1677¶	30,534 ± 2433	37,764 ± 3771

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. $^\dagger P<0.05$, $^{\dagger\dagger} P<0.01$, $^{\dagger\dagger\dagger} P<0.001$ versus control I (Student's or Aspin–Welch t-test). $^\P P<0.05$, $^{\P\P} P<0.01$, $^{\P\P\P} P<0.001$ versus control II (Dunnett or Steel test).
ND: not detected.

this difference are not clear, but it is possible that the oral administration of EPA-E is of a too low dose to suppress the hepatic expression of Δ6-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 hepatocyte hypertrophy as seen by histology and morphometry in the KK-A^y 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 PPARγ 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 MUFAs of TG in SCD-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 SCD, 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 SCD 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 HFHSD 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 hypertriglyceridemic 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 SCD 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 [8,52–54]. 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
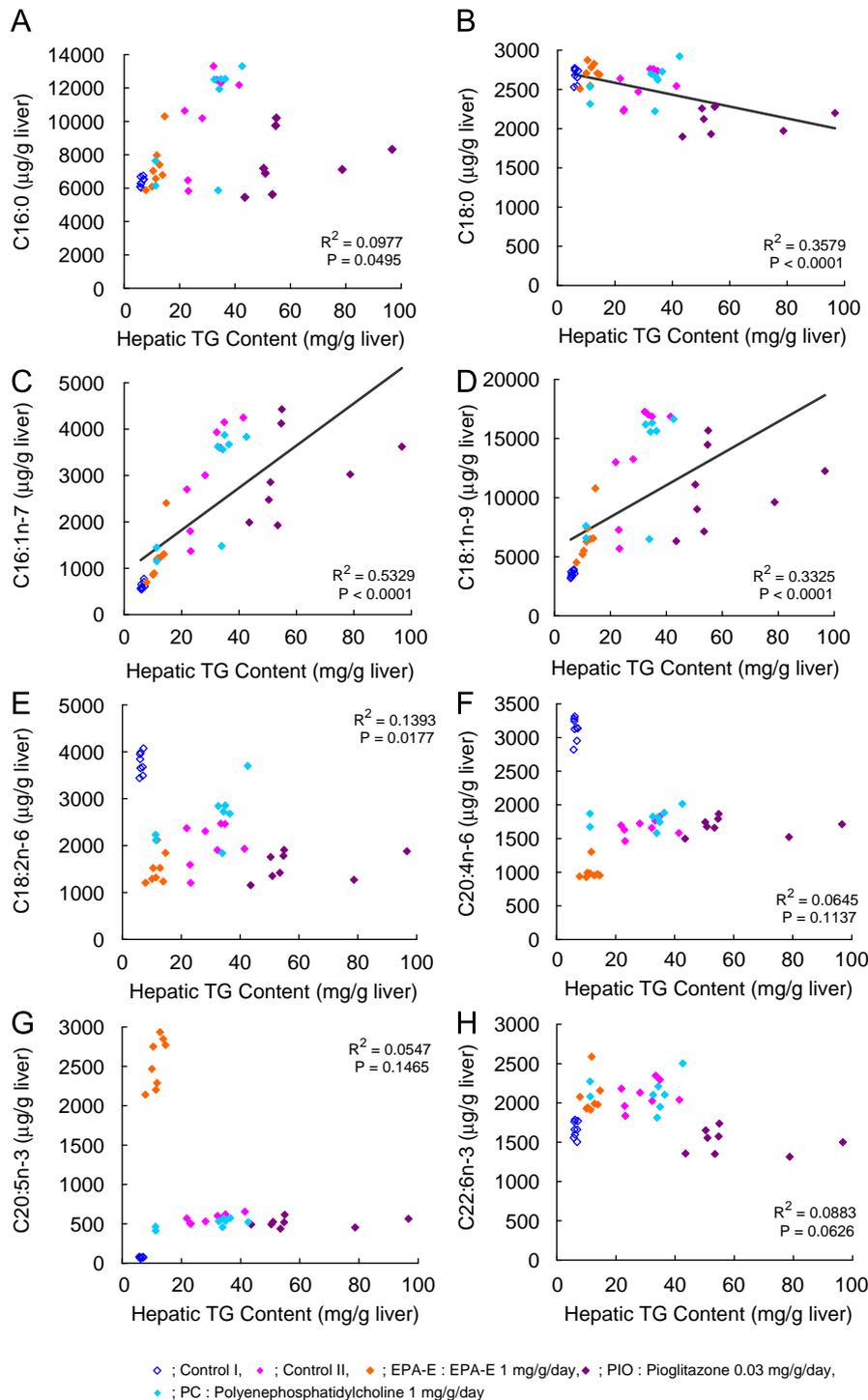
Fig. 5. The relationship between hepatic fatty acid concentration and hepatic TG content. Mice were fed STD or HFHSD for 2 weeks, and EPA-E (1 mg/g/day), PIO (0.03 mg/g/day), or PC (1 mg/g/day) was administered by gavage for 2 weeks in HFHSD-fed mice ($n = 8$ for each group). Total hepatic lipids were 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.

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
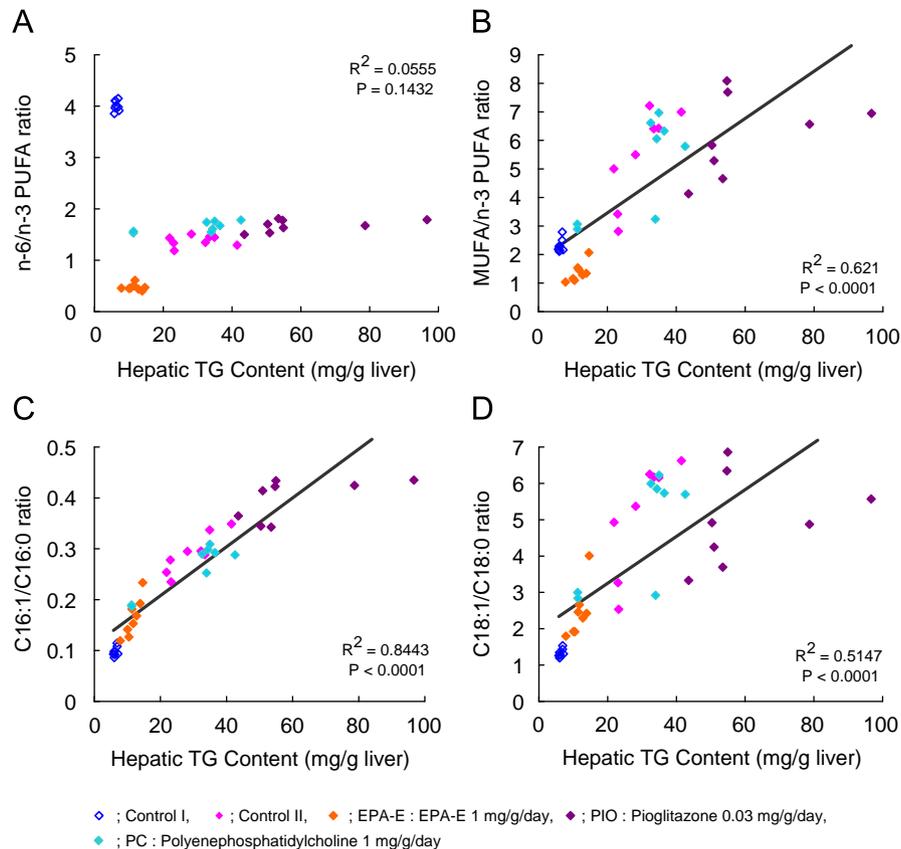
Fig. 6. The relationship between the ratio of hepatic fatty acid concentrations and hepatic TG content. Mice were fed STD or HFHSD for 2 weeks, and EPA-E (1 mg/g/day), PIO (0.03 mg/g/day), or PC (1 mg/g/day) was administered by gavage for 2 weeks in HFHSD-fed mice ($n = 8$ for each group). Total hepatic lipids were analyzed by gas–liquid chromatography. Correlations between hepatic TG content and the ratio of the fatty acids n-6/n-3 PUFA (A), MUFA/n-3 PUFA (B), C16:1/C16:0 (C), or C18:1/C18:0 (D) 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.

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