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Dietary Effects of Oxidized Eicosapentaenoic Acid (EPA) and Intact EPA on Hepatic Steatosis Induced by a High-sucrose Diet and Liver-X-receptor α Agonist in Mice

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Abstract: Numerous studies have shown that dietary omega-3 polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA), improve lipid metabolism. The beneficial effects of PUFA-derived oxidation products have been increasingly reported. However, EPA is easily oxidized in food products and in the human body, generating various derivatives of oxidized EPA (oxEPA), such that these oxidation products may partially contribute to EPA's effect. We previously reported that oxEPA was more potent than intact EPA in reducing liver-X-receptor α (LXRα)-induced cellular triacylglycerol (TG) accumulation. However, the *in vivo* hypolipidemic effects of oxEPA remain unclear. In the present study, we evaluated the effect of oral administration of EPA and oxEPA on hepatic steatosis in mice induced by a high-sucrose diet and a synthetic LXRα agonist, TO-901317. Both EPA and oxEPA reduced TG accumulation in the liver and plasma biomarkers of liver injury. Furthermore, they suppressed the expression of lipogenic genes, but not β-oxidation genes, in a similar pattern as the biomarkers. Our results suggest that oxEPA and intact EPA suppress *de novo* lipogenesis to ameliorate hepatic steatosis.

Key words: eicosapentaenoic acid, hepatic steatosis, oxidized lipids, SREBP-1c, triacylglycerol

1 INTRODUCTION

Recently, the prevalence of lipid metabolic disorders associated with obesity and diabetes has been dramatically increased worldwide because of the consumption of unbalanced diets and lack of exercise[1)]. Ectopic fat accumulation has attracted a great deal of public attention among lipid metabolism disorders. It is associated with an increased risk for detrimental metabolic conditions, such as nonalcoholic fatty liver disease (NAFLD)[2)]. Furthermore, the subsequent oxidative stress and inflammation induced by excessive lipid accumulation can cause nonalcoholic steatohepatitis (NASH), which has a significant risk of progressing to more severe liver diseases, including hepatic cirrhosis and hepatocellular carcinoma[3-5)]. The rise in the number of patients with NASH has become a serious problem in recent years. Effective therapies have not yet been established for this disease. Therefore, a suppression of hepatic lipogenesis may be as important as decreased fat intake and increased energy consumption in order to prevent NASH development.

One potentially useful treatment for such diseases is eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid (PUFA), which has been clinically used as a pharmaceutical agent and dietary supplement due to its multiple health benefits, including the lowering of serum and hepatic triacylglycerol (TG) levels[6, 7)]. EPA suppresses lipogenesis through the antagonization of liver-X-receptor α (LXRα) and by decreasing the expression of sterol-regulatory element binding protein-1c (SREBP-1c)[8, 9)], which is the key transcription factor in lipogenic gene regulation in

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the liver[10, 11]. In addition, EPA can promote β-oxidation through the activation of peroxisome proliferator-activated receptor α (PPARα)[12, 13] and can resolve inflammation by acting as a lipid mediator[14, 15]. Because of its multiple health benefits, EPA is thought to prevent and improve lipid metabolic disorders such as NASH.

Although the diverse effects of EPA have been established, the biological functions of EPA-derived autoxidation products are poorly understood. Since EPA has five carbon double bonds in its structure, it is readily oxidized to various oxidation products in food and the human body[16, 17]. Lipid oxidation has been proposed to be harmful to human health and food quality. However, the favorable biological functions of PUFA-derived oxidation products have increasingly been investigated. For example, oxidized docosahexaenoic acid (DHA) and EPA can suppress oxidative stress[18] and inflammation[19–21]. We previously revealed that in hepatocytes, oxidized EPA (oxEPA) reduced LXRα-induced cellular TG accumulation more potently than intact EPA[22]. However, the *in vivo* effect of dietary oxEPA on lipid metabolism remains unknown.

A high-sucrose diet can promote *de novo* lipogenesis through the activation of transcription factors which regulate the expression of enzymes associated with fatty acid synthesis, leading to obesity, diabetes, and fatty liver disease in animal models[23–26]. However, a high-sucrose diet could potentially be insufficient to induce hepatic steatosis in our preliminary experimental condition. Therefore, to compare effects of dietary EPA and oxEPA on hepatic steatosis, we employed a mouse model fed a high-sucrose diet in combination with the administration of a synthetic LXRα agonist to induce severe short-term hepatic lipogenesis[27]. In this model, oxEPA as well as intact EPA reduced TG accumulation in the liver via the suppression of lipogenic gene expression.

2 EXPERIMENTAL

2.1 Chemicals

EPA, polyoxyethylene (20) sorbitan monolaurate (Tween 20), and dimethyl sulfoxide (DMSO) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). A synthetic LXRα agonist, TO-901317, was purchased from Cayman Chemicals (Ann Arbor, MI, USA).

2.2 Preparation of oxEPA

We had previously determined that 4-h oxEPA was the most effective in suppressing lipid synthesis and accumulation in the hepatocellular carcinoma cell line HepG2[22]. To prepare 4-h oxEPA, EPA was incubated in brown glass tubes at 40℃ for 4 h. After incubation, the prepared oxEPA was diluted in ethanol and stored at −80℃ until use. We confirmed the derivative composition of the EPA oxidation products in the obtained oxEPA by liquid chromatography-mass spectrometry (LC-MS), as reported previously[22].

2.3 Animals and treatments

Eight-week-old male ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were kept in individual cages in a temperature-controlled facility with a constant 12-h light/dark cycle. All experimental animal protocols were approved by the Animal Experimentation Committee of Kyoto University for the care and use of experimental animals. After a week habituation period, the mice were randomly divided into 4 experimental groups (n = 6/group). The mice in the standard diet (SD) group were fed an AIN-93G diet containing 10 wt% sucrose. The high-sucrose and TO-901317 (HS + TO) group was fed a high-sucrose diet that consisted of a modified AIN-93G diet containing 40 wt% sucrose (Table 1) and was orally administered 0.5 mg/mouse TO-901317. The EPA and oxEPA groups were fed the high-sucrose diet and daily administered TO-901317 and either 5 mg/mouse EPA or oxEPA by oral gavage, respectively. EPA and oxEPA were dissolved in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (v/v) and 6% DMSO (v/v). Body weight and food intake were monitored throughout the experiment. After 2 weeks of treatment, mice were fasted for 12 h and then euthanized under isoflurane anesthesia. Blood was collected from the postcaval vein with a heparin-coated syringe, and centrifuged at 400 × *g* for 15 min at 4℃ to prepare the plasma samples. The harvested tissues were weighed and immediately frozen in liquid nitrogen. Aliquots of liver tissues were stored in RNA Later Solution (Life Technologies, Carlsbad, CA, USA) for RNA isolation. All samples were stored at −80℃ until further analysis.

Table 1 Composition of standard and high-sucrose diet.

Ingredient (wt%)	Standard diet	High-sucrose diet
Cornstarch	39.7486	10
Casein	20	20
Maltodextrin	13.2	13.2
Sucrose	10	39.7486
Soybean oil	7	7
Cellulose	5	5
Mineral Mix	3.5	3.5
Vitamin Mix	1	1
L-cystine	0.3	0.3
Choline bitartrate	0.25	0.25
Dibutylhydroxytoluene	0.0014	0.0014
Total	100	100
Total energy (kcal/g)	3.6621	3.7603
Sucrose (kcal%)	10.5678	40.9087

2.3.1 Measurement of plasma biochemical parameters

The plasma levels of TG, non-esterified fatty acids (NEFA), total cholesterol (T-Chol), high-density lipoprotein-Chol (HDL-Chol), free-Chol (F-Chol), glucose, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using commercial enzymatic kits (TG-E, NEFA C, T-Cho E, HDL-C E, F-Cho E, Glu C II, and Transaminase C II, respectively; Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer's instructions. The levels of non HDL-Chol were calculated by subtracting levels of HDL-Chol from those of T-Chol.

2.3.2 Measurement of hepatic TG and T-Chol

Liver sample aliquots (approximately 50 mg) were homogenized in PBS on ice with the handy homogenizer (T10 basic ULTRA-TURRAX, IKA). Hepatic lipids were extracted from the homogenates with chloroform/methanol (2:1, v/v). The lipid samples were dissolved in methanol containing 50% Triton X-100, followed by evaporation under a nitrogen stream. The levels of TG and T-Chol were measured using commercial kits as described above.

2.3.3 RNA extraction and quantitative real time reverse transcription polymerase chain reaction (RT-PCR)

Liver tissues stored in RNA Later Solution were washed in PBS and homogenized with the handy homogenizer in Sepasol®-RNA I Super G (Nacalai Tesque, Inc.). The total RNA was extracted from the homogenate by phenol-chloroform extraction and transcribed to complementary DNA using SuperScript RNase II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). To quantify gene expression associated with lipid metabolism, real time PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) with a thermal cycler (Bio-Rad Laboratories). Table 2 shows the primer pairs used for each gene quantification. The thermal cycle program consisted of 15 min at 95℃ and 43 cycles of 15 sec at 95℃ and 30 sec at 60℃. The values for the relative expression were normalized against the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an internal standard, and were quantified using the ΔΔCt method.

2.4 Statistical analysis

Experimental data are reported as the mean ± standard error of the mean (SEM). Statistical analyses were conducted with a one-way ANOVA followed by the Tukey–Kramer test to identify statistically significant differences. Statistical analyses were conducted in Stat View (SAS institute, Cary, NC, USA).

3 RESULTS

3.1 LC–MS analysis of 4-h oxEPA

As previously reported, several ions at mass to charge ratio (m/z) values of 317, 333, 349, 365, 381, and 397 were detected in the 4-h oxEPA (data not shown)[22]. These ions and values corresponded to EPA oxidation products, gaining 1, 2, 3, 4, 5, or 6 oxygen atoms, respectively. After a 4-h incubation, the ion of monohydroxy-EPA, at *m/z* 317, was most abundant among the EPA oxidation products. 5-, 11-, and 18-HEPE were identified in the total intensity of the molecular ion $[M\text{-}H]^-$ at *m/z* 317 at 4.9%, 14.1%, and 12.3%, respectively, using commercially available standards. Approximately 4% of total HEPE was identified in the 4-h oxEPA. Other numerous small ion peaks, which may indicate the secondary oxidation products, including aldehyde and ketone, but they were not identified. The remainder of the 4-h oxEPA sample is almost non-oxidized EPA.

3.2 Effect of EPA and oxEPA on tissue weight

While the total calorie intake during the treatment was not significantly different among the 4 groups, the body weight gain was higher in the HS + TO, EPA, and oxEPA groups than in the SD group (Table 3). The liver weight in the HS + TO group was significantly higher than the SD group. The liver weight in the EPA and oxEPA groups tended to decrease, although this was not statistically significant different (Table 4). The weights of the gastrocnemius muscle and white adipose tissue (WAT), including the epididymal, mesenteric, and perirenal WAT, was not significantly different among the groups.

Table 2 Real time RT-PCR primers used for quantification of mRNAs.

Gene name	Accession number	Forward (from 5' to 3')	Reverse (from 5' to 3')
Acc	NM_133360	AAACTGCAGGTATCCCAACTCTTC	CTGTGGAACATTTAAGATACGTTTCGAAAA
Acox1	NM_015729	ACCTTCACTTGGGCATGTTC	TTCCAAGCCTCGAAGATGAG
Cpt1a	NM_013495	CTCCGCCTGAGCCATGAAG	CACCAGTGATGCCATTCT
Fas	NM_007988	CCTGGAACGAGAACACGATCT	AGACGTGTCACTCCTGGACTTG
Gapdh	NM_008084	CGTCCCGTAGACAAAATGGT	TGCCGTGAGTGGAGTCATAC
Pgc1a	NM_008904	GAAGTGGTGTAGCGACCAATC	AATGAGGGCAATCCGTCTTCA
Ppara	NM_011144	GTACGGTGTGTATGAAGCCATC	GCCGTACGCGATCAGCAT
Scd1	NM_009127	ACAGTCCAGGGCCAACGGT	GGCACCTTACACAGCCAGTT
Srebp1c	NM_011480	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGCCCAG

Table 3 Weight gain and total calorie intake during 2 weeks feeding.

	SD	HS+TO	EPA	oxEPA
Weight gain (g)	4.6 ± 0.6[a]	6.8 ± 0.6[b]	6.9 ± 0.5[b]	7.1 ± 0.5[b]
Total calorie intake (kcal)	247.3 ± 6.9	262.9 ± 5.1	267.0 ± 5.9	263.8 ± 6.2

The data are shown as the mean ± SEM (n = 6/group).

Values with different letters are significantly different ($p < 0.05$, Tukey-Kramer test).

Table 4 Tissue ratios of mice after 2 weeks feeding.

Tissues (g/100 g body weight)	SD	HS+TO	EPA	oxEPA
Liver	3.9 ± 0.1[a]	9.4 ± 1.1[b]	6.9 ± 0.7[a,b]	7.1 ± 0.8[b]
Total WAT	5.4 ± 0.5	4.1 ± 0.3	4.6 ± 0.2	5.0 ± 0.5
Epididymal WAT	2.6 ± 0.2	2.1 ± 0.1	2.5 ± 0.1	2.6 ± 0.2
Mesenteric WAT	1.2 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
Perirenal WAT	1.5 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.2
Gastrocremius muscle	1.9 ± 0.1	1.7 ± 0.2	1.6 ± 0.1	1.6 ± 0.1

The data are shown as the mean ± SEM (n = 6/group).

Values with different letters are significantly different ($p < 0.05$, Tukey-Kramer test).

Table 5 Parameters of plasma in mice after 2 weeks of feeding.

	SD	HS+TO	EPA	oxEPA
TG (mg/dL)	156.9 ± 8.4[b]	39.8 ± 4.5[a]	89.8 ± 33.5[a,b]	148.3 ± 35.5[b]
NEFA (mEq/L)	2.5 ± 0.1[b]	1.1 ± 0.1[a]	2.1 ± 0.5[a,b]	2.7 ± 0.4[b]
T-Chol (mg/dL)	131.3 ± 14.1[a]	221.7 ± 17.4[b]	202.2 ± 15.7[b]	198.7 ± 17.6[b]
HDL-Chol (mg/dL)	107.4 ± 12.0[a]	139.3 ± 10.4[a,b]	142.3 ± 6.5[b]	133.5 ± 6.6[a,b]
non HDL-Chol (mg/dL)	23.9 ± 4.2[a]	82.4 ± 7.9[b]	60.0 ± 10.7[a,b]	65.2 ± 13.3[b]
F-Chol (mg/dL)	33.4 ± 3.7[a]	56.3 ± 5.8[b]	51.6 ± 3.4[a,b]	53.9 ± 5.3[b]
ALT (IU/L)	128.8 ± 3.2[a]	164.2 ± 11.9[b]	135.2 ± 3.6[a,b]	146.7 ± 9.2[a,b]
AST (IU/L)	188.4 ± 10.0[a]	357.8 ± 47.7[b]	251.4 ± 24.8[a,b]	284.3 ± 44.7[a,b]
Glucose (mg/dL)	155.8 ± 17.4	227.1 ± 16.9	204.9 ± 27.2	208.1 ± 16.5

The data are shown as the mean ± SEM (n = 6/group).

Values with different letters are significantly different ($p < 0.05$, Tukey-Kramer test).

3.3 Effect of EPA and oxEPA on plasma parameters

Table 5 shows the major biochemical parameters measured in the plasma. The plasma concentrations of TG and NEFA were significantly lower in the HS + TO group. This reduction was reversed by oxEPA treatment. The EPA treatment showed a similar effect on plasma TG and NEFA levels to that of oxEPA, although this result was not statistically significant. The T-Chol levels were significantly higher in the HS + TO, EPA, and oxEPA groups than in the SD group. The HDL-Chol levels were significantly higher in the EPA group, while the non HDL- and F-Chol levels were higher in the HS + TO and oxEPA groups than in the SD group. The ALT and AST plasma activities were significantly higher in the HS + TO group than in the SD group. This increase tended to be suppressed by the EPA and oxEPA treatments. Among the 4 groups, there were no significant differences in the plasma glucose levels.

3.4 Effect of EPA and oxEPA on liver lipid content

The hepatic TG levels were markedly higher in the HS + TO group than in the SD group (Fig. 1A). This increase in the hepatic TG levels induced by a high-sucrose diet and TO-901317 was significantly inhibited in the oxEPA group. The EPA treatment also suppressed TG accumulation. However, resulting TG levels were not significantly different from the HS + TO group's TG levels. The hepatic content of T-Chol was significantly lower in the HS + TO, EPA, and oxEPA groups than in the SD group (Fig. 1B).

3.5 Effect of EPA and oxEPA on lipid metabolism-associated gene expression in liver

We evaluated the hepatic gene expression of SREBP-1c

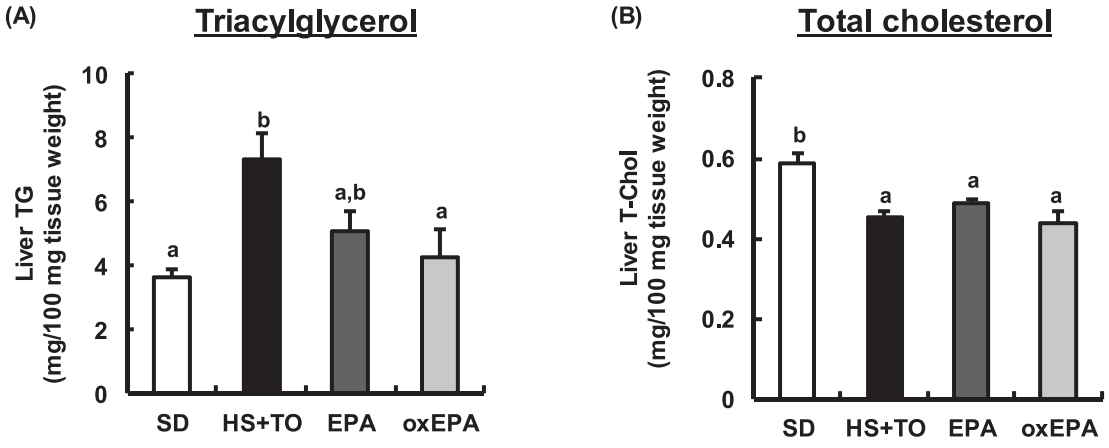

Fig. 1 Effect of eicosapentaenoic acid (EPA) and oxidized EPA (oxEPA) on lipid content in liver tissue. The hepatic content of triacylglycerol (**A**) and total cholesterol (**B**) were measured at the end of the treatment period. The data are shown as the mean ± SEM (n = 6/group). Values with different letters are significantly different ($p < 0.05$).

and its target genes, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1)[10]. The expression levels of FAS and SCD1 mRNA were significantly higher in the HS + TO group than in the SD group, this increase was significantly suppressed in the EPA group (Fig. 2A). The oxEPA treatment showed lower expression of FAS and SCD1 genes than the HS + TO treatment did, although this was not significant.

We also evaluated the hepatic expression of genes associated with fatty acid β-oxidation: acyl-CoA oxidase 1 (ACOX1), carnitine palmitoyltransferase 1A (CPT1A), PPARγ coactivator 1α (PGC1α), and PPARα[28]. There were no significant differences in the mRNA expression levels of ACOX1, CPT1A, and PPARα among the 4 groups (Fig. 2B). The PGC1α mRNA expression levels were significantly lower in the EPA group than in the SD group.

4 DISCUSSION

These studies demonstrate that the increase in the hepatic TG content induced by a high sucrose diet and LXRα agonist TO-901317 was suppressed in the oxEPA and EPA groups (Fig. 1A). In contrast, the plasma TG and NEFA levels were decreased in the HS + TO group, and this decrease was reversed in the oxEPA group (Table 4). In the HS + TO group, the secretion of very low-density lipoprotein (VLDL), which transports TG from the liver to peripheral tissues, may be impaired due to hepatic steatosis[29,30]. Treatment with oxEPA could ameliorate this liver damage, which is supported by the oxEPA-induced suppression of the ALT and AST increases (Table 5), and then, might improve VLDL secretion. While the gene expression of the β-oxidation enzymes was not markedly affected, the higher FAS and SCD1 mRNA expression levels induced by a high-sucrose diet and TO-901317 administration tended to be suppressed by oxEPA treatment, similar to EPA treatment (Fig. 2A and 2B). Our results suggest that EPA and oxEPA can ameliorate hepatic steatosis through the suppression of lipogenic genes.

Previously, we reported that oxidized EPA at an early stage of autoxidation and hydroxy-EPA (HEPE) were more potent than intact EPA in reducing LXRα-induced cellular TG accumulation through the suppression of SREBP-1c expression[22]. In this study, intact EPA and oxEPA also suppressed the expression of lipogenic genes, but not β-oxidation genes (Fig. 2A and 2B). However, SREBP-1c mRNA expression levels did not change in the EPA or oxEPA group compared with that in the HS + TO group. Hepatic steatosis was induced in mice by a high-sucrose diet and LXRα agonist administration, in which differs from previous experiments using cultured cells[22]. Therefore, we speculate that the hypolipidemic effect of EPA and oxEPA in the present experimental conditions may occur through the regulation of SREBP-1c. In addition, this hypolipidemic effect may also be due to the regulation of other transcription factors which control glucose and lipid metabolism, such as hepatic nuclear factor 4α (HNF4α) and carbohydrate response element binding protein (ChREBP). These factors respond to glucose levels and promote glycolysis and lipogenesis[31,32].

Recently, the beneficial effects of PUFA-derived oxidation products have been reported. A_4/J_4-neuroprostanes, derived from DHA during peroxidation, ameliorate endothelial inflammation[18,19]. OxEPA has also been shown to inhibit leukocyte–endothelial interactions[21]. Previously, we demonstrated that 18-HEPE is the most effective in suppressing SREBP-1c expression and its target gene mRNAs in hepatocytes[22]. The beneficial effects of omega-3 PUFAs may depend in part on their oxidized form. In this study,

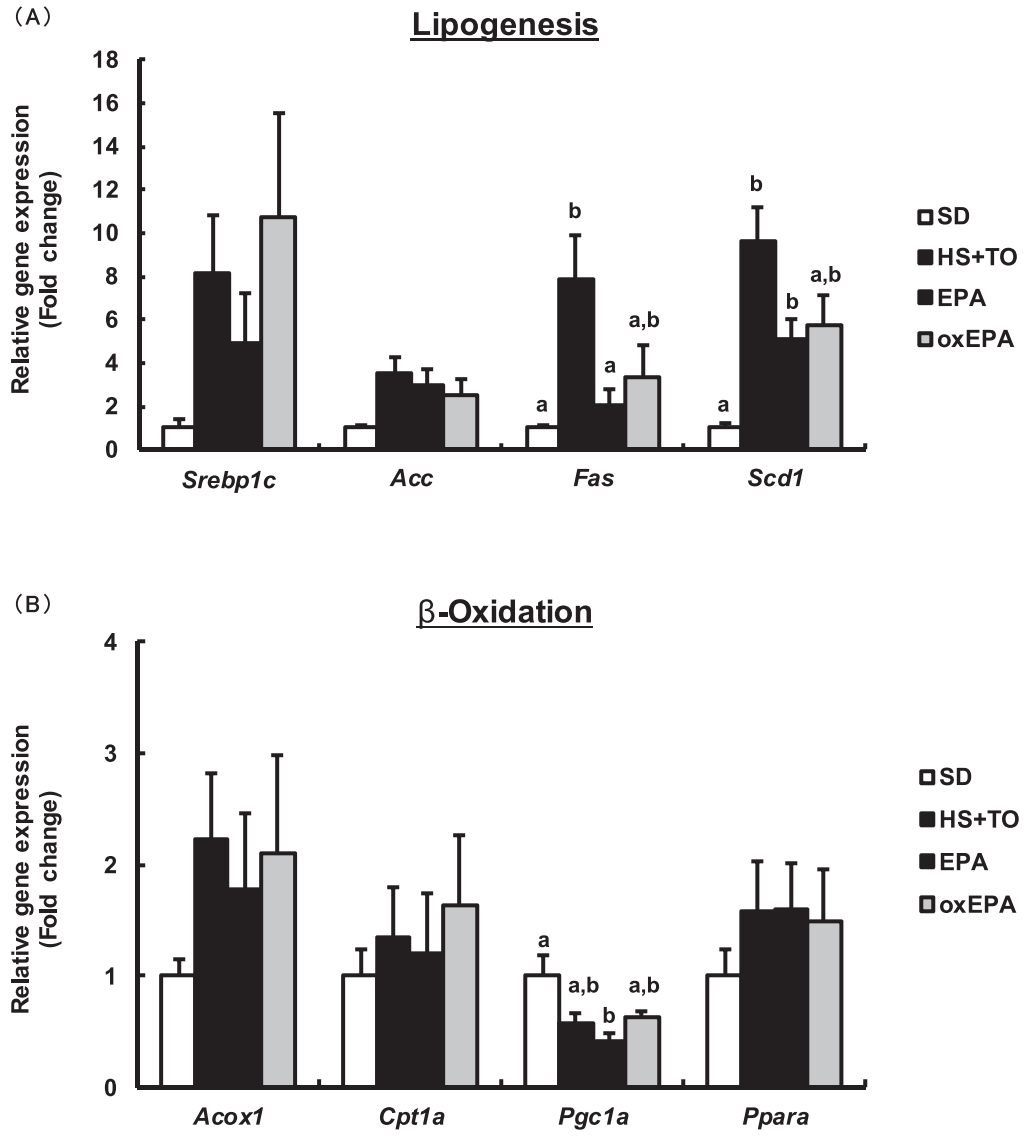

Fig. 2 Effect of eicosapentaenoic acid (EPA) and oxidized EPA (oxEPA) on expression of genes associated with lipid metabolism.
The expression of genes associated with lipogenesis (**A**) and β-oxidation (**B**) in liver tissue was quantified by real time reverse transcription polymerase chain reaction. The expression levels are presented as fold induction relative to the SD group. The data are shown as the mean ± SEM (n = 4−6/group). Values with different letters are significantly different ($p < 0.05$).

4-h oxEPA was employed to evaluate its effect on hepatic steatosis in an animal model based on our previous result[22]. Although 4-h oxEPA contains mainly non-oxidized EPA, HEPE is found at relatively higher levels during the oxidation period. Our results with dietary oxEPA were influenced by EPA. However, oxidation products such as HEPE may partly contribute to EPA's hypolipidemic effects in the liver. Therefore, by regulating the autoxidation of PUFAs, these biological activities may be more effectively utilized to improve health.

Excessive lipid accumulation in the liver induces oxidative stress and inflammation, which could cause NAFLD and NASH[2]. To prevent the development and progression of hepatic steatosis, the suppression of lipid synthesis may be an effective strategy, as well as a reduced fat intake and increased fatty acid catabolism. Our data demonstrated that the oral administration of oxEPA and EPA suppressed *de novo* lipogenesis in the liver and could prevent the hepatic dysfunction caused by lipid metabolism disorders.

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