Studies on Nutraceutical Properties of Modified Fatty Acids by Autoxidation and Lactic Acid Bacterial Metabolism

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GENERAL INTRODUCTION

Recently, the prevalence of metabolic syndrome has been dramatically increased worldwide because of the consumption of unbalanced diets and lack of exercise. Metabolic syndrome is a cluster of the medical conditions, including obesity, a high blood glucose level, a high blood triacylglycerol (TG) level, a low high density lipoprotein-cholesterol level, and high blood pressure. A person who has at least three of these metabolic risk factors can be diagnosed with metabolic syndrome. It causes multiple diseases such as lipid metabolic disorders and cardiovascular diseases. Ectopic fat accumulation has attracted a great deal of public attention among lipid metabolism disorders and been associated with an increased risk for detrimental metabolic conditions, such as nonalcoholic fatty liver disease (NAFLD). Liver is the major organ of energy metabolism and detoxification and tends to be damaged by lipotoxicity and oxidative stress. 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. 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. Numerous dietary ingredients which have hypolipidemic effects have been investigated.

Polyunsaturated fatty acids (PUFAs) are known to have diverse biological function associated with lipid metabolism. Linoleic acid (LA) is omega-6 PUFA involved in the biosynthesis of several lipid mediators, including prostaglandins, leukotrienes, and thromboxanes to regulate inflammation. Although LA is essential fatty acid, its excessive intake can cause allergy and cancer. α–Linolenic acid (αLA) is one of omega-3 PUFAs essential for biosynthesis of eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA). Since they have multiple health benefits, including suppression of allergy, neuronal protection, and improvement of lipid metabolism disorders, omega-3 PUFAs are commercially utilized for dietary supplements. Especially, EPA has a potent hypolipidemic effect through suppression of lipid synthesis and promotion of energy production. In addition, EPA is converted to novel anti-inflammatory lipid mediators, resolvins to promote the resolution of the inflammatory response.

While the biological functions of dietary PUFAs have been investigated well, modified fatty acids derived from the PUFAs produced through autoxidation in food and human body is poorly understood. The lipid oxidation is proposed to cause unfavorable flavor and dietary indiscretion to decrease food quality. The lipid oxidation products and reactive oxygen species damage biomolecules such as proteins, nucleic acids, and lipids, and then impair cellular biological functions. Therefore, it is proposed that lipid oxidation must be prevented because of its harmfulness. However, the biological benefits of PUFA-derived oxidation products have been gradually uncovered. Previous study revealed that oxidized DHA and EPA can suppress oxidative stress and inflammation. In addition, oxidized EPA (oxEPA) reduced LXRα-induced cellular TG accumulation more potently than intact EPA in hepatocytes.

The modified fatty acids can also be produced by microbial metabolism. A gut lactic acid bacterium *Lactobacillus plantarum* converts LA to conjugated LA through multi-enzymatic reaction in saturation metabolism. *L. plantarum* which is the representative lactic acid bacterium utilized for the food fermentation exists generally in human gastrointestinal tract. The efficient production of metabolic intermediates such as hydroxy and oxo fatty acids derived from PUFAs including LA, αLA, γ-linolenic acid, and arachidonic acid is achieved by application of the metabolic pathway. A part of the fatty acid metabolites could be detected in host organs, suggesting that they affect host health.

Recent studies reported that the hydroxy and oxo fatty acids derived from LA provided the beneficial effects on immune system and lipid metabolism. These data indicate that the fatty acid metabolites generated by bacterial flora in gastrointestinal tract may contribute to human health.

The aim of this study is to investigate the beneficial effects of PUFA-derived modified fatty acids generated by autoxidation and microbial metabolism on lipid metabolism and oxidative stress in hepatocytes. In chapter 1, the *in vivo* effects of oxEPA were elucidated in hepatic steatosis induced by a high-sucrose and liver-X-receptor agonist in ICR mice. In chapter 2, the effect of the fatty acid metabolites generated by *L. plantarum* on cellular TG levels was evaluated by using human hepatocellular carcinoma cell line HepG2. In chapter 3, the antioxidative effect of the fatty acid metabolites generated by *L. plantarum* was investigated in HepG2 cells and ICR mice. As the results of this study, the hypolipidemic effect of oxEPA was confirmed *in vivo.* Then, a modified fatty acid which had the most hypolipidemic and antioxidative effect was found among the bacterial fatty acid metabolites. This study supports the hypothesis that the modified fatty acid generated in dietary foods and human body may contribute the biological activities of their precursors.

Chapter 1: Dietary effects of oxidized eicosapentaenoic acid (EPA) on hepatic steatosis induced by a high-sucrose diet and liver-X-receptor α agonist in mice

INTRODUCTION

PUFAs are known to have diverse biological function associated with lipid metabolism. In particular, EPA, which is an omega-3 PUFA enriched in marine products, has been clinically used as a pharmaceutical agent and dietary supplement due to its multiple health benefits, including the lowering of serum and hepatic TG levels. 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) which is the key transcription factor in lipogenic gene regulation in the liver. In addition, EPA can promote β-oxidation through the activation of peroxisome proliferator-activated receptor α (PPARα) and can resolve inflammation by acting as a lipid mediator. Because of its multiple health benefits, EPA is thought to prevent and improve lipid metabolic disorders such as hepatic steatosis.

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. 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 been increasingly investigated. For example, oxidized DHA and EPA can suppress oxidative stress and inflammation. Previously, it was revealed that in hepatocytes, oxidized EPA (oxEPA) reduced LXRα-induced cellular TG accumulation more potently than intact EPA. It suggested that the hypolipidemic effect of EPA could be augmented by modification of its chemical structure and polarity through autoxidation. 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. However, a high-sucrose diet could potentially be insufficient to induce hepatic steatosis in the preliminary experimental condition. In this chapter, to compare effects of dietary EPA and oxEPA on hepatic steatosis, a mouse model fed a high-sucrose diet was combined with the oral administration of a synthetic LXRα agonist to induce severe short-term hepatic lipogenesis. In this model, oxEPA as well as intact EPA reduced TG accumulation in the liver via the suppression of lipogenic gene expression.

MATERIALS AND METHODS

Chemicals and reagents

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).

Preparation of oxEPA

EPA was incubated in brown glass tubes at 40°C for 4 h to use in this chapter, because 4-h oxEPA was the most effective in suppressing lipid synthesis and accumulation in the hepatocellular carcinoma cell line HepG2. After incubation, the prepared oxEPA was diluted in ethanol and stored at −80°C until use. The derivative composition of the EPA oxidation products in the obtained oxEPA was determined by liquid chromatography-mass spectrometry (LC-MS), as reported previously.

Animal study

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 1-1**) 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 two weeks of treatment, mice were fasted for 12 hours 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°C 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°C until further analysis.

Table 1-1 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

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.

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.

RNA extraction and real time 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 by RT-PCR 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 1-2** shows the primer pairs used for each gene quantification. The thermal cycle program consisted of 15 min at 95°C and 43 cycles of 15 sec at 95°C and 30 sec at 60°C. 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.

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).

Table 1-2 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	CTGTGGAACATTTAAGATACGTTTCGAAA
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

RESULTS

LC-MS analysis of 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 (**Fig. 1-1**). 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 $[M-H]^-$ 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.

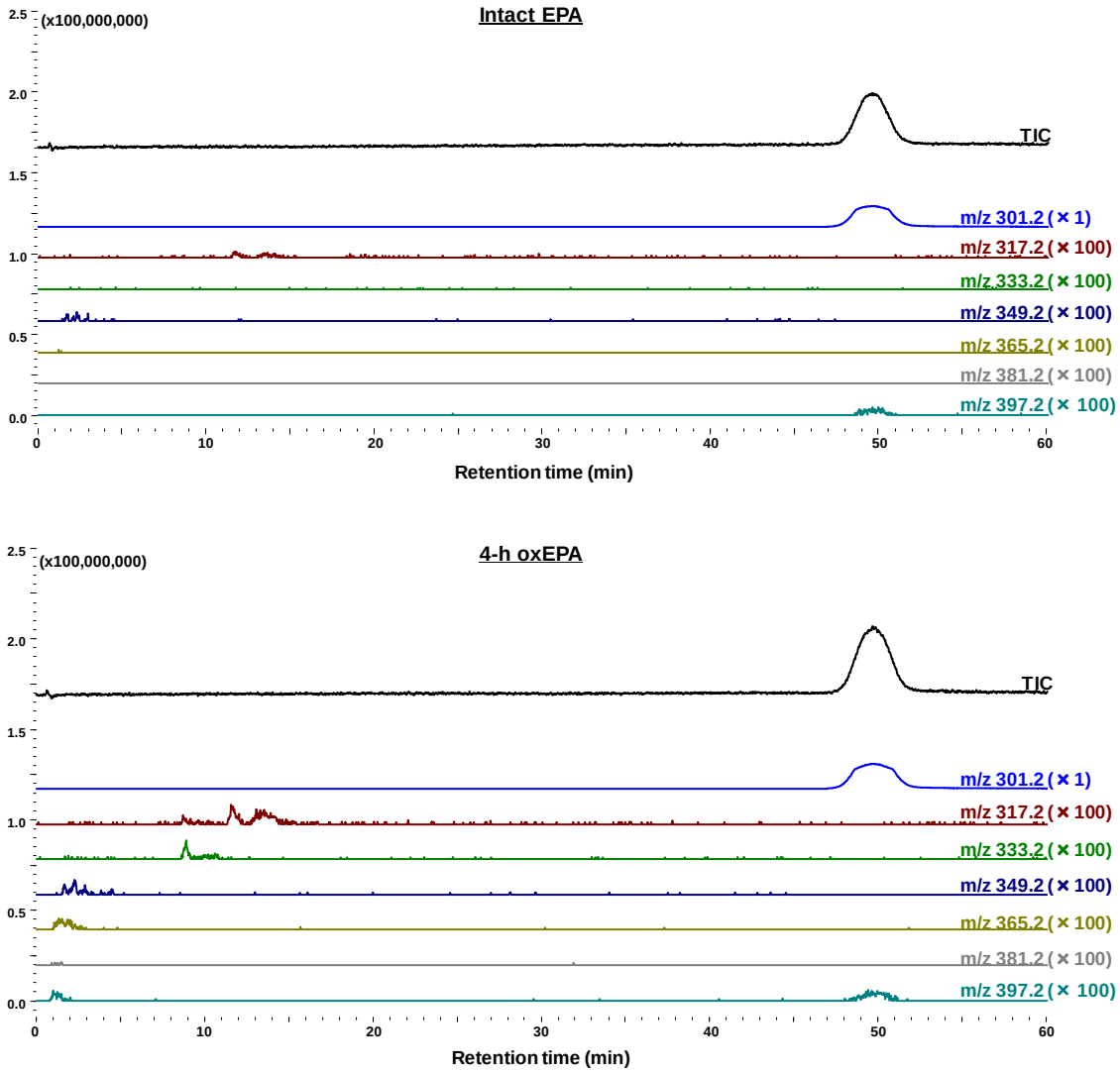

Figure 1-1 Total and selected ion chromatograms of intact eicosapentaenoic acid (EPA) and 4-h oxidized EPA (oxEPA). The ion intensities of intact EPA (m/z 301.2) and the EPA oxidation products (m/z 317.2, 333.2, 349.2, 365.2, 381.2, and 397.2) were detected by LC-MS.

Effect of EPA and oxEPA on tissue weight

While the total calorie intake during the treatment was not significantly between the 4 groups, the body weight gain was higher in the HS+TO, EPA, and oxEPA groups than in the SD group (**Table 1-1**). 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 there was no significantly

difference. The weights of the gastrocnemius muscle and white adipose tissue (WAT), including the epididymal, mesenteric, and perirenal WAT, was not significantly different between the groups.

Table 1-3 Weight gain, total calorie intake and tissue weight after 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
Liver (g/100 g BW)	3.9 ± 0.1^{a}	9.4 ± 1.1^{b}	$6.9 \pm 0.7^{a, b}$	7.1 ± 0.8^{b}
Total WAT (g/100 g BW)	5.4 ± 0.5	4.1 ± 0.3	4.6 ± 0.2	5.0 ± 0.5
Epididymal WAT (g/100 g BW)	2.6 ± 0.2	2.1 ± 0.1	2.5 ± 0.1	2.6 ± 0.2
Mesenteric WAT (g/100 g BW)	1.2 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
Perirenal WAT (g/100 g BW)	1.5 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.2
Gastrocremius muscle (g/100 g BW)	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).

Effect of EPA and oxEPA on plasma biochemical parameters

Table 1-4 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.

Table 1-4 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).

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. 1-2A**). 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. 1-2B**).

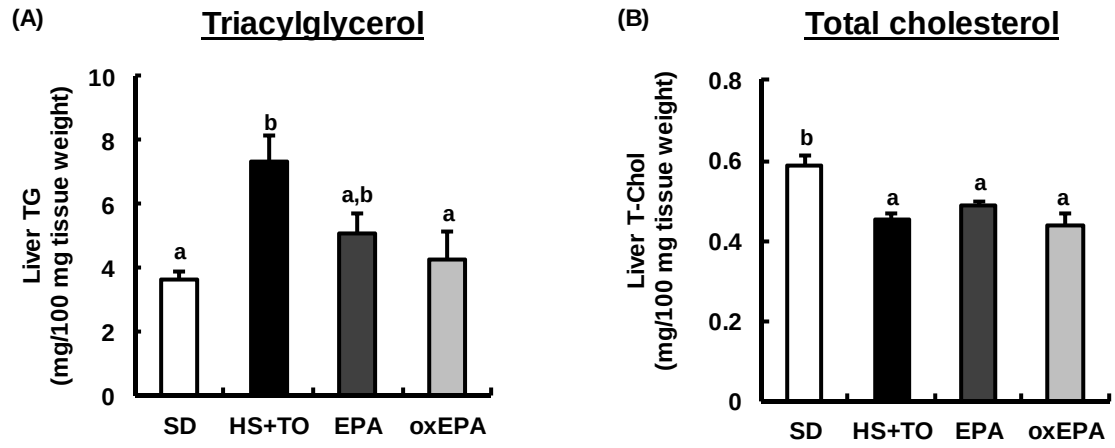

Figure 1-2 Effect of eicosapentaenoic acid (EPA) and oxidized EPA (oxEPA) on lipid content in liver tissue. The hepatic content of triacylglycerol **(A)** and total cholesterol **(B)** were measured at the end of the treatment period. The data are shown as the mean ± SEM (n = 6/group). Values with different letters are significantly different ($p < 0.05$).

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

In this chapter, the hepatic gene expression of SREBP-1c and its target genes, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1) were evaluated. 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. 1-3A**). The oxEPA treatment showed lower expression of FAS and SCD1 genes than the HS+TO treatment did, although this was not significant.

The hepatic expression of genes associated with fatty acid β-oxidation, such as acyl-CoA oxidase 1 (ACOX1), carnitine palmitoyltransferase 1A (CPT1A), PPARγ coactivator 1α (PGC1α), and PPARα, was also evaluated . There were no significant differences in the mRNA expression levels of ACOX1, CPT1A, and PPARα among the 4 groups (**Fig. 1-3B**). The PGC1α mRNA expression levels were significantly lower in the EPA group than in the SD group.

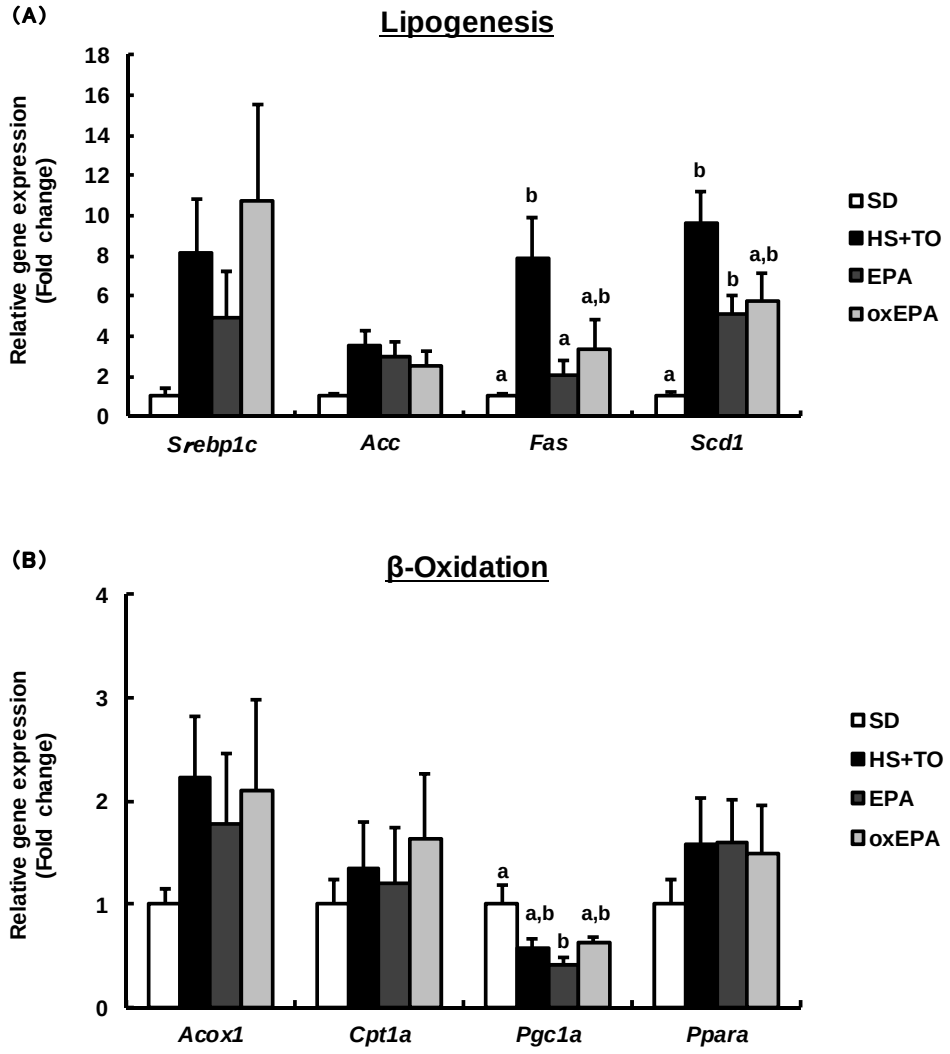

Figure 1-3 Effect of eicosapentaenoic acid (EPA) and oxidized EPA (oxEPA) on expression of genes associated with lipid metabolism. The expression of genes associated with lipogenesis **(A)** and β-oxidation **(B)** in liver tissue was quantified by real time RT-PCR. The expression levels are presented 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 ($p < 0.05$).

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. 1-2A)**. 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 1-3)**. 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. Treatment with oxEPA could ameliorate this liver damage, which is supported by the oxEPA-induced suppression of the ALT and AST increases (**Table 1-4**), 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. 1-3A** and **1-3B)**. The results in this chapter suggest that EPA and oxEPA can ameliorate hepatic steatosis through the suppression of lipogenic genes.

Previously, it was 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. In this chapter, intact EPA and oxEPA also suppressed the expression of lipogenic genes, but not β-oxidation genes (**Fig. 1-3A** and **1-3B**). 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. Therefore, it was speculated 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.

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. It was also shown that oxEPA inhibited leukocyte−endothelial interactions. Previously, it was demonstrated that 18-HEPE is the most effective in suppressing SREBP-1c expression and its target gene mRNAs in hepatocytes. The beneficial effects of omega-3 PUFAs may depend in part on their oxidized form. In this chapter, 4-h oxEPA was employed to evaluate its effect on hepatic steatosis in an animal model based on the previous result. Although 4-h oxEPA contains mainly non-oxidized EPA, HEPE is found at relatively higher levels during the oxidation period. Therefore, oxidation products such as HEPE may partly contribute to EPA's hypolipidemic effects in the liver. 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 hepatic steatosis. Moreover, hepatic steatosis is the risk factor of more severe diseases, such as nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, and cardiovascular disease. To prevent the development and progression of these high mortality diseases, the suppression of lipid synthesis may be an effective strategy, as well as a reduced fat intake and increased fatty acid catabolism. In this chapter, it was 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. These results support the hypothesis that the hypolipidemic effect of EPA was augmented by modification of the chemical structure through autoxidation.

Chapter 2: Effects of fatty acid metabolites generated from long chain fatty acid by a gut lactic acid bacterium *Lactobacillus plantarum* on cellular triacylglycerol synthesis

Recently, the increase population of lipid metabolism disorders, including hepatic steatosis, is a

serious problem worldwide. EPA has been utilized as a therapeutic agent and dietary supplement because of its potent hypolipidemic effect. EPA reduces serum and hepatic TG levels through suppression of lipogenic enzyme expression regulated by LXRα and promotion of β-oxidation enzyme expression regulated by PPARα. Furthermore, it was previously revealed that *trans* geometric isomers of EPA and oxEPA more potently reduce LXRα-induced cellular TG than intact EPA. In chapter 1, it was shown that oxEPA ameliorated hepatic steatosis induced by a high-sucrose diet and a synthetic LXRα agonist. These results suggest that oxEPA generated by autoxidation may partially contribute the hypolipidemic effect of dietary EPA. However, EPA oxidation products are inconvenient for clinical or commercial use because of their instability and complexity. Therefore, this chapter focused on the metabolic intermediates derived from PUFAs generated by *Lactobacillus plantarum*. It is one of the representative lactic acid bacteria which is utilized for the food fermentation and exists generally in human gastrointestinal tract. Kishino et al revealed that *L. plantarum* converts LA to conjugated LA and oleic acid through multi-enzymatic reactions in the host gastrointestinal tract. Additionally, the effective production of the various kinds of metabolic intermediates, including hydroxy and oxo fatty acids derived from PUFAs, are achieved by the application of the enzymes in the metabolic pathway in *L. plantarum*. Since these fatty acid metabolites can be detected in host organs, they could be involved in host health conditions. Actually, a part of the fatty acid metabolites generated by *L. plantarum* have been reported to improve intestinal epithelial barrier impairment, promote adipogenesis, and control immune system. It was also found that the fatty acid metabolites such as 10-hydroxy-*cis*-12-octadecenoic acid (HYA) and 10-oxo-*cis*-12-octadecenoic acid (KetoA) derived from LA, and 10-hydroxy-*cis*-6-*cis*-12-octadecadienoic acid (γHYA) and 10-oxo-*cis*-6-*cis*-12-octadecadienoic acid (γKetoA) derived from γLA reduced TG levels in hepatocytes. They reduced the expression of

SREBP-1c and its target genes through inhibition of LXRα activity to suppress lipid synthesis. However, the hypolipidemic effects of other fatty acid metabolites generated by *L. plantarum* are still unknown.

To investigate the hypolipidemic effect of the hydroxy and oxo fatty acids generated by *L. plantarum*, lipogenic gene expression, cellular TG levels and SREBP-1 maturation in HepG2 cells treated with each fatty acid metabolite were evaluated. As a result, a novel potent hypolipidemic metabolite was firstly found in this chapter. It was confirmed that the metabolite significantly decreased SREBP-1c gene expression and cellular TG levels in a dose-dependent manner. Additionally, the metabolite suppressed the lipogenic gene expression and SREBP-1 maturation.

Chapter 3: Effects of the fatty acid metabolites generated by a gut lactic acid bacterium *Lactobacillus plantarum* on cellular antioxidative response

Oxidative stress is due to an imbalance between production of reactive oxygen species (ROS) and antioxidative mechanisms. Since excessive ROS and oxidation products damage biomolecules such as proteins, nucleic acids, and lipids, impairing their biological functions, oxidative stress has been implicated in multiple diseases including NAFLD/NASH. Excessive lipid accumulation in liver leads to NAFLD, and subsequent oxidative stress and inflammation promote the development of NASH. In recent years, the increasing population of NAFLD/NASH becomes a serious problem worldwide, because NASH has a significant risk of progressing to more severe liver diseases including hepatic cirrhosis and hepatocellular carcinoma. To prevent the development of these lethal pathologies, not only improvement of lipid metabolism but also suppression of oxidative stress would be efficient.

Cellular defense mechanisms against oxidative stress are naturally present in the human body.

On such mechanism is the nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway, which plays a central role in cellular antioxidative responses. Under normal conditions, protein expression of the transcription factor Nrf2 is suppressed by binding to the Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, which leads to Nrf2 ubiquitin-dependent proteasomal degradation. Under oxidative stress, electrophilic xenobiotics or oxidation products cause conformational changes in Keap1 through modification of cysteine residues, leading to the release Nrf2 and thus preventing it from degradation. Nrf2 is then transported into the nucleus where it binds to the ARE in the promoter region of antioxidative and phase II detoxification enzyme genes to induce their expression. Since these enzymes quench ROS and eliminate electrophilic substances, Nrf2 activators are expected to ameliorate diseases associated with oxidative stress. Interestingly, many natural dietary ingredients have been previously identified as Nrf2 activators, including sulforaphane in broccoli sprouts and curcumin in turmeric.

The oxidation products (or metabolites) of PUFAs such as nitro linoleic acids, 4-hydroxynonenal, and 15-deoxy-$\Delta^{12,\ 14}$-prostaglandin J_2 have been reported to be endogenous inducers of Nrf2 activation. Additionally, 9-oxo-*trans*-7-octadecenoic acid derived from green alga, *Ulva lactuca*, has been reported to activate the Nrf2-ARE pathway in human neuroblastoma IMR-32 cells. These reports indicate that derivatives of long chain fatty acids can activate the Nrf2-ARE pathway.

This chapter is focused on the metabolic intermediates derived from PUFAs generated by gut microorganisms *L. plantarum* as potential Nrf2 activators. A part of the fatty acid intermediates can be detected in host organs, and their biological functions have been gradually uncovered. For instance, the fatty acid metabolites generated by *L. plantarum* have been reported to improve intestinal epithelial barrier impairment, promote adipogenesis, and control immune system. In previous study

and the chapter 2, it was also found that a part of the hydroxy and oxo fatty acids suppressed lipogenesis. However, their effects on cellular antioxidative response are still unknown.

In this chapter, the effect of hydroxy and oxo fatty acids generated by *L. plantarum* on cellular antioxidative responses regulated by the Nrf2-ARE pathway was investigated. As a result, it was found a part of the fatty acid metabolites generated by *L. plantarum* provided the potent cytoprotective effects against oxidative stress through activation of the Nrf2-ARE pathway. Furthermore, it was confirmed that one of the antioxidative metabolites induced the gene and protein expression of antioxidative enzymes *in vivo*.

SUMMARY AND COCLUSION

The present study demonstrated the beneficial effects of diverse kinds of modified fatty acids generated by autoxidation and bacterial metabolism on lipid metabolism and antioxidative response. The *in vivo* effect of 4-h oxEPA on lipid metabolism in ICR mice fed a high-sucrose diet and treated orally with LXRα agonist TO-901317 was evaluated in chapter 1. It was confirmed that intact and oxEPA suppressed hepatic steatosis. In chapter 2 and 3, to elucidate the biological activities of modified fatty acids which are different in the location of double bonds and hydroxyl/carbonyl groups, various molecules of the fatty acid metabolites derived from PUFAs generated by the enzymes in the metabolic pathway in a gut lactic acid bacterium *L. plantarum* were screened and clarified the effects on lipid synthesis and antioxidative response. As a result, the metabolites which had the potent effects on lipid metabolism and oxidative stress were newly found.

In conclusion, these data demonstrated that the modified fatty acids generated by autoxidation and microbial metabolites can provide more potent bioactivities than their precursors. The modified fatty acids could be used as a therapeutic agent for lipid metabolism disorder and oxidative stress.