

**Studies on the Ameliorating Effects of
Oxygenated Fatty Acids on Lipid Metabolism**

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GENERAL INTRODUCTION

Nowadays, dysfunction or imbalance of lipid metabolism is believed as the cause of many lifestyle related diseases, such as obesity, fatty liver and atherosclerosis. These diseases consequently are the main risk factors of cardiovascular disease (CVD) which is the leading cause of mortality worldwide. The prevalence of CVD has not increased only in the elder, but also in the midlife and the young adulthood due to the changes of the lifestyle in our societies [1, 2]. Hence, the improvement of lipid metabolism is essential to prevent or ameliorate many diseases. One of the most important factors that are concerned as the therapeutic target of CVD is triacylglycerol (TG) [3, 4].

TG is a dense form in which to store the energy. It circulates in the plasma in lipoproteins and also presents as lipid droplets within cells. Most tissues are involved in TG metabolism, but adipose tissue, skeletal muscle and liver are quantitatively more important than other tissues as shown in Figure 1 [3]. In the case of adipose tissue, adipocyte hypertrophy that occurs when TG synthesis (esterification) exceeds TG breakdown (lipolysis), resulting in elevated TG storage, primarily contributes to the development of obesity and it is also subsequent by many pathologies [5]. Whereas in the liver fatty acids were re-esterified within the endoplasmic reticulum (ER) to produce TG that will be secreted as very-low-density lipoprotein (VLDL) [3]. With respect to the importance of lipid metabolism in liver, many studies described that the symptoms of positive imbalance of TG metabolism in liver, such as high TG content, hepatosteatosis and leakage of liver enzyme, can utilize as the risk marker of CVD. [6-12] Therefore, ameliorating the dysfunctions of lipid metabolism in the adipose tissue and the liver is essential to abate diverse lifestyle related diseases, particularly CVD.

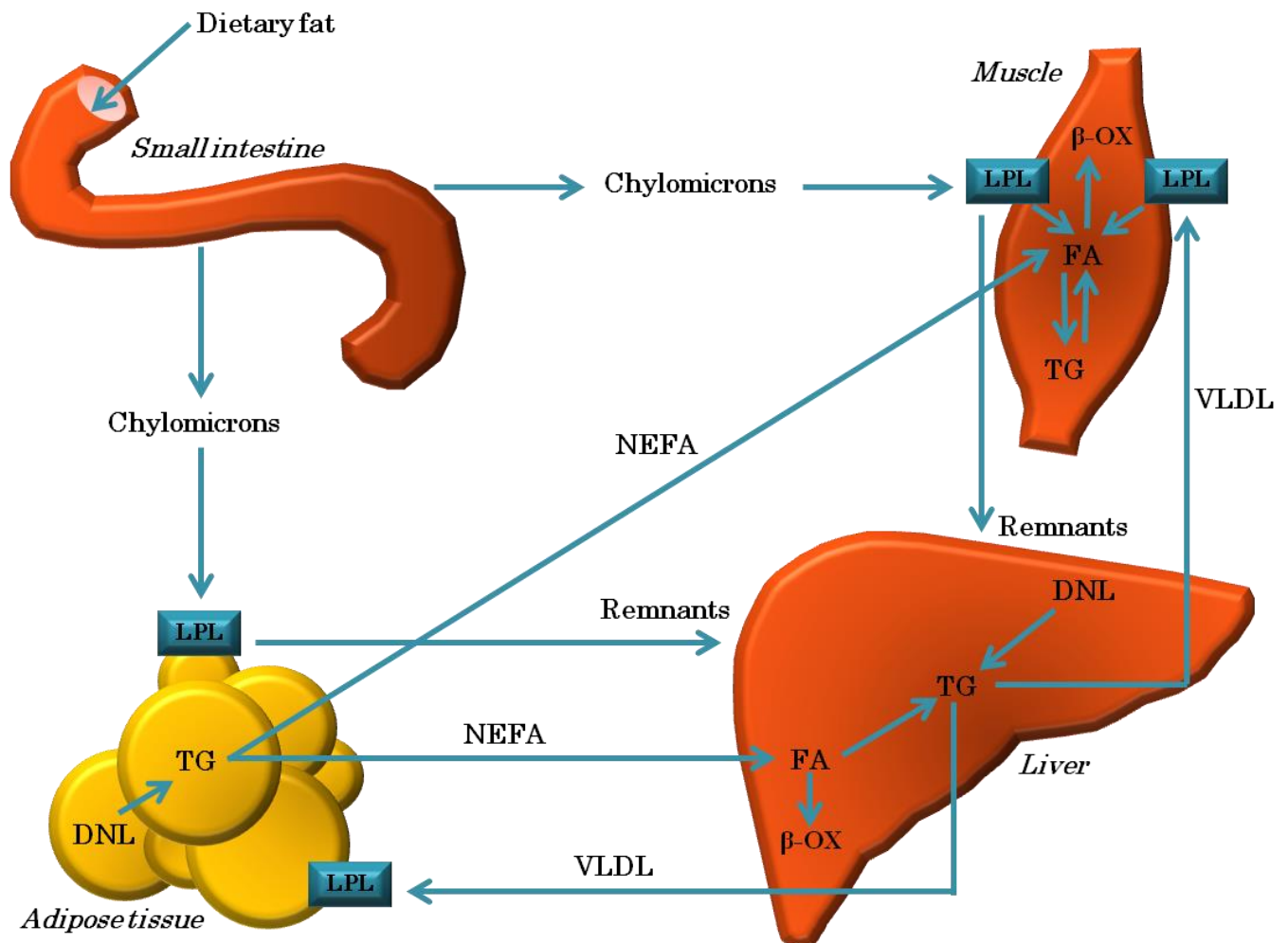


Figure 1. Lipid metabolism among gut, adipose tissue, skeletal muscle and liver. Note that adipose tissue lipoprotein lipase (LPL) also releases non-esterified fatty acids (NEFA) into the plasma, making further fatty acids available for uptake by muscle and liver. DNL, de novo lipogenesis; FA, fatty acids; β -OX, β -oxidation; TG, triacylglycerol; VLDL, very-low-density lipoprotein.

Generally, diet from marine source shows many benefits for health. Omega-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from marine sources, have been developed commercially as dietary supplements due to their various health benefits, particularly their hypolipidemic and anti-obesity effects [11, 12]. EPA has TG-reducing effects in normolipidemic [13] and in hyperlipidemic subjects [14]. However, EPA in the diet is easily oxidized at room temperature and several types of oxidized EPA (OEPA) derivatives are generated. It has been proposed that oxidation products of omega-3 fatty acids, especially EPA and DHA, play crucial roles in the palliative effects via many mechanisms particularly those that are cardiovascular-related [15-18].

On the other hand, some PUFAs, such as linoleic acid (LA) and linolenic acid (LNA), cannot suppress hepatic lipogenesis and TG accumulation in mouse adipocytes (3T3-L1) [19, 20]. However, it is suspicious that their oxygenated forms might involve in many benefits in our health. For example, hydroxy- and oxo-fatty acids act as ligands for PPAR γ [21]; oxo-fatty acids discovered in tomato juice are potent PPAR α activators and decrease the amount of TG in obese diabetic mice [22]. Recently, Ogawa et al. discovered many species of intermediates from the metabolic pathway of gut bacterium *Lactobacillus plantarum*. Interestingly, these intermediates are hydroxy-fatty acids, oxo-fatty acids, conjugated fatty acids, and partially fatty acids [23-27]. *L. plantarum* has been used as a probiotic. Its biotherapeutic applications have been extensively accepted, such as decreased pain and constipation associated with irritable bowel syndrome, and reduced bloating, flatulence and incidence of diarrhea in daycare centers. Moreover, it also exerts positive effect on immunity in HIV⁺ children [28, 29]. Nevertheless, the benefits of *L. plantarum* are still ambiguous in their mechanisms. Thus, the new-finding oxygenated fatty acids fermented by *L. plantarum* from

unsaturated acids like LA were predicted to exert their physiological functions, especially the improvement in lipid metabolism.

The aims of this study were to examine the properties and bioactivities of oxygenated fatty acids from various sources, including the autoxidation of EPA and the fermentation from *L. plantarum*, on lipid metabolism in human hepatocellular liver carcinoma cell line (HepG2), 3T3-L1 preadipocytes and diabetic KK-Ay mice fed with high fat diet. In this study, for the first time the ameliorating effects of OEPA and oxygenated fatty acids from *L. plantarum* fermentation on TG accumulation were investigated. The importances of this study are not only the preferable effects on lipid metabolism of oxygenated fatty acids from marine sources compared to their intact form, but the data also suggested that the efficiency of each oxygenated fatty acid depends on the chemical structure, particularly the type, the location and the number of functional group and double bond.

**Chapter 1: Oxidized eicosapentaenoic acids more potently reduce
LXR α -induced cellular triacylglycerol via suppression of SREBP-1c,
PGC-1 β and GPA than its intact form**

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide. Concernedly, the alarming rise in the prevalence of CVD is not being increased only in the elder, but the incidence is also being followed in the childhood and the young adulthood [64, 65]. Recent lifestyle and environment alterations, such as an excessive consumption of saturated fat and carbohydrate and a lack of physical activity, have been enormously raising the prevalence of obesity and metabolic syndrome which enable to progress to CVD [66-69]. Triacylglycerol (TG), in either the serum or the liver, is a major risk factor for CVD [1, 2, 6]. With respect to the importance of hepatic TG levels, nonalcoholic fatty liver disease (NAFLD) is highly associated with CVD [7-10]. Several prospective epidemiological studies recently demonstrated that both an increased liver enzyme concentration in the serum [30, 31] and hepatic steatosis determined by ultrasound [9, 10] are able to predict the development of CVD independent of alcohol consumption or traditional CVD risk markers, such as serum LDL cholesterol concentrations. In addition, Rijzewijk et al. demonstrated that type 2 diabetes mellitus patients with high liver TG content showed a decreased myocardial perfusion compared with similar diabetes patients with low liver TG content [6]. Therefore, the dysfunction of hepatic lipid metabolism has been of concern as a therapeutic target of CVD.

Omega-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA), have been developed commercially as dietary supplements due to their various health benefits, particularly their ameliorating effect on CVD [11, 12]. EPA has TG-reducing effects in normolipidemic [13] and in hyperlipidemic subjects [14]. It has been proposed that EPA decreases TG through the regulation of peroxisome proliferator-activated receptor α (PPAR α) and sterol regulatory element-binding protein (SREBP)-1, which govern hepatic fatty acid (FA) catabolism and synthesis, respectively [32].

Sterol regulatory element (SRE)-binding proteins (SREBPs) are transcription factors that central to the regulation of lipid metabolism. The three SREBP isoforms (SREBP-1a, -1c, and -2) have overlapping target genes and show the differential expression across tissues as shown in Figure 1-1 [33]. SREBP-1c is the major isoform expressed in the liver and in the other tissues involved in energy homeostasis [34]. It was emphasized that the dysregulation of SREBP-1c has been implicated in the pathogenesis of hepatic steatosis and dyslipidemia which are closely related to atherosclerosis and CVD [35, 36]. SREBPs are synthesized as precursor proteins that are inserted into the endoplasmic reticulum (ER) membrane. After proteolytic processing in the Golgi, the active form accumulates in the nucleus where it binds SRE in promoters of many genes that involve in fatty acid and TG synthesis (Fig. 1-2). The transcription of the SREBP-1c gene is induced by insulin [37, 38] and oxysterols through liver X receptor α (LXR α) [39].

Glycerol-3-phosphate acyltransferase (GPA) is supposed to be a rate-limiting step in TG and phospholipid biosynthesis. It catalyzed the first step in glycerophospholipid synthesis by acting as the esterification of glycerol-2-phosphate in the sn-1 position with a fatty acyl-CoA to form 1-acylglycerol-3-phosphate (lysophosphatidic acid). Lysophosphatidic acid is further esterified by 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) to form 1,2-diacylglycerol-3-phosphate (phosphatidic acid), which is the precursor of TG and phospholipids [40]. The study by Lewin et al. showed that mice deficient in mitochondrial GPA have diminished myocardial TG accumulation during lipogenic diet [41]. Therefore, a decrease of the expression of GPA in the liver might prevent hepatic steatosis and dyslipidemia.

A recent study demonstrated that peroxisome proliferator-activated receptor γ coactivator 1 β (PGC-1 β) is a potent activator of mitochondrial gene expression, including genes regulating the β -oxidation of fatty acids, but has relatively little ability to

stimulate the program of gluconeogenesis [42]. Furthermore, PGC-1 β has also been reported to co-activate the LXR and SREBP families and to elevate circulating TG and cholesterol in VLDL particles [43]. As mentioned above, PGC-1 β might be a cofactor connecting the regulation of fatty acids with the ameliorating effect on TG accumulation in the liver.

Oxidation products of omega-3 fatty acids, especially EPA and DHA, play crucial roles in the palliative effects via many mechanisms especially those that are cardiovascular-related. A study by Majkova et al. showed that components of oxidized docosahexaenoic acid (DHA) can alleviate the endothelial dysfunction caused by coplanar PCB77 [15]. Furthermore, oxidized EPA (OEPA) has been shown to inhibit leukocyte-endothelial interactions by potently activating PPAR α in endothelial cells, and to do so to a much greater extent than native EPA. Although native EPA activates PPAR α about half as well as OEPA, unlike EPA, OEPA has effects on leukocyte-endothelial interactions *in vitro* and *in vivo* [16]. 5-HEPE, a metabolite produced from EPA in human neutrophils and eosinophils, has been shown to be a potent agonist for G protein-coupled receptor (GPR) 119, which results in a reduction in food intake and in body weight gain in rats [17], and to enhance glucose-dependent insulin secretion [18].

However, the effects of OEPA on many processes are still obscure. Therefore, the aim of this study is to elucidate the mechanism of 4-24 h OEPAs which are composed of various species of oxidation products on lipid metabolism, particularly via the LXR α and SREBP-1c pathway, which plays an important role in lipid metabolism in liver cells (Fig. 1-2). Surprisingly, the result shows that OEPA significantly down-regulates the expression of lipogenic genes, which results in the suppression of hepatocellular TG more than EPA.

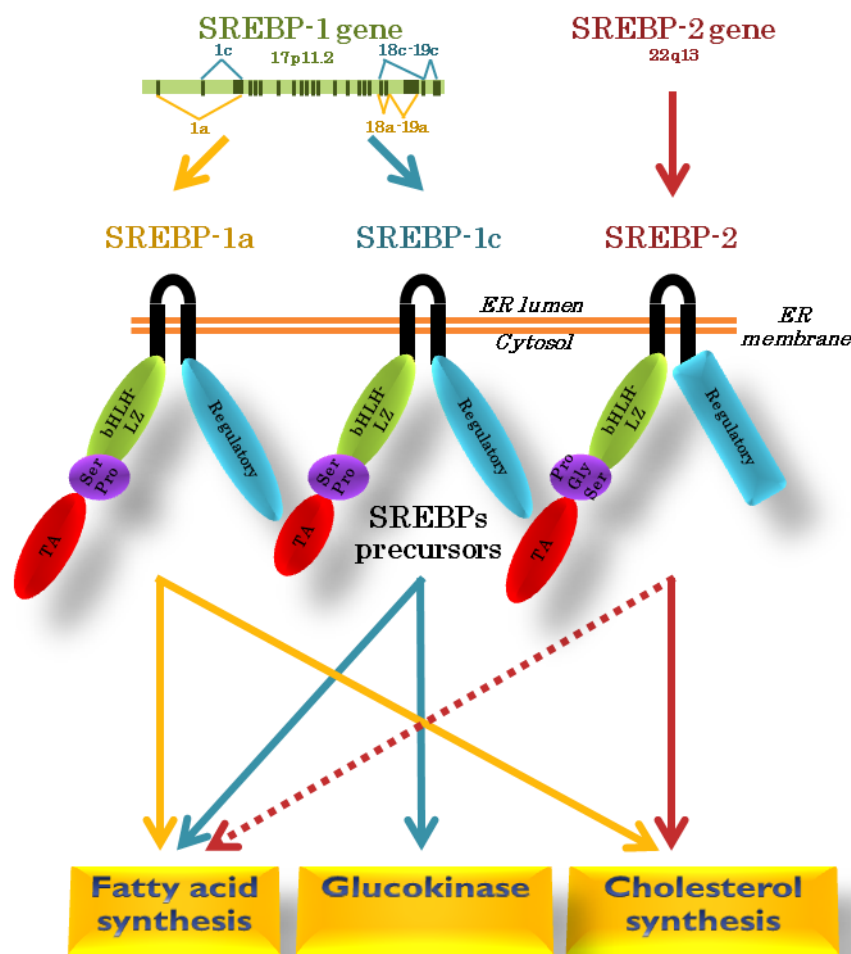


Figure 1-1. SREBPs genes, structures and functions. SREBPs family is composed of three members: SREBP-1a and 1c produced from a single gene named SREBP-1 located on human chromosome 17p11.2 and SREBP-2 from a separate gene named SREBP-2 located on human chromosome 22q13. SREBP-1a and 1c transcripts are produced through the use of alternative transcription start sites and differed in their first exon (exon 1a and exon 1c). The other exons are common to both isoforms. In humans, alternative splicing in the 3' end have also been described (exon 18a and 19a or exon 18c and 19c). SREBP-1a regulates both fatty acid and cholesterol syntheses, while previously SREBP-2 functions not only cholesterol synthesis, but also modestly regulates fatty acid synthesis. SREBP-1c does not affect cholesterol synthesis, yet it controls both lipogenic genes and glucokinase.

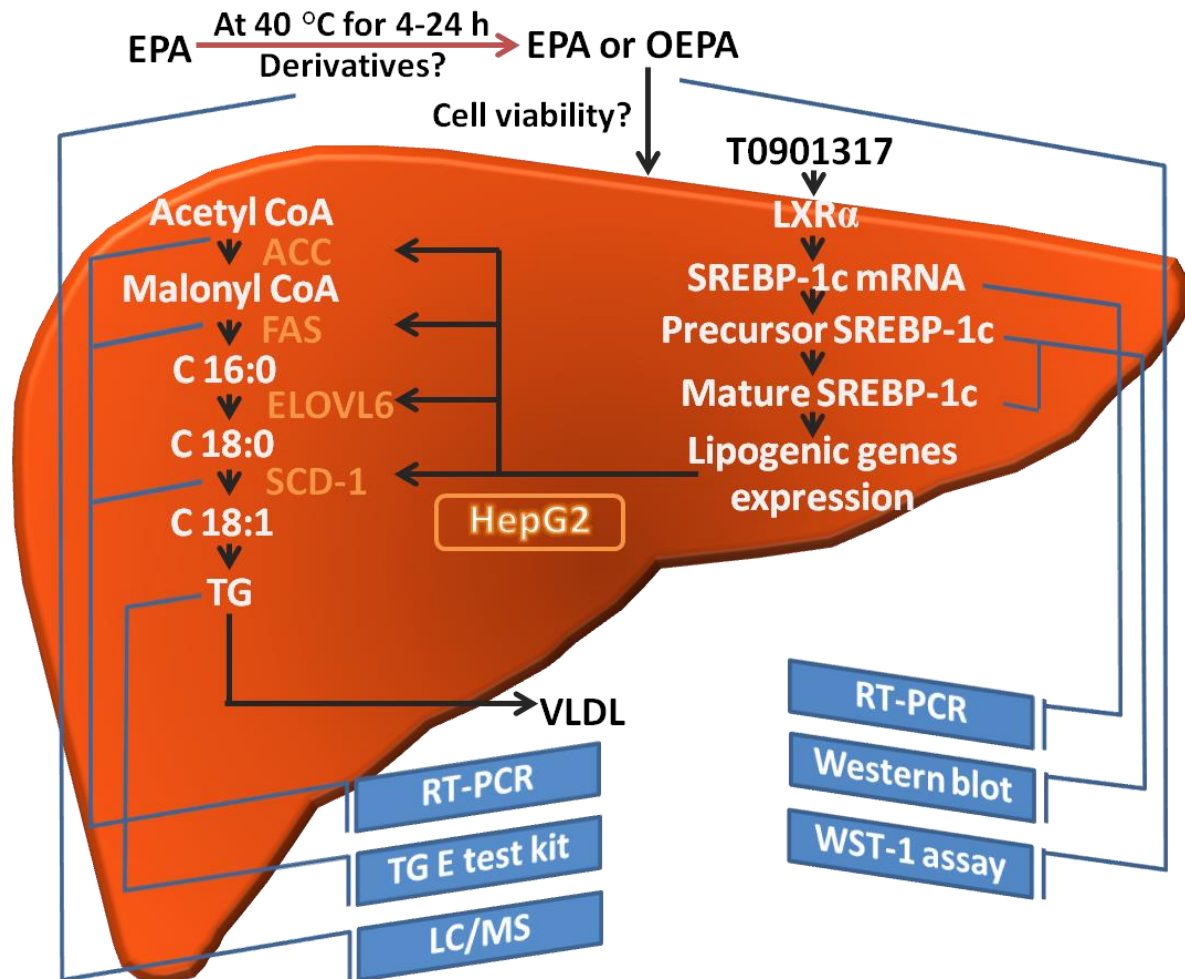


Figure 1-2. The LXRα and SREBP-1c pathway in lipogenesis in the liver cells including the experimental plan to elucidate the mechanism of each fatty acid step by step

MATERIALS AND METHODS

I. Chemicals and reagents

Eicosapentaenoic acid and DL- α -tocopherol (vitamin E) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). T0901317, ($\pm$)-5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid (($\pm$)-5-HEPE), ($\pm$)-11-hydroxy-5Z,8Z,12E,14Z,17Z-eicosapentaenoic acid (($\pm$)-11-HEPE), and ($\pm$)-18-hydroxy-5Z,8Z,11Z,14Z,16E-eicosapentaenoic acid (($\pm$)-18-HEPE) were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

II. Preparation of OEPA

EPA solution (equivalent of 10 mg) was added into brown glass tubes and the diluted ethanol supernatant was gently evaporated under a N₂ stream. The prepared tubes were incubated in a 40°C water bath for 4-24 h. After incubation, OEPA was adjusted to a concentration of 100 mM by diluting with ethanol. All samples were stored at -80°C to preserve the quality of the OEPA derivatives.

III. OEPA and HEPE analyses by liquid chromatography-mass spectrometry (LC-MS)

For analyzing derivatives of EPA, OEPA and HEPEs, a prominence HPLC system coupled to a LCMS-IT-TOF spectrometer equipped with an electrospray ionization interface (Shimadzu, Kyoto, Japan) was used. A TSK gel ODS-100Z column (2.0 $\times$ 50 mm, 3 μ m, Tosoh, Tokyo, Japan) was eluted with methanol:water:acetate (70:30:0.01, v/v) at a flow rate 0.2 ml/min. The MS was operated with the following conditions: probe voltage of 1.50 kV, CDL temperature of 200°C, block heater temperature of 200°C, nebulizer gas flow of 1.5 L/min, ion accumulation time of 50 msec,

MS range of m/z 250 to 450, and CID parameters were follows: energy, 50%; collision gas 50%.

IV. Cell culture

HepG2 cells (JCRB 1054; Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and antibiotics (100 unit/ml penicillin and 100 μ g/ml streptomycin; Gibco Life Technologies Corp, Grand Island, NY, USA) at 37°C in a humidified atmosphere in the presence of 5% CO₂.

V. Cell viability analysis

Cell viability was assessed by the WST-1 method. HepG2 cells were plated in 96-well culture plates at a density of 1.0×10^4 cells/well in 100 μ l DMEM containing 10% fetal bovine serum and antibiotics as detailed above, and were incubated at 37°C for 24 h. Each fatty acid was then added to HepG2 cells with T0901317 (10 nM) and/or vitamin E (10 μ M) in serum-free medium containing 0.1% BSA (Sigma-Aldrich, Co., St. Louis, MO, USA). The final ethanol concentration was 0.3%. After incubation for 24 h at 37°C, 10 μ l WST-1 solution (Dojindo Laboratories, Co., Kumamoto, Japan) was added to each well to evaluate cell viability. After incubation for 100 min at 37°C, cell viability was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA) at a wavelength of 450 nm.

VI. Lipid extraction and quantification of TG

HepG2 cells were plated on 6-well plates at 5.0×10^5 cells/ml for 24 h in DMEM supplemented with 10% fetal bovine serum and antibiotics as mentioned above. The cells were then treated with EPA, OEPA and/or T0901317 (10 nM) in the presence of

vitamin E (10 μ M) in serum-supplemented medium. Fatty acids, vitamin E and T0901317 were dissolved in ethanol (final ethanol concentration of 0.3%). After incubation for 48 h, lipids were extracted from cells with chloroform-methanol (2:1, v/v). Reference control cells were extracted for cellular lipids before incubation (zero time control). Collected supernatants were evaporated gently under a N₂ stream, and TG was quantified using a TG E-test kit (Wako Pure Chemical Industries, Osaka, Japan).

VII. Determination of mRNA expression levels by real-time RT-PCR

HepG2 cells were seeded in 12-well plates at 2.0×10^5 cells/ml in DMEM supplemented with 10% fetal bovine serum and antibiotics. After 24 h of incubation, each fatty acid was added to HepG2 cells with T0901317 (10 nM) in the presence of vitamin E (10 μ M) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.3%. After 24 h of incubation, total RNA was extracted from the cells using Sepasol reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. RNAs were treated with RNase-free DNase (Promega, Madison, WI, USA) to remove contaminating genomic DNA. After inactivating DNase by adding DNase stop solution (Promega, Madison, WI, USA) and heating at 65°C for 10 min, each RNA was transcribed to cDNA using SuperScript RNase II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamers at 25°C for 10 min and then at 42°C for 50 min. The reactions were stopped by incubation at 70°C for 15 min. To quantify the mRNA expression levels, real-time quantitative RT-PCR was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primers used for the quantification of each gene are listed in Table 1-1. Primer pairs were selected to yield gene-specific single amplicons based on analyses by melting curves and by agarose gel electrophoresis. The thermal cycling conditions were as follows: 15 min at 95°C for one cycle, followed by

amplification of the cDNA for 43 cycles with melting for 15 s at 95°C and with annealing and extension for 30 s at 60°C. Values were normalized against 18s rRNA as an endogenous internal standard.

Table 1-1. Real-time RT-PCR primers used for the quantification of human mRNAs

Gene name	Reference or Accession Number	Forward (from 5' to 3')	Reverse (from 5' to 3')
SREBP-1c	[44]	GGAGGGGTAGGGCCAACGGCCT	CATGTCTTCGAAAGTGCAATCC
SCD-1	NM_005063	TGGTTTCACTTGGAGCTGTG	GGCCTTGGAGACTTTCTTCC
FAS	[44]	CAGGGACAACCTGGAGTTCT	CTGTGGTCCCCTTGATGAGT
ACC1	NM_198834	ATCCCGTACCTTCTTCTACTG	CCCAAACATAAGCCTTCACTG
ABCA1	[45]	GCAAGGCTACCAGTTACATTTG	GTCAGAAACATCACCTCCTG
GPA	NM_020918	TTGGGTTTGCGGAATGTTAT	GGCAGAACCATCAGGGTTTA
PGC-1β	NM_133263	TGACTCCGAGCTCTTCCAG	CGAAGCTGAGGTGCATGATA
18S	[44]	TAAGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC

VIII. Cell fractionation and immunoblotting

HepG2 cells were plated in 6-well plates at 5.0×10^5 cells/ml for 24 h in DMEM supplemented with 10% fetal bovine serum and antibiotics as detailed above. The cells were then treated with EPA, OEPA and/or T0901317 (10 nM) in the presence of vitamin E (10 μ M) in serum-free medium containing 0.1% BSA. After incubation for 24 h, membrane fractions and nuclear extracts from cells were prepared by the method of Hannah et al. [46]. Briefly, cells were harvested by scraping and the cell suspensions were centrifuged at $1,000 \times g$ for 5 min at 4°C. The cell pellets were resuspended in buffer A (250 mM sucrose, 10 mM Hepes-KOH at pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA) containing protease inhibitors (Complete Mini Protease Inhibitor tablet, Roche, Mannheim, Germany). The cell suspensions were

passed through a 23-gauge needle 20 times and were centrifuged at $1,000 \times g$ for 5 min at 4°C. The pellets were resuspended in 40 μ l Buffer B (20 mM Hepes-KOH at pH 7.6, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA and protease inhibitors). The suspensions were rotated at 4°C for 1 h and were then centrifuged at $10^5 \times g$ for 15 min at 4°C. The resulting supernatant is designated as the nuclear extract fraction. The supernatant of the original $1,000 \times g$ spin was centrifuged at $10^4 \times g$ for 15 min at 4°C after which the pellet was dissolved in 25 μ l SDS lysis buffer (10 mM Tris-HCl at pH 6.8, 100 mM NaCl, 1% (w/v) SDS, 1 mM sodium EDTA, 1 mM sodium EGTA, and protease inhibitors) and is designated as the membrane fraction. The concentration of soluble proteins in the supernatant was quantified using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For immunoblot analysis, given amounts of membrane fractions (25 μ g) and nuclear extracts (10 μ g) were separated by 7% and 10% SDS-PAGE, respectively. Protein bands were transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). The filters were probed with a rabbit polyclonal anti-SREBP-1 antibody (H-160, 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound antibodies were visualized with alkaline phosphatase-conjugated anti-rabbit IgG (1:500 dilution for membrane fractions and 1:1,000 dilution for nuclear extracts; Cell Signaling Technology, Danvers, MA). The bands were visualized with the substrate, chemi-lumione L (Nacalai Tesque, Kyoto, Japan) using a FUJIFILM visualizer (LAS-3000, Fujifilm Corporation, Tokyo, Japan).

IX. Statistical analysis

Data are reported as means $\pm$ SD. Statistical analyses were carried out by one-way ANOVA with Dunnett's F-test to identify significant difference using Stat View software (SAS Institute, Cary, NC, USA).

RESULTS

I. Changes in derivatives of EPA oxidation products after autoxidation

First, OEPAs were prepared from EPA (10 mg) by autoxidation. $[M-H]^-$ at the mass to charge ratio (m/z) 301.2 presented as the molecular ion of EPA and m/z 317.2-397.2 were presumably derived from EPA oxidation products. The intensity of the molecular ion of EPA at m/z 301.2 decreased continuously after autoxidation and remained around 50% of the pre-incubation level of EPA at 24 h under these experimental conditions. After oxidation for 4 h, several new ions that were oxidative products of EPA appeared. The molecular ion $[M-H]^-$ of hydroxy-EPA (HEPE) at m/z 317.2 reached a peak at 4 h incubation and then gradually decreased. HEPE was the major EPA oxidation product at 4 and 8 h. The ion at m/z 333.2, known as hydroperoxy-EPA, appeared after 4 h of autoxidation and increased gradually from 8 to 24 h. On the other hand, the ion at m/z 349.2 was detected at 8 h and after that it reduced gradually. The ions at m/z 365.2 and 397.2 were found at 4 and 8 h, respectively, and then they tended to increase gradually (Fig. 1-3).

II. Effects of each fatty acid on HepG2 cell viability

To ascertain the effects of each compound on cell viability, various concentrations of fatty acids and T0901317 were added to HepG2 liver cells in culture. Figure 1-4 shows the results of the viability assay after 24 h of treatment. The cytotoxic effect of EPA weakened depending on the oxidation time. EPA, 4 h OEPA, 8 h OEPA and 16 h OEPA at 30 and 60 μ M significantly reduced cell viability to around 24%, 34%, 34% and 66% of vehicle-treated control cells, respectively. The 24 h OEPA at 30 μ M, but not at 60 μ M, had no cytotoxic effect on HepG2 cells under these experimental conditions. Notwithstanding, when HepG2 cells were co-incubated with 10 μ M vitamin E, the

cytotoxicity of EPA and OEPA was eliminated, which corresponded with the study of Caputo et al. [47].

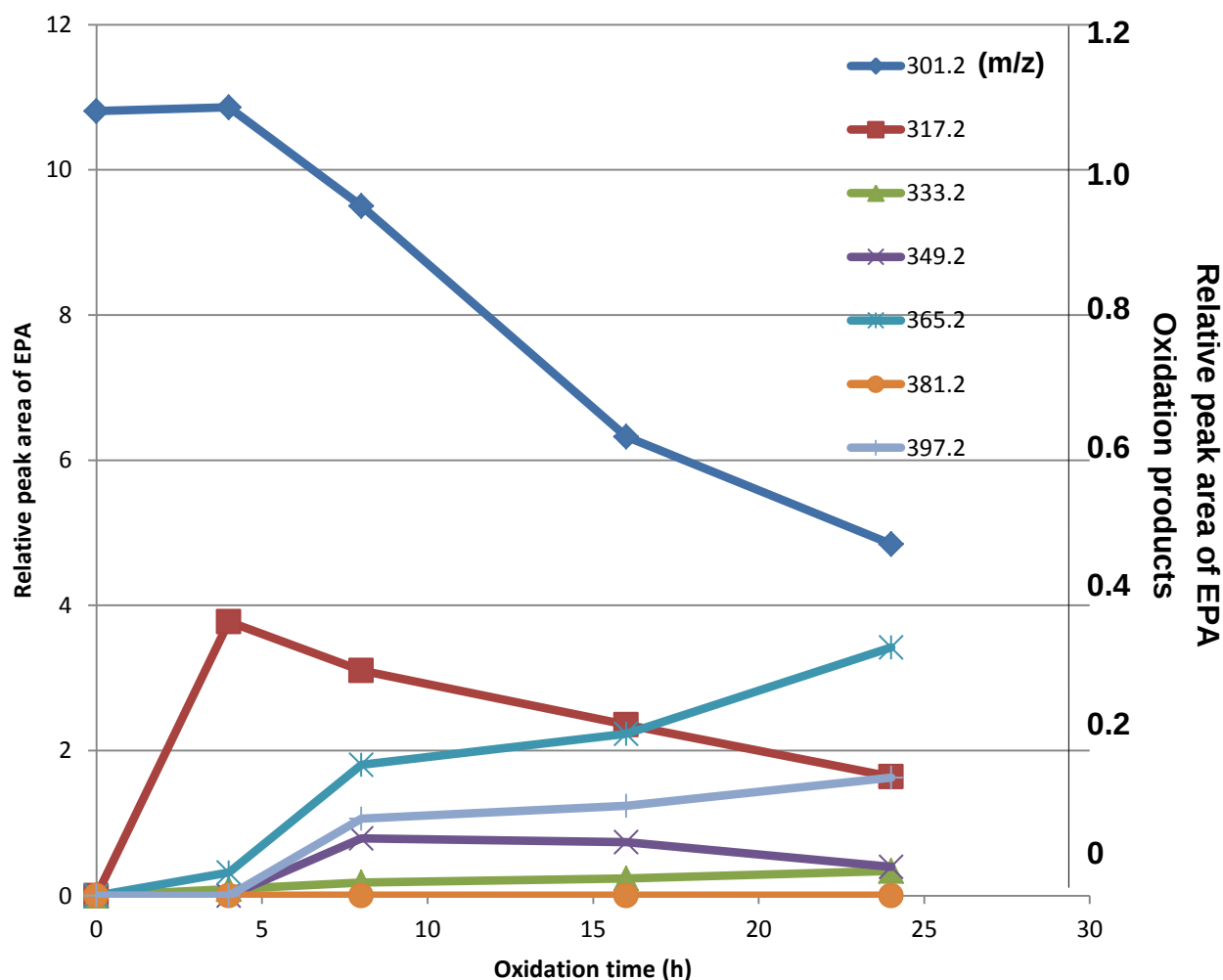


Figure 1-3. Ion intensities derived from EPA and EPA oxidation products analyzed by LC/MS. m/z 301.2 was derived from EPA and m/z 317.2-397.2 were presumably derived from hydroxy- and hydroperoxy-EPAs. Each oxidized EPA was prepared from EPA (10 mg) incubated in 40°C water bath for 4, 8, 16 or 24 h.

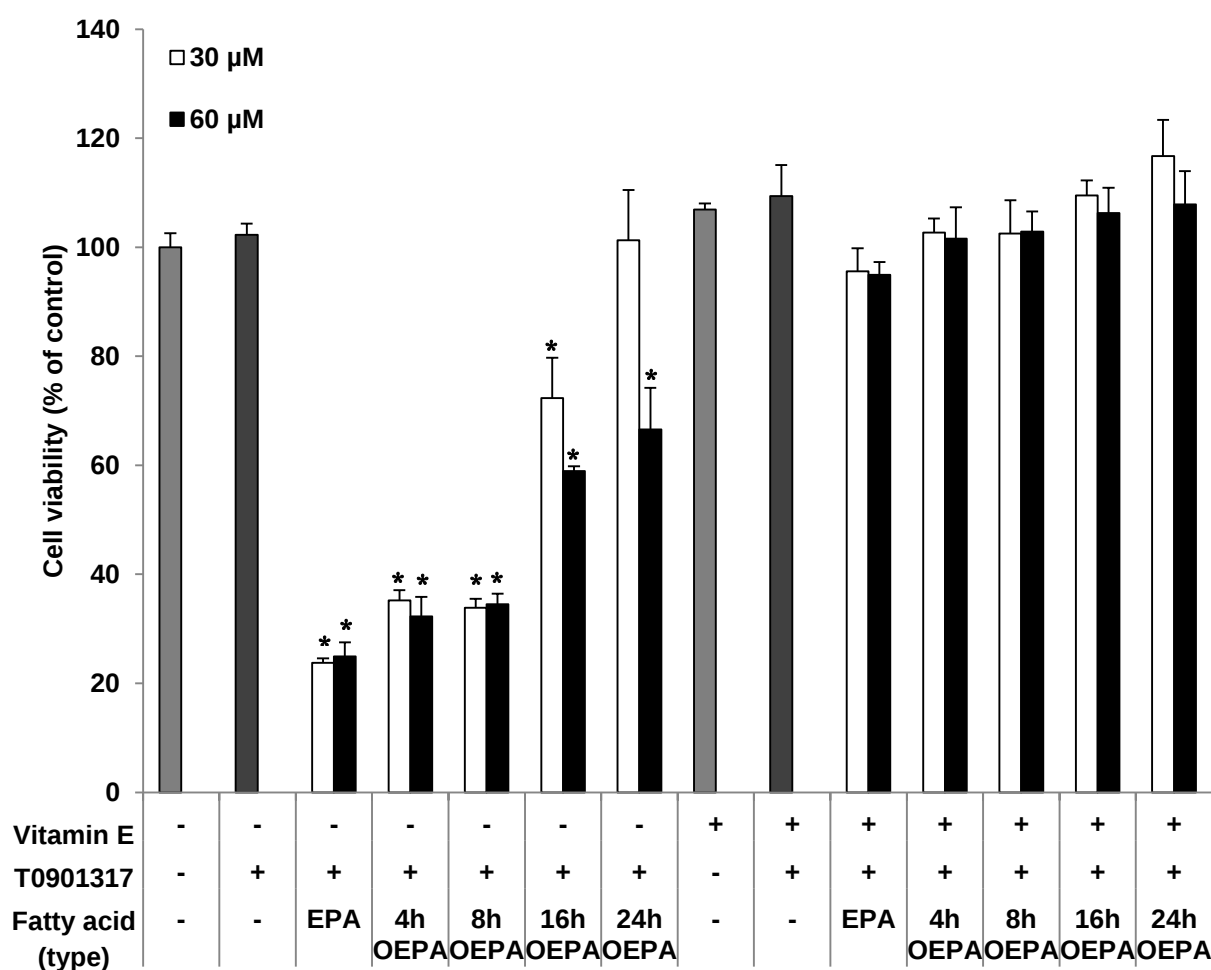


Figure 1-4. Effects of EPA and OEPA on cell viability as determined by the water soluble tetrazolium (WST)-1 assay. Each fatty acid (30 or 60 μM) was added to HepG2 cells with T0901317 (10 nM) and/or vitamin E (10 μM) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.3%. After 24 h incubation, the WST-1 solution was added to the cells. The absorbance at 450 nm corresponds to cell viability. Expression levels are presented as relative percentage to vehicle control (ethanol). Data are reported as means ± SD (n = 4/group). * A significant difference from the vehicle control group (p < 0.05).

III. OEPA suppresses TG synthesis in HepG2 cells

The effects of EPA and each OEPA on TG synthesis in T0901317-induced HepG2 cells were then examined. While the cells were being treated with each compound, vitamin E was simultaneously added in FBS-free medium containing 0.1% BSA since EPA has very severe cytotoxic effects, as shown in Figure 1-4. T0901317 significantly augmented the cellular levels of TG (about 3200 $\mu\text{g}/\text{mg}$ protein) after 48 h of incubation. Consistent with a previous study [17], EPA significantly reduced TG synthesis of T0901317-treated HepG2 cells to 2400 $\mu\text{g}/\text{mg}$ protein. Surprisingly, treatment with OEPA for 4, 8 and 16 h significantly inhibited the cellular TG content of HepG2 cells more than did EPA (Fig. 1-5). Moreover, the 24 h OEPA, which has around 50% the ion intensity at m/z 301.2 to intact EPA (Fig. 1-3), showed almost the same levels of TG suppression as did intact EPA (Fig. 1-5). These findings indicate that HEPEs might play a crucial role in the effect of EPA on the inhibition of cellular TG synthesis in HepG2 liver cells.

IV. OEPA decreases SREBP-1c mRNA expression and maturation more effectively than EPA

To determine whether the hypolipogenic effect of OEPA on HepG2 cells is due to SREBP-1c, the mRNA expression levels of SREBP-1c and SREBP-1 protein levels were determined. The expression of SREBP-1c mRNA in cells treated with 10 nM T0901317, a synthetic LXR agonist, was 9.4-fold higher than in vehicle-treated control cells (Fig. 1-6). Treatment with EPA and 4-24 h OEPA significantly down-regulated SREBP-1c mRNA expression stimulated by T0901317. In agreement with the result of TG accumulation, the suppressive effect of OEPA at 4 and 8 h was statistically stronger than EPA. The 4 h OEPA treated cells showed the least SREBP-1c mRNA expression as also noted in the effect on TG synthesis in hepatocytes. Furthermore, the 24 h OEPA

reduced SREBP-1c expression to the same level as treatment with EPA (similar to Figure 1-5).

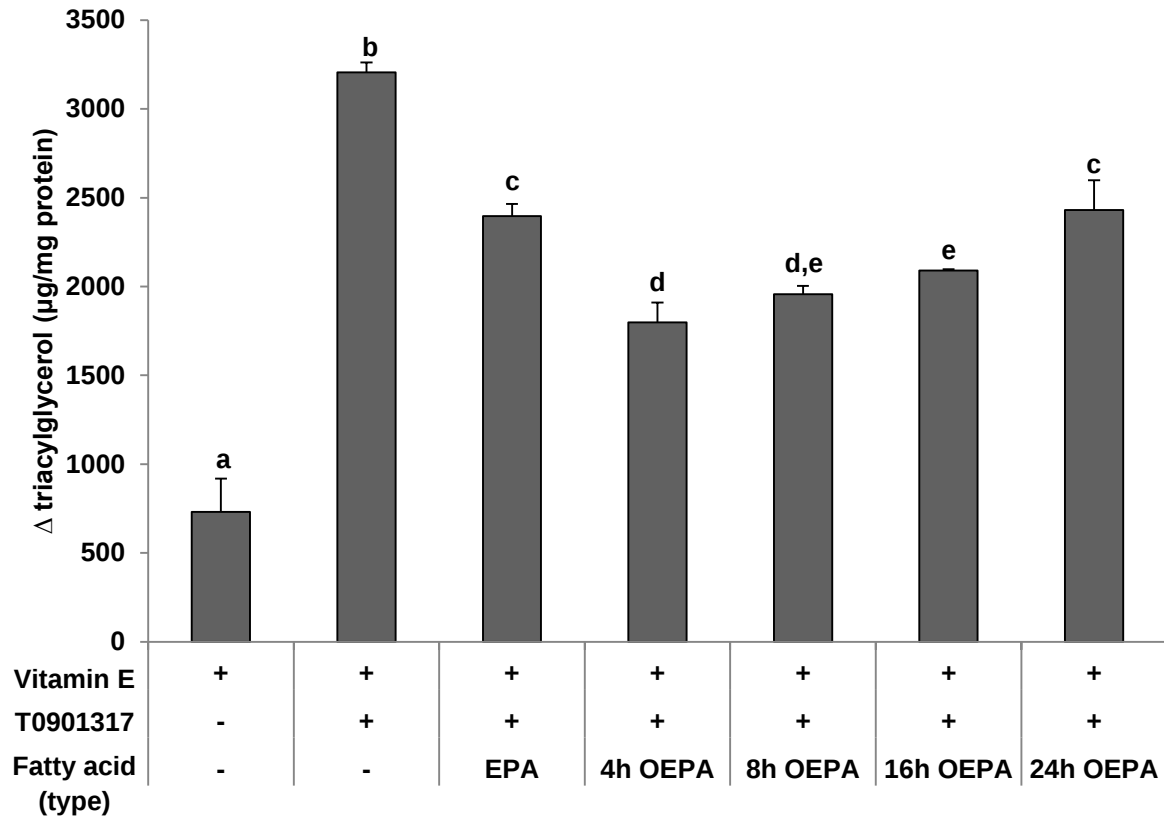


Figure 1-5. Effects of EPA and OEPA on TG synthesis in HepG2 cells. Each fatty acid (60 μ M) was added to HepG2 cells with T0901317 (10 nM) and vitamin E (10 μ M) as noted in serum-free medium containing 0.1% BSA for 48 h. The final ethanol concentration was 0.3%. The increased TG levels per mg protein (TG levels after 48 h of incubation minus the levels of reference cells) are shown. Data are reported as means $\pm$ SD (n = 3-4/group). Values with different letters are significantly different ($p < 0.05$).

To investigate the effect of these fatty acids on SREBP-1 protein levels, the full-length precursor form in cell membranes (125 kDa) and the cleaved mature form (68 kDa) in nuclear extracts were estimated by immunoblotting. Because the antibody used cannot distinguish between the SREBP-1c and -1a isoforms, the general term SREBP-1

was used to refer to the results. Representative blots are shown in Figure 1-7A and B. T0901317 increased the levels of both the precursor and the mature forms of SREBP-1.

The 4-16 h OEPA as well as EPA significantly decreased the T0901317 induction of both the precursor and mature forms of SREBP-1. On the other hand, the 24 h OEPA significantly differentiated only mature SREBP-1. Corresponding to SREBP-1c mRNA expression, the precursor form of SREBP-1 was down-regulated by treatment with the 4 and 8 h OEPA more significantly than EPA. Interestingly, the mature form of SREBP-1 was also inhibited by the 4 h OEPA more significantly than EPA and the vehicle control.

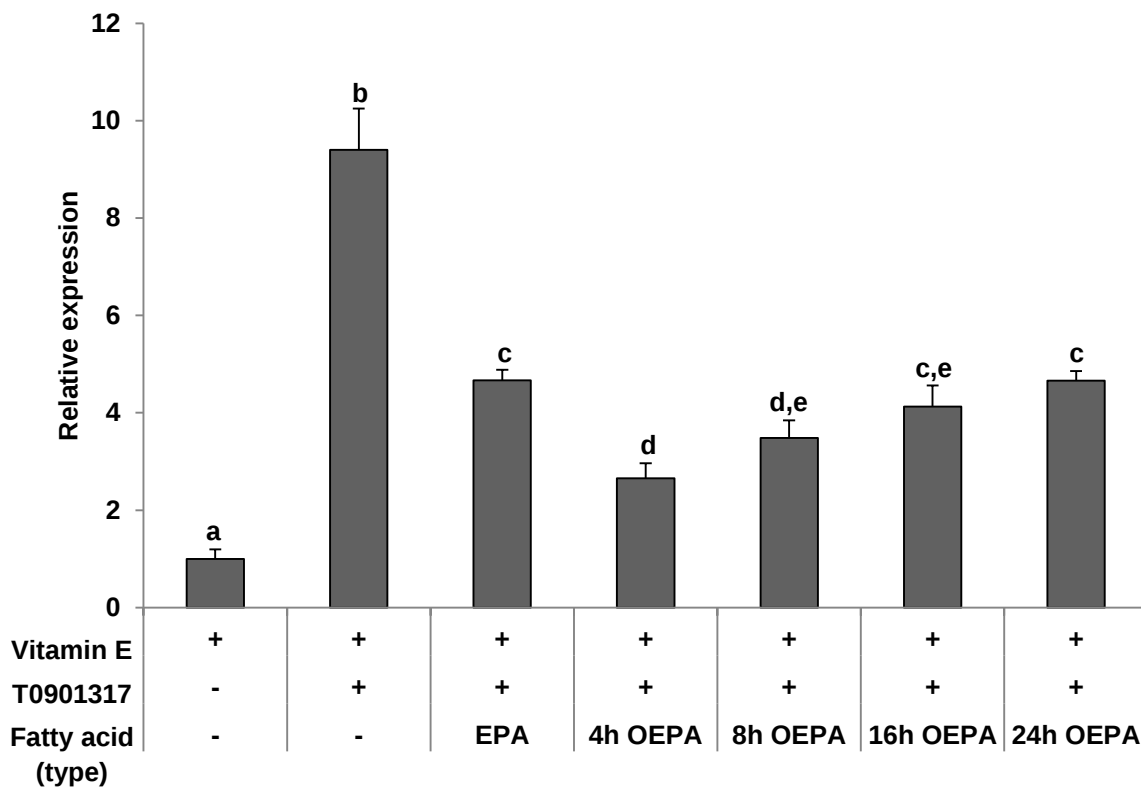
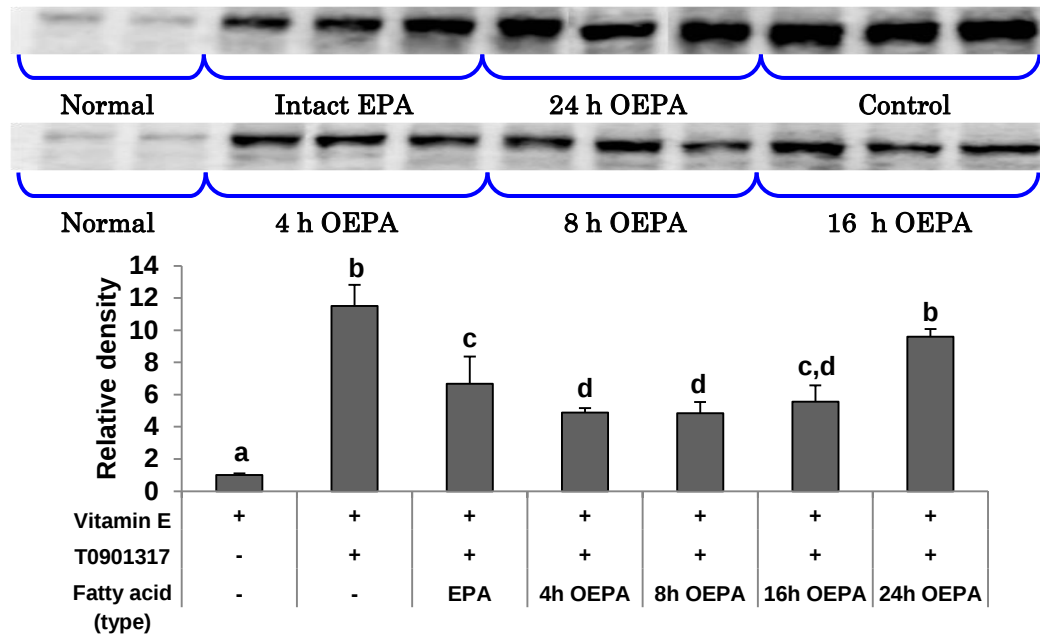


Figure 1-6. Effect on SREBP-1c mRNA expression. Each fatty acid (60 μ M) was added to HepG2 cells with T0901317 (10 nM) and vitamin E (10 μ M) as noted in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.3%. After 24 h incubation, SREBP-1c mRNA levels were quantified by real-time quantitative PCR. Each value of SREBP-1c mRNA was adjusted by that of 18s rRNA (internal control).

Expression levels are presented as -fold induction relative to the vehicle control (vitamin E and ethanol). Data are reported as means $\pm$ SD (n = 3/group). Values with different letters are significantly different (p < 0.05).

(A) Precursor SREBP-1



(B) Mature SREBP-1

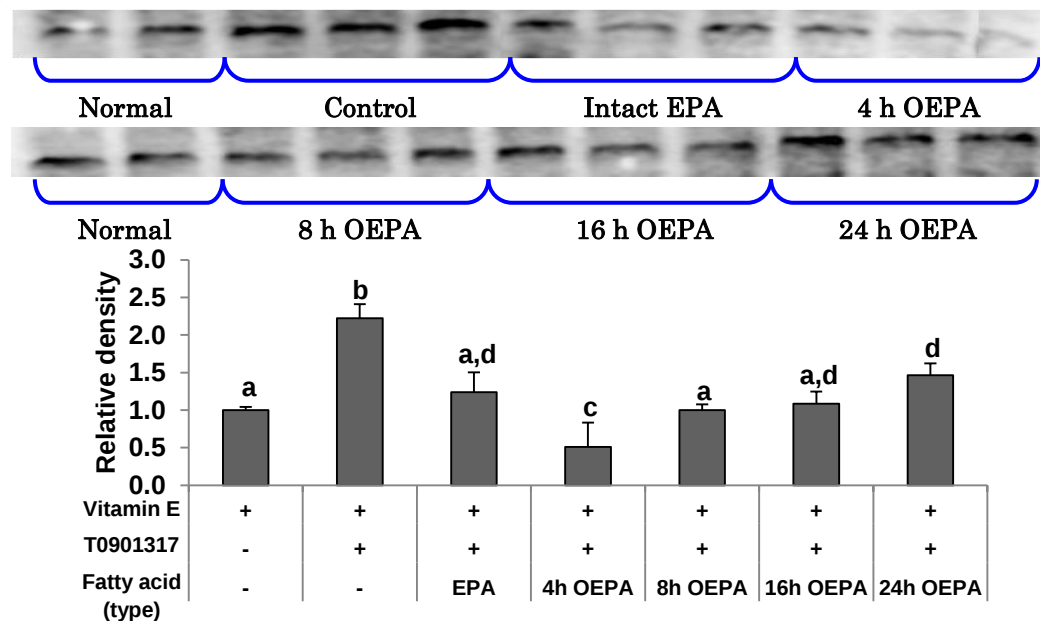


Figure 1-7. Effects on precursor (A) and mature (B) SREBP-1 protein expression. Each fatty acid was added to HepG2 cells with T0901317 (10 nM) and vitamin E (10 μ M) as

noted in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.3%. The data represent the mean -fold change of the precursor and the mature forms of SREBP-1 from the vehicle control (ethanol). Data are reported as means $\pm$ SD (n = 3-4/group). Values with different letters are significantly different ($p < 0.05$).

V. Regulation of mRNA levels of lipogenic genes by OEPA

To further elucidate whether the more efficient effect on the reduction of hepatic TG synthesis by OEPA compared to EPA is due simply to the suppression of SREBP-1c or also to other pathways that might be involved, SREBP-1c target genes and other lipid metabolism related genes were examined. Treatment with EPA or 4 h OEPA significantly decreased the expression of Acetyl CoA carboxylase (ACC), Fatty acid synthase (FAS) and Stearoyl-coenzyme A desaturase-1 (SCD1) mRNAs (Fig. 1-8A, B, C). Corresponding to the aforementioned results, treatment with 4 h OEPA significantly decreased ACC and FAS expression more than the vehicle control while treatment with EPA reduced the expression of these genes to the same level as the vehicle control (Fig. 1-8A, B). Moreover, 4 h OEPA treatment significantly down-regulated the T0901317-induced expression of SCD1 more than did EPA (Fig. 1-8C). GPA, an enzyme located in the endoplasmic reticulum and the mitochondrial membrane that is required for TG synthesis in the glycerol phosphate pathway, which is regulated by SREBP-1c, was significantly weakened by 4 h OEPA compared to LXR α agonist-induced cells, while EPA did not affect the increase of GPA induced by T0901317 (Fig. 1-8D). In agreement with the decreased expression of lipogenic target genes of SREBP-1c, mRNA expression levels of ATP-binding cassette transporter A1 (ABCA1), the other target gene of LXR that is related to cholesterol transport, was significantly decreased by 4 h OEPA, but was not significantly changed by EPA (Fig. 1-8E). It was unexpectedly found that after the 4 h OEPA treatment, the expression of PGC-1 β , the co-activator of LXR α and the

SREBP families, was significantly down-regulated in the T0901317-induced group. In contrast, EPA did not affect the expression of PGC-1 β (Fig. 1-8F).

VI. The members of HEPEs contained in EPA oxidation products after autoxidation

From the results mentioned above, the 4 and 8 h OEPAs, which contain high portions of HEPEs (m/z 317.2), showed dominant suppressive effects on the lipogenesis pathway in HepG2 hepatic cells induced by the synthetic LXR α agonist among EPA and other OEPAs which contain lower portions of HEPEs. For this reason, the standards of HEPEs, including 5-, 11- and 18-HEPEs, were utilized to identify the kinds of HEPEs that occurred in the autoxidized process by LC-MS (Fig. 1-9). The results showed that 5-, 11- and 18-HEPEs might be members of HEPEs in OEPA due to their correlate retention time between peaks of the standards and HEPEs derivative in 4 h OEPA (Fig. 1-10A). Based on their peak area, 5-HEPE is the major member of HEPEs in 4 h OEPA (25.0%). Besides, 4 h OEPA also has 11-HEPE and 18-HEPE in the proportion of 14.1% and 12.3%, respectively, comparing with total peaks area of ion at m/z 318.4 (Fig. 1-10B).

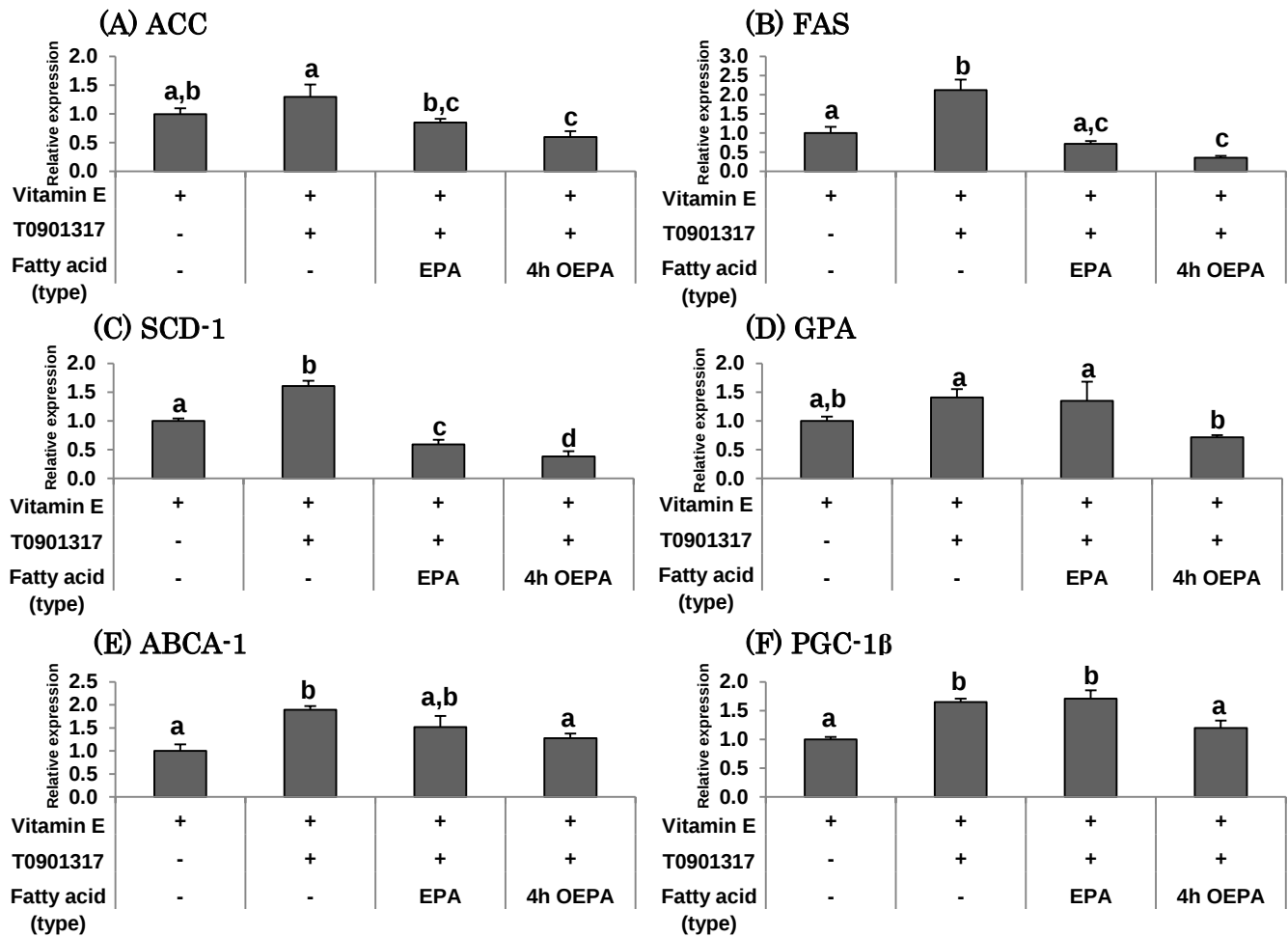


Figure 1-8. Effects of EPA and OEPA on the expression of acetyl-CoA carboxylase-1 (ACC; A), fatty acid synthase (FAS; B), stearoyl-coenzyme A desaturase-1 (SCD-1; C), glycerol-3-phosphate acyltransferase (GPA; D), ATP-binding cassette sub-family A member 1 (ABCA1; E) and peroxisome proliferator-activated receptor γ co-activator 1 β (PGC-1 β ; F) mRNAs. Each fatty acid (60 μ M) was added to HepG2 cells with T0901317 (10 nM) and vitamin E (10 μ M) as noted in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.3%. After 24 h incubation, ACC, FAS, SCD-1, GPA, ABCA1 and PGC-1 β mRNA levels were quantified by real-time quantitative PCR. Each value of ACC, FAS, SCD-1, GPA, ABCA1 and PGC-1 β mRNAs was adjusted by that of 18s rRNA (internal control). Expression levels are presented as -fold induction relative to the vehicle control (vitamin E and ethanol). Data are reported as means $\pm$ SD (n = 3/group). Values with different letters are significantly different (p < 0.05).

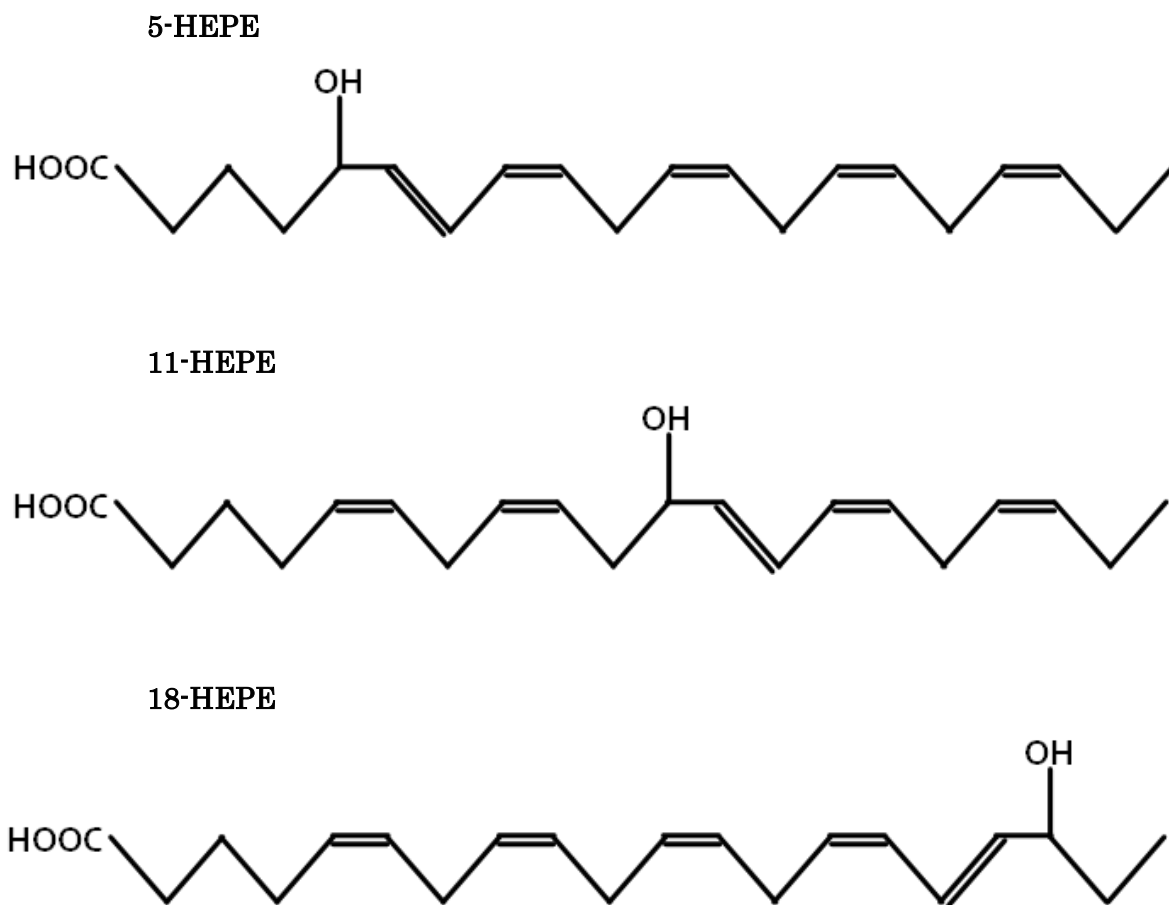
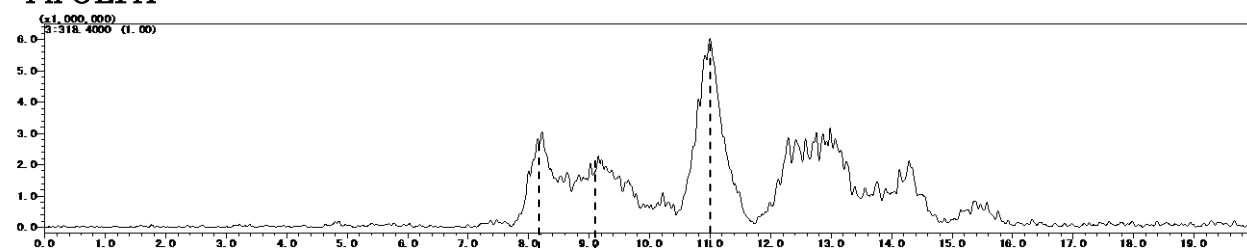


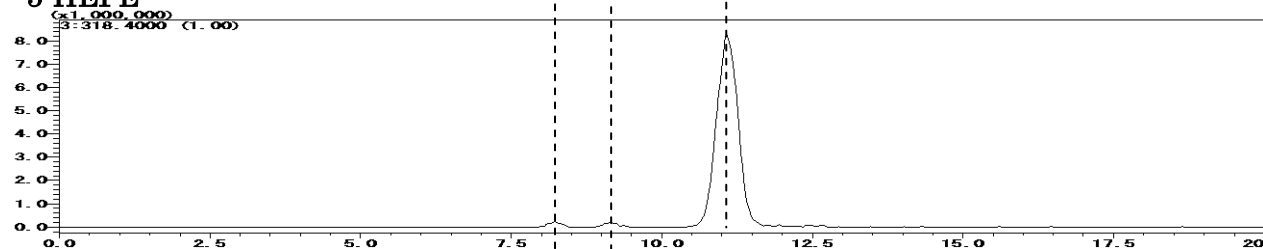
Figure 1-9. The chemical structures of the standards of HEPES, including 5-, 11- and 18- HEPES.

(A) Selected ion chromatograms of m/z 318.4 (HEPEs) in 4 h OEPA compared with purified HEPEs

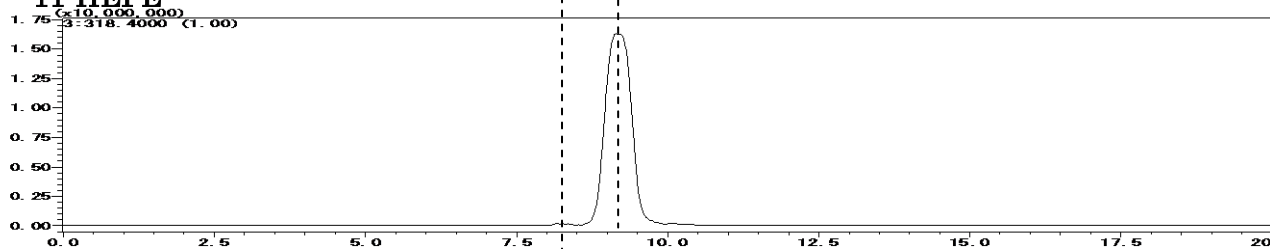
4 h OEPA



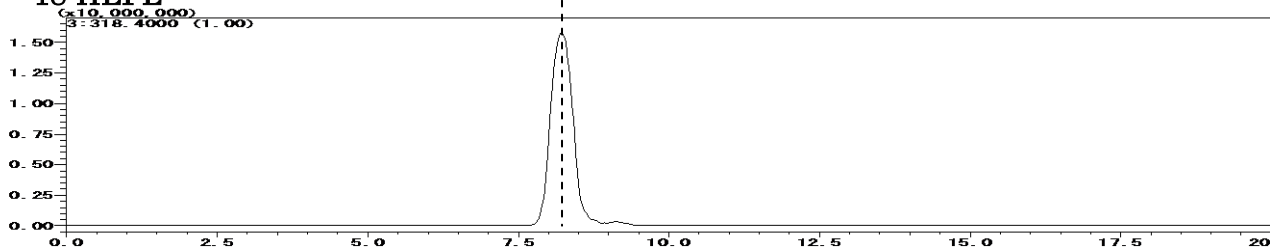
5-HEPE



11-HEPE



18-HEPE



(B) Percentage of peaks area of each type of HEPEs in 318.4 m/z (hydroxyl EPA) of 4 h OEPA

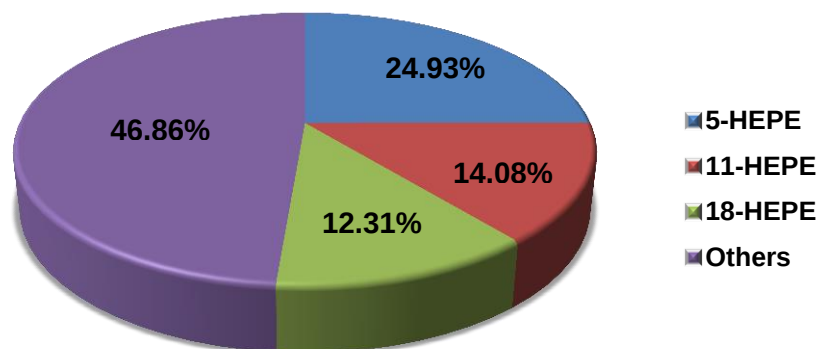


Figure 1-10. Various kinds of the standards of HEPEs were compared with HEPEs derived from autoxidized EPA. A: The retention time of 4 h OEPA, 5-HEPE, 11-HEPE, and 18-HEPE were allegorized. B: Ion intensities percentage of each type of HEPE contained in hydroxy EPA (m/z 317.2) derived from 4 h OEPA was measured by using the graph area from the specific retention time. Retention time and ion intensities in 4 h OEPA and HEPEs were analyzed by LC-MS.

VII. Regulation of lipogenic gene mRNA levels by 5-, 11- and 18-HEPEs

These standard HEPEs were then treated to HepG2 cells to confirm whether those HEPEs are involved in the TG reduction of OEPAs. At both 30 and 60 μ M concentrations, all fatty acids significantly decreased the augmentation of SREBP-1c expression caused by the LXR α agonist. It was noteworthy that at a 60 μ M concentration, 5-HEPE and 18-HEPE significantly reduced SREBP-1c expression more than did EPA (Fig. 1-11A). The expression of SREBP-1c target genes also had a tendency for the same responses. All fatty acids significantly down-regulated the T0901317-induced increase of ACC. At 30 μ M, expression of ACC in the 18-HEPE treated group was significantly lower than in the EPA-treated group. In addition, 60 μ M 5-HEPE or 18-HEPE significantly reduced the expression of ACC more than did EPA (Fig. 1-11B). Expression of SCD1 at both 30 μ M and 60 μ M 18-HEPE was significantly lower than was elicited by EPA at similar concentrations (Fig. 1-11D). On the other hand, all fatty acids significantly down-regulated the T0901317-induced expression of FAS, but there was no significant different between any of the fatty acids at the same concentrations (Fig. 1-11C). Among all experimental groups, only treatment with 60 μ M 18-HEPE significantly decreased GPA expression compared with T0901317-stimulated cells. Interestingly, cells which were treated with all types of HEPEs in this experiment had significantly reduced expression of GPA more than the EPA-treated cells (Fig. 1-

11E). Similar to the 4 h OEPA treatment, 18-HEPE at a high dose can significantly decrease the expression of PGC-1 β (Fig. 1-11F).

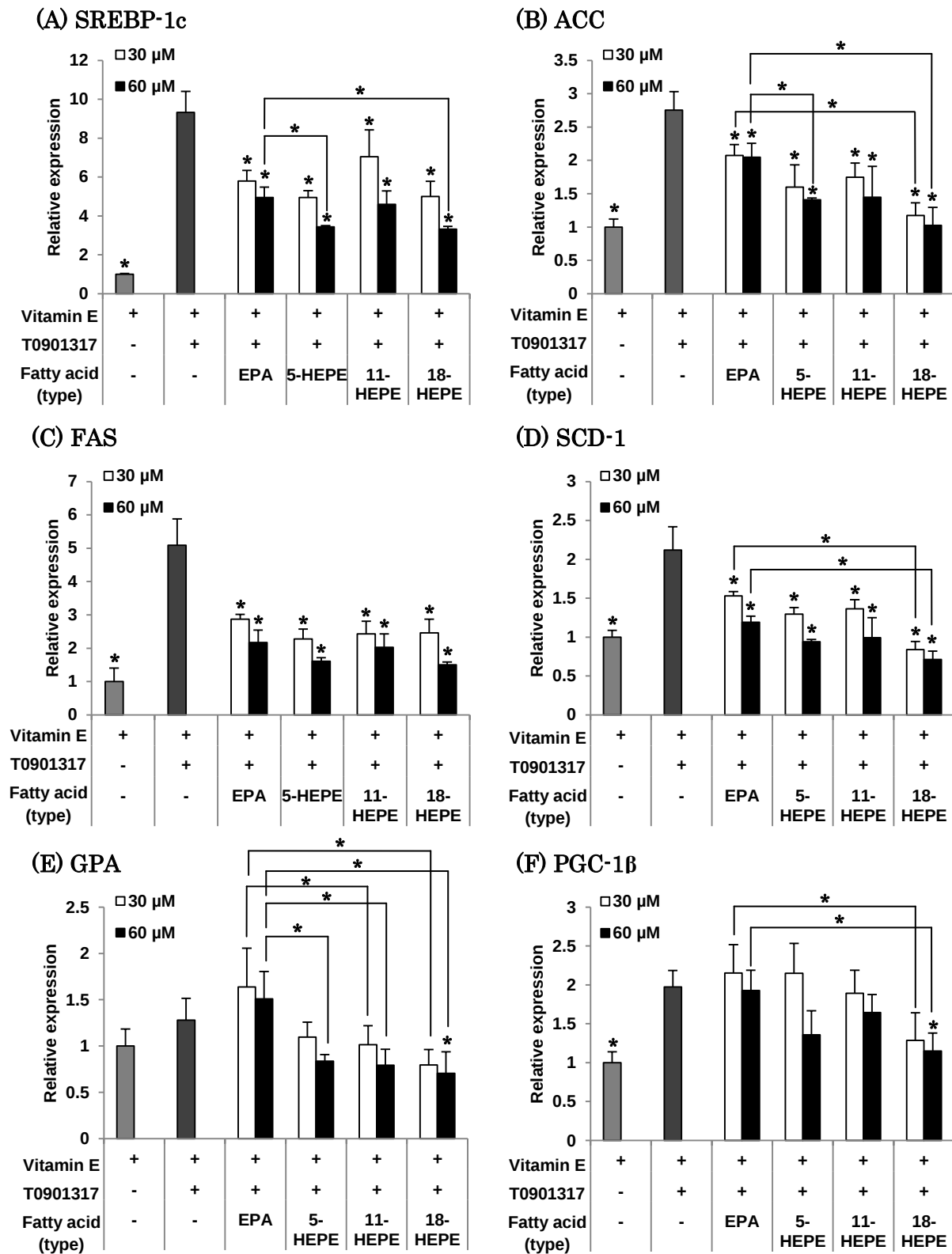


Figure 1-11. Effects of EPA, 5-HEPE, 11-HEPE and 18-HEPE on the expression of sterol-regulatory element binding protein-1c (SREBP-1c; A), acetyl-CoA carboxylase-1 (ACC; B), fatty acid synthase (FAS; C), stearoyl-coenzyme A desaturase-1 (SCD-1; D), glycerol-3-phosphate acyltransferase (GPA; E) and peroxisome proliferator-activated receptor γ coactivator 1 β (PGC-1 β ; F) mRNAs. Each fatty acid (60 μ M) was added to HepG2 cells with T0901317 (10 nM) and vitamin E (10 μ M) as noted in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.3%. After 24 h incubation, SREBP-1c, ACC, FAS, SCD-1, GPA and PGC-1 β mRNA levels were quantified by real-time quantitative PCR. The value of each mRNA was adjusted by that of 18s rRNA (internal control). Expression levels are presented as -fold induction relative to the vehicle control (vitamin E and ethanol). Data are reported as means $\pm$ SD (n = 3/group). * A significant difference from the T0901317-induced control group (p < 0.05).

DISCUSSION

Previous studies showed that oxidation products of EPA and DHA alleviate some pathways related to CVD [15-18]. In this study, the hypolipidemic effect of OEPA on hepatic cells was emphasized. The hypolipidemic effect of PUFAs, including EPA, is attributable both to a decrease in lipogenesis and an increase in fatty acid catabolism through the regulation of SREBP-1 and PPAR α , respectively [32]. Nevertheless, it is well known that PPAR α weakly expresses and has very low function in HepG2 cells, because the cells are originated from hepatoma and some kinds of genes, such as PPAR α , is mutated [48]. Thus, this study focused on the regulation of lipogenesis via SREBP-1 by OEPA.

First of all, it is noteworthy that 4-16 h OEPA significantly decreased the T0901317-induced accumulation of TG in liver cells more than did intact EPA. In this experiment, 4 h OEPA was the most effective agent which was able to suppress TG accumulation. In accordance with that result, the T0901317 induced expression of SREBP-1c, a major transcription factor that activates the expression of lipogenic genes, was significantly down-regulated by 4-8 h OEPA more than EPA. Not only the expression levels of SREBP-1c mRNA, but the protein levels of SREBP-1c were also significantly inhibited by OEPA more than by EPA. It has been well established that PUFAs suppress SREBP-1c pathway via competing with LXR α ligands in the activation of the ligand binding domain [49-51]. It has been shown that trans isomers of EPA (TEPA), similar to EPA, reduces the expression of SREBP-1c through LXR α pathway [49]. Thus, OEPA might decrease the expression of SREBP-1c by competing with LXR α ligands more effectively than EPA.

SREBP-1c target genes were also evaluated after simultaneously treating HepG2 cells with T0901317, and EPA or 4 h OEPA. As expected, ACC, FAS and SCD-1 mRNA

expression in the 4 h OEPA-, but not EPA-, treated group was significantly lower than the vehicle control group. Furthermore, 4 h OEPA significantly decreased SCD-1 more than did EPA. Surprisingly, 4 h OEPA significantly diminished the T0901317-induced expression of GPA, but EPA did not. In agreement with a previous finding, the TEPA significantly impaired GPA mRNA expression as well [49]. GPA is the rate limiting enzyme required for the *de novo* synthesis of TG and phospholipids [52, 53]. Hence, a decrease of GPA expression in the liver of OEPA might implicate in its preferable hypolipidemic effect. Deletion of the gene encoding SREBP-1c has been shown to result in the failure of T0901317-induced GPA mRNA expression in animal studies [54, 55]. However, the previous and present studies illustrate that EPA, which can significantly down-regulate T0901317-induced SREBP-1c expression, cannot abate GPA expression [46]. Therefore, it can be hypothesized that not only SREBP-1c, but also other factors, might regulate GPA expression. Ericsson et al. [56] demonstrated that GPA promoter-luciferase reporter genes are stimulated by the co-expression of SREBP-1a. Importantly, that increase was attenuated when either a dominant negative form of nuclear factor- κ B (NF- κ B) was cotransfected into the cells or when the GPA promoter contained mutations in the putative binding sites for SREBP-1a or NF- κ B. Taken together, modification of the molecular structure of EPA, such as OEPA and TEPA, might be necessary for it to be involved in GPA down-regulation via SREBP-1a or NF- κ B.

These results could be also attributable in part to the suppression of PGC-1 β expression by 4 h OEPA (Fig. 1-8F), because PGC-1 β plays dual roles in modulating hepatic fatty acid metabolism and regulating either fatty acid oxidation or *de novo* fatty acid synthesis by co-activating SREBP-1 and LXR α [43, 57]. Present data support the concept that PGC-1 β is important for the full induction of lipogenic genes regulated by SREBP-1 and LXR α , and thus, PGC-1 β provides a therapeutic target for the metabolic syndrome. It was shown that PGC-1 β is a key regulator of hepatic lipogenesis and

lipoprotein secretion in response to dietary intake of saturated fats [43]. In contrast, it has minimal effect on the expression of gluconeogenic genes [42]. The expression of PGC-1 β in the liver is strongly induced by dietary fats, likely through direct regulation by fatty acids in hepatocytes. Hence, the effective inhibition of the cellular TG synthesis by OEPA might be caused by the reduction of PGC-1 β expression. Notwithstanding, the mechanism by which OEPA decreases PGC-1 β mRNA is unclear, but the reduction of mRNA expression of PGC-1 β by OEPA is consistent with the results of previous report concerning about TEPA [49]. Thus, OEPA and TEPA might suppress the expression of PGC-1 β by the same mechanism.

All the aforementioned results confirm that treatment with 4 h OEPA is the most effective in suppressing the hepatic lipogenesis pathway among OEPAs incubated for different oxidation times in this experiment. Interestingly, these inhibition trends are associated with the change in intensity of the ion at m/z 317.2, which appeared as HEPEs in OEPA. Therefore, it was assumed that HEPEs, but not every oxidation product, might be the most effective oxidation product which plays a crucial role in lipid accumulation in the liver through SREBP-1c pathway inhibition.

It was presumed that HEPEs in the 4 h OEPA includes 5-, 11- and 18-HEPEs by LC/MS analysis compared with HEPEs standards. Similar to the effect of 4 h OEPA, 5- and 18-HEPEs also significantly suppressed the T0901317-induced expression of SREBP-1c, ACC and SCD-1 more than did EPA, while purified 11-HEPE showed almost the same inhibition level as EPA. In this study, 18-HEPE was the most effective in suppressing the expression of SREBP-1c and its target gene mRNAs. Especially, only 18-HEPE significantly down-regulated GPA and PGC-1 β compared with T0901317-treated cells.

Of interest, 18-HEPE can be converted from EPA via acetylated cyclooxygenase 2 (COX-2) in vascular endothelial cells after treatment at local sites of inflammation with aspirin, and is then rapidly converted by activated 5-lipoxygenase (5-LOX) in human polymorphonuclear (PMN) leukocytes to insert molecular oxygen and in subsequent steps through 5(6) epoxide formation to bioactive Resolvin E1 (RvE1) and 15-epi-lipoxin (LX) A₅, the aspirin-triggered lipid mediators hydrolyzed from 5*R*,6-epoxy-15*R*-HEPE [58, 59]. These newly identified chemical mediators appear to exert potent inflammatory and pro-resolving actions both *in vitro* and *in vivo* as proposed by many studies [60, 61]. In addition, EPA can be directly metabolized to 5-HEPE in human neutrophils and eosinophils by activated 5-LOX, which has significantly decreased biological effects compared to arachidonic acid-derived metabolites [62, 63]. Kogure et al. demonstrated that 5-HEPE is a potent agonist for GPR119 and enhances glucose-dependent insulin secretion [18]. This study shows for the first time that the location of the hydroxy group on the carbon backbone of HEPES is an interesting factor that influences their performance in the regulation of the lipogenesis pathway.

In summary, these data suggest that OEPA augments the ameliorating effect of EPA on lipogenesis in liver cells via the suppression of lipogenic genes related to SREBP-1c. In addition, the preferable ameliorating effect of OEPA on lipogenesis might be due to the decreases of the expressions of PGC-1 β and GPA by OEPA, meanwhile EPA does not significantly differentiate those expressions (Fig. 1-12). The findings provide insight into the importance of the location and the number of hydroxy groups of OEPA in the hypolipidemic effect. This study also suggests that oxidation products as food components might contribute to the beneficial effects of EPA on lipid metabolism in the liver resulting in the prevention of CVD. However, further *in vivo* experiments are necessary to ensure the effects of dietary OEPA on lipid metabolism.

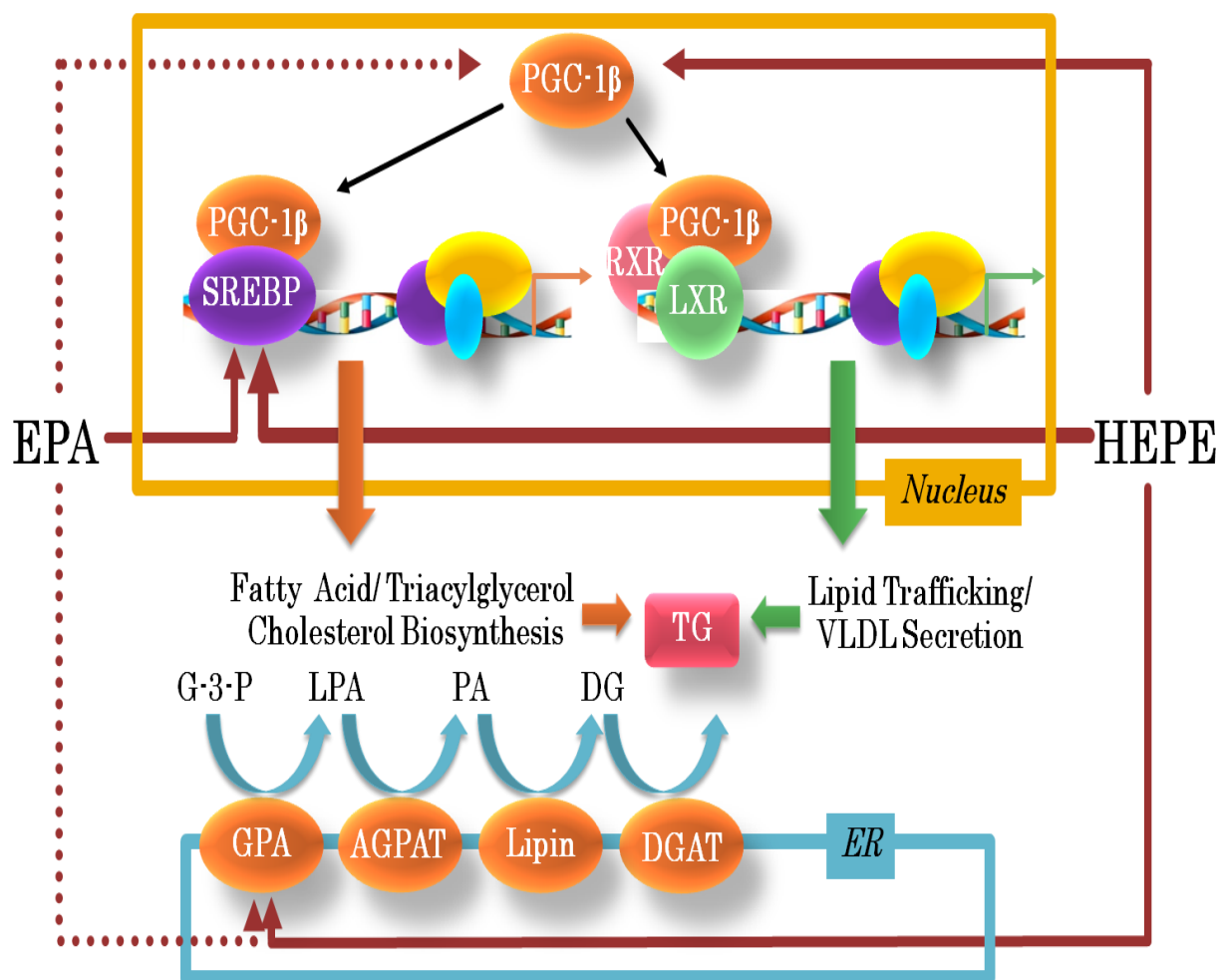


Figure 1-12. The mechanism of ameliorating effects of EPA and HEPE on TG accumulation in hepatic cells. G-3-P, glycerol-3-phosphate; LPA, lysophosphatidate; PA, phosphatidate; DG, diacylglycerol; TG, triacylglycerol; VLDL, very-low-density lipoprotein; GPA, glycerol-3-phosphate acyltransferase; AGPAT, acylglycerol-3-phosphate acyltransferase; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum.

Chapter 2: Oxygenated fatty acids fermented by *Lactobacillus*
plantarum decrease LXR-induced SREBP-1c expression resulting in
triacylglycerol reduction

INTRODUCTION

Lactobacillus plantarum is a non-pathogenic gram-positive bacterium naturally existing in human and other mammals' saliva and gastrointestinal tract. As a member of the lactic acid bacteria, it is commonly used in food fermentation, such as vegetable, fish, and cheese [78-80]. Being used as a probiotic, its biotherapeutic applications have been increasingly recognized, such as reduced incidence of diarrhea in daycare centers, decreased pain and constipation associated with irritable bowel syndrome, reduced bloating, flatulence, and capacity to exert positive effect on immunity in HIV⁺ children [28, 29]. The benefits of *L. plantarum* are still ambiguous in their mechanisms. Nevertheless, those might be explained by which microorganisms, including *L. plantarum*, produce various PUFAs and catalyst them into unique molecular species beyond common PUFAs, which are difficult to gain from plants or animals, and their functions have been attracting much attention for improving health and for developing new chemical materials. It has been recently revealed that the metabolic pathway of gut bacterium *L. plantarum* generates hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially fatty acids as intermediates [23-27]. These fatty acid intermediates are predicted to exert physiological functions. For example, hydroxy- and oxo-fatty acids act as ligands for PPAR γ [21]; oxo-fatty acids discovered in tomato juice are potent PPAR α activators and decrease the amount of TG in obese diabetic mice [22]. The dysfunction of hepatic lipid metabolism has been of concern as a therapeutic target of cardiovascular disease (CVD). Sterol regulatory element (SRE)-binding proteins (SREBPs) are transcription factors integral to the maintenance of lipid homeostasis. This study focuses on SREBP-1c pathway, because it is the major isoform expressed in the liver and in tissues involved in energy homeostasis [34] (Fig. 2-1). Hence, it is interesting to investigate the effects of each oxygenated fatty acid on SREBP-1c pathway.

Acetyl-CoA carboxylases 1 and 2 (ACC1 and ACC2), the target genes of SREBP-1c, catalyze the carboxylation of acetyl-CoA to malonyl-CoA, the substrate for the fatty acid synthesis and the regulator of fatty acid oxidation [71-74]. They are potential targets for treatment of human diseases of obesity, diabetes, cancer, and including CVD. In the cytosol, acetyl-CoA is carboxylated to malonyl-CoA by ACC1 and utilized through fatty acid synthase (FAS) reactions to generate palmitate, which is utilized in the synthesis of TG and very low-density lipoprotein (VLDL). On the other hand, the malonyl-CoA, generated by ACC2 at the mitochondrial membrane, functions as an inhibitor of the activity of carnitine/palmitoyl-transferase 1 (CPT1) that results in an inhibition of the transfer of the fatty acyl group through the carnitine/palmitoyl shuttle system to inside the mitochondria for β -oxidation [4]. Moreover, Castle et al. suggested that ACC2, in addition to ACC1, may play a role in lipogenesis, since it is also capable of contributing of fatty acid synthesis in human adipose tissue [75]. The aforementioned mechanism is supported by Harada et al. study illustrating that liver-specific ACC1-deficient mice still showed hepatic de novo lipogenesis [76] in parallel with the observation of Oh et al. [77] that ACC2^{-/-} mice showed not only increased fatty-acid oxidation but also decreased fat levels in adipose tissue. Hence, a reduction of ACC2 leads to an increase of the β -oxidation of fatty acids and a decrease of lipid accumulation simultaneously, meanwhile ACC1 involves only in lipogenesis.

The aim of this study was to clarify the effects of new oxygenated fatty acids, including oxo- and hydroxy-fatty acids, produced by *L. plantarum* on TG synthesis comparing with EPA and LA both *in vitro* and *in vivo*. The structure-related functions of oxygenated fatty acids on LXRA and SREBP-1c pathway, which plays an important role in lipid metabolism in HepG2 liver cells, were also attempted to elucidate (Fig. 2-1). Interestingly, among hydroxy- and oxo-octadecenoic and octadecadienoic acids (ODAs and ODDAs) that were screened in this experiment, 10-hydroxy-12*Z*-18:1 (HYA), 6*Z*-10-

hydroxy-12Z-18:2 (γ HYA), 10-keto-12Z-18:1 (KetoA), and 6Z-10-keto-12Z-18:2 (γ KetoA) showed an outstanding reduction of TG accumulation in HepG2 cells. Furthermore, these findings also describe the importance of chemical structure in the fatty acids' functions in lipid metabolism.

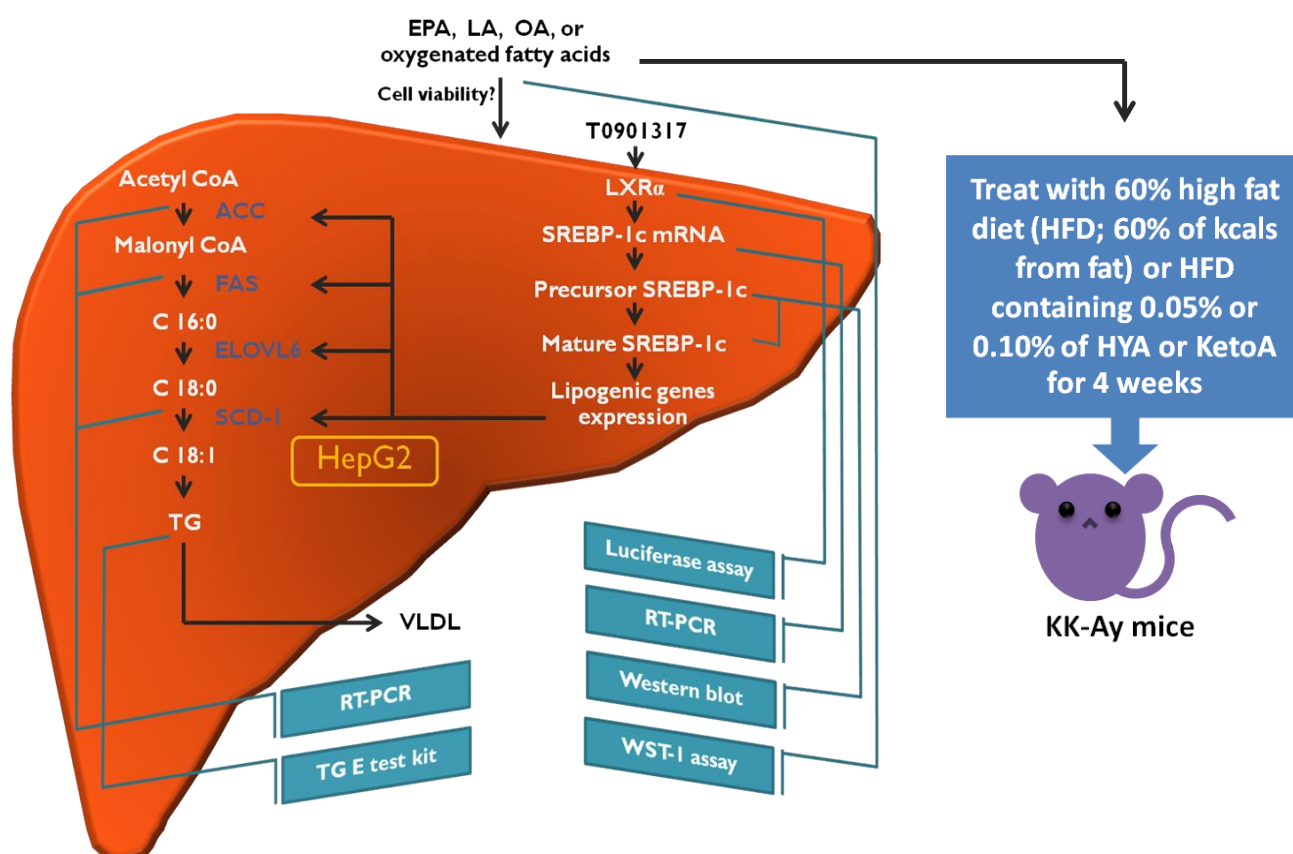


Figure 2-1. The LXRα and SREBP-1c pathway in liogenesis in the liver cells including the experimental plan both *in vitro* and *in vivo* to elucidate the mechanism of each fatty acid step by step.

MATERIALS AND METHODS

I. Chemicals and reagents

Various species of hydroxy- and oxo-ODAs and ODDAs, which are the intermediates of the metabolic pathway of *L. plantarum*, were extracted from the incubation of substrates, including oleic acid (OA), linoleic acid (LA), α linoleic acid (α LA), γ linoleic acid (γ LA), 12-hydroxy-18:0, and ricinoleic acid (RA), with the enzymes, named CLA-HY, CLA-DH, CLA-DC and CLA-ER, that exist in both the membrane and soluble fractions of *L. plantarum* as detailed in previous studies [23-27]. The chemical structures of these oxygenated fatty acids are demonstrated on Figure 2-2. Eicosapentaenoic acid, linoleic acid, oleic acid and DL- α -tocopherol (vitamin E) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). T0901317 was obtained from Cayman Chemicals (Ann Arbor, MI, USA).

II. Cell culture

HepG2 cells (JCRB 1054; Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM medium (Invitrogen, Carlsbad, CA) containing 10 % fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics (100 unit/ml penicillin and 100 μ g/ml streptomycin; Gibco Life Technologies Corp, Grand Island, NY, USA) at 37°C in a humidified atmosphere in the presence of 5% CO₂.

III. Cell viability analysis

Cell viability was assessed by the WST-1 method. HepG2 cells were plated in 96-well culture plates at a density of 1.0×10^4 cells/well in 100 μ l DMEM containing 10% fetal bovine serum and antibiotics as detailed above, and were incubated at 37 °C for 24 h. Each fatty acid was then added to HepG2 cells with T0901317 (10 nM) in serum-free

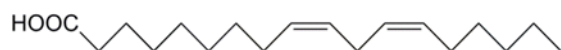
medium containing 0.1% BSA. The final ethanol concentration was 0.4%. After incubation for 24 h at 37 °C, 10 µl WST-1 solution (Dojindo Laboratories, Co., Kumamoto, Japan) was added to each well to evaluate cell viability. After incubation for 100 min at 37 °C, cell viability was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA) at a wavelength of 450 nm.

IV. Determination of mRNA expression levels by real-time RT-PCR

HepG2 cells were seeded in 12-well plates at 2.0×10^5 cells/ml in DMEM supplemented with 10% fetal bovine serum and antibiotics. After 24 h of incubation, each fatty acid was added to HepG2 cells with T0901317 (10 nM) in serum-free medium containing 0.1% BSA (Sigma-Aldrich, Co., St. Louis, MO, USA). The final ethanol concentration was 0.2-0.4%. After 24 h of incubation, total RNA was extracted from the cells using Sepasol reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. RNAs were treated with RNase-free DNase (Promega, Madison, WI) to remove contaminating genomic DNA. After inactivating DNase by adding DNase stop solution (Promega, Madison, WI, USA) and heating at 65°C for 10 min, each RNA was transcribed to cDNA using SuperScript RNase II reverse transcriptase (Invitrogen, Carlsbad, CA) with random hexamers at 25°C for 10 min and then at 42°C for 50 min. The reactions were stopped by incubation at 70°C for 15 min.

Regarding an animal experiment, total RNA was prepared from livers using Sepasol reagent (Nacalai Tesque, Kyoto, Japan) in accordance with the manufacturer's protocol. Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions using a thermal cycler (Takara PCR Thermal Cycler SP; Takara, Shiga, Japan)

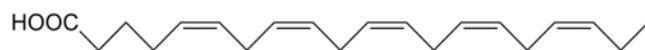
Linoleic acid (LA; 18:2)



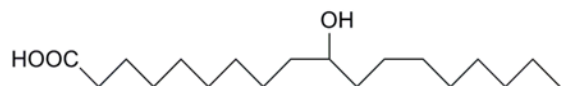
Oleic acid (OA, 18:1)



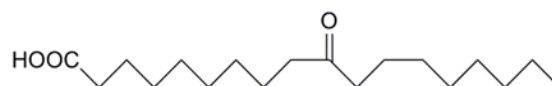
Eicosapentaenoic acid (EPA; 20:5)



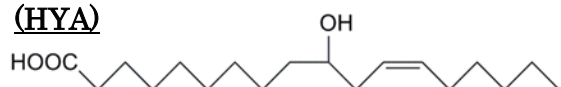
10-hydroxy-18:0 (HYB)



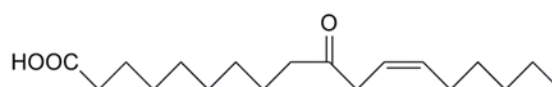
10-keto-18:0 (KetoB)



10-hydroxy-12Z-18:1 (HYA)



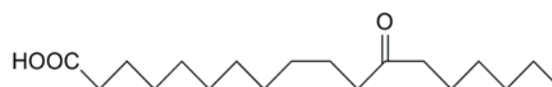
10-keto-12Z-18:1 (KetoA)



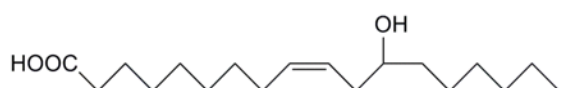
12-hydroxy-18:0



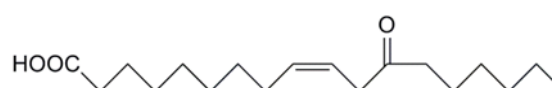
12-keto-18:0



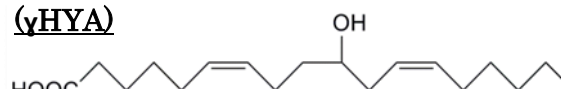
9Z-12-hydroxy-18:1 (RA)



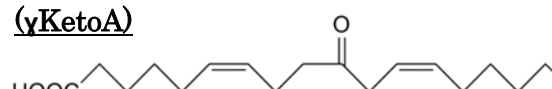
9Z-12-keto-18:1 (KetoRA)



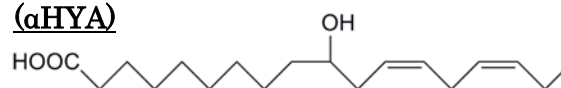
6Z-10-hydroxy-12Z-18:2 (γHYA)



6Z-10-keto-12Z-18:2 (γKetoA)



10-hydroxy-12Z,15Z-18:2 (αHYA)



10-keto-12Z,15Z-18:2 (αKetoA)

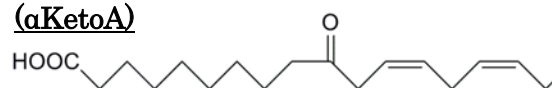


Figure 2-2. The chemical structures of EPA, OA, LA, and oxygenated fatty acid intermediates from metabolic pathway of *L. plantarum*.

To quantify the mRNA expression levels, real-time quantitative RT-PCR was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primers used for the quantification of each human and mouse gene are listed in Table 2-1 and 2-2, respectively. Primer pairs were selected to yield gene-specific single amplicons based on analyses by melting curves and by agarose gel electrophoresis. The thermal cycling conditions were as follows: 15 min at 95°C for one cycle, followed by amplification of the cDNA for 43 cycles with melting for 15 s at 95°C and with annealing and extension for 30 s at 60°C. Values were normalized against 18s or GADPH rRNA as an endogenous internal standard in human or mouse sample, respectively.

Table 2-1. Real-time RT-PCR primers used for the quantification of human mRNAs

Gene name	Reference or Accession Number	Forward (from 5' to 3')	Reverse (from 5' to 3')
SREBP-1c	[44]	GGAGGGGTAGGGCCAACGGCCT	CATGTCTTCGAAAGTGCAATCC
SCD-1	NM_005063	TGGTTTCACTTGGAGCTGTG	GGCCTTGGAGACTTTCTTCC
FAS	[44]	ACAGGGACAACCTGGAGTTCT	CTGTGGTCCCACCTTGATGAGT
ACC1	NM_198834	ATCCCGTACCTTCTTCTACTG	CCCAAACATAAGCCTTCACTG
ACC2	NM_001093	CTCTGACCATGTTTCGTTCTC	ATCTTCATCACCTCCATCTC
18s	[44]	TAAGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC

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

Gene name	Reference or Accession Number	Forward (from 5' to 3')	Reverse (from 5' to 3')
SREBP-1c	NM_011480	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGCCCAG
ACC1	[75]	AAACTGCAGGTATCCCAACTCTTC	CTGTGGAACATTTAAGATACG TTTCGAAAA
ACC2	[75]	GACGCCCGAGGATCTGAAG	GGGACAGGGACGTACTGATC
GADPH	NM_008084.2	CGTCCCGTAGACAAAATGGT	TGCCGTGAGTGGAGTCATAC

V. Cell fractionation and immunoblotting

HepG2 cells were plated in 6-well plates at 5.0×10^5 cells/ml for 24 h in DMEM supplemented with 10% fetal bovine serum and antibiotics as detailed above. The cells were then treated with EPA, HYA, γ HYA, KetoA, γ KetoA and/or T0901317 (10 nM) in serum-free medium containing 0.1% BSA. After incubation for 24 h, membrane fractions and nuclear extracts from cells were prepared by the method of Hannah et al. [46]. Briefly, cells were harvested by scraping and the cell suspensions were centrifuged at $1,000 \times g$ for 5min at 4°C. The cell pellets were resuspended in buffer A (250 mM sucrose, 10 mM Hepes-KOH at pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA) containing protease inhibitors (Complete Mini Protease Inhibitor tablet, Roche, Mannheim, Germany). The cell suspensions were passed through a 23-gauge needle 20 times and were centrifuged at $1,000 \times g$ for 5 min at 4°C. The $1,000 \times g$ pellets were resuspended in 40 ul Buffer B (20 mM Hepes-KOH at pH 7.6, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA and protease inhibitors). The suspensions were rotated at 4°C for 1 h and were then centrifuged at $10^5 \times g$ for 15 min at 4°C. The resulting supernatant is designated as the

nuclear extract fraction. The supernatant of the original $1,000 \times g$ spin was centrifuged at $10^4 \times g$ for 15 min at 4°C after which the pellet was dissolved in 25 μl SDS lysis buffer (10 mM Tris-HCl at pH 6.8, 100 mM NaCl, 1% (w/v) SDS, 1 mM sodium EDTA, 1 mM sodium EGTA, and protease inhibitors) and was designated as the membrane fraction.

Regarding *in vivo* study, the mice livers were fractionated by using nuclear/cytosol fractionation kit (BioVision, Mountain View, CA, USA) following protocols provided by the manufacturer.

The concentration of soluble proteins in the supernatant was quantified using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For immunoblot analysis, given amounts of membrane fractions (HepG2 cells, 25 μg ; mice livers, 40 μg) and nuclear extracts (HepG2 cells, 20 μg ; mice livers, 10 μg) were separated by 7% and 10% SDS-PAGE, respectively. Protein bands were transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). The filters were probed with a rabbit polyclonal anti-SREBP-1 antibody (H-160, 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound antibodies were visualized with alkaline phosphatase-conjugated anti-rabbit IgG (1:500 and 1:1,000 dilutions for membrane fractions and nuclear extracts consecutively; Cell Signaling Technology, Danvers, MA). The bands were visualized with the substrate, chemi-lumione L (Nacalai Tesque, Kyoto, Japan) using a FUJIFILM visualizer (LAS-3000, Fujifilm Corporation, Tokyo, Japan).

VI. Luciferase reporter assay

HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics as detailed above at 37°C . The luciferase ligand assay was performed using the dual luciferase system (Promega, Madison, WI), as previously described [81]. For LXR α activity assay, p3xIR1-tk-Luc, pCMX-hLXR α and pRL-CMV were transfected

into HepG2 cells. Briefly, transfections into HepG2 cultured cells in 10 cm dishes were performed using LipofectAMINE (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. After transfection for 4 h, the transfected cells were seeded into 96-well plates in the medium containing EPA, LA, HYA, γ HYA, KetoA, γ KetoA, and/or T0901317 (500 nM). After 24 h of incubation, luciferase activity was measured.

VII. Lipid extraction and quantification of TG

HepG2 cells were plated on 6-well plates at 5.0×10^5 cells/ml for 24 h in DMEM supplemented with 10% fetal bovine serum and antibiotics as mentioned above. The cells were then treated with EPA, LA, HYA, γ HYA, KetoA, γ KetoA, and/or T0901317 (10 nM) in the presence of vitamin E (10 μ M) in serum-supplemented medium. Fatty acids, vitamin E and T0901317 were dissolved in ethanol (final ethanol concentration of 0.3%). After incubation for 48 h, lipids were extracted from cells with chloroform-methanol (2:1, v/v). Reference control cells were extracted for cellular lipids before incubation (zero time control). Collected supernatants were evaporated gently under a N₂ stream, and TG was quantified using a TG E-test kit (Wako Pure Chemical Industries, Osaka, Japan).

VIII. Animal study

All mice were maintained in a temperature-controlled (23°C) facility with a constant 12 hour light/dark cycle and were given water *ad libitum*. Five-week-old male KK-Ay (CLEA Japan, Tokyo, Japan) mice were maintained for 4 weeks either on a 60% HFD (D12492 research diet, MO USA) or on the HFD containing 0.05% or 0.1% Keto A or HYA. The energy intake of all mice was adjusted by pair-feeding. Four weeks after feeding, these mice were fasted 5 h before sacrificed under isoflurane anesthesia and liver tissues for RNA isolation and protein extraction were immediately excised. The

animal care procedures and methods were performed according to the guidelines of Kyoto University for the use and care of laboratory animals.

IX. Statistical analysis

Data are reported as means $\pm$ SD. *In vitro* study statistical analyses were carried out by one-way ANOVA with Scheffe's F-test to identify significant difference using Stat View software (SAS Institute, Cary, NC, USA). On the other hand, *in vivo* study statistical analyses were performed using one-way ANOVA with Dunnett's F-test to identify significant difference between control and the others using the similar software.

RESULTS

I. Effects of each fatty acid on HepG2 cell viability

L. plantarum fermented unsaturated fatty acids like LA and produced diverse species of oxygenated fatty acids which are different in the locations of double bond, hydroxy group and ketone group (Fig. 2-2). To determine the effects of each compound on cell viability, various concentrations of fatty acids and T0901317 were added to HepG2 liver cells in culture. Figure 2-3 shows the result of the viability assay after 24 h of treatment. There are no significant cytotoxic effects by each fatty acid compared with control cells, excluding 60 μ M EPA, HYB and 12-hydroxy-18:0. Corresponding to this experiment, EPA, which is famous in its hypolipidemic effect, is well-known for its high cytotoxic effect [47].

II. HYA, γ HYA, KetoA and γ KetoA effectively reduce SREBP-1c mRNA expression

To investigate whether these oxygenated fatty acids diminish the expression of SREBP-1c compared to LA and EPA, HepG2 hepatic cells were treated with T0901317, synthesis LXR α agonist, resulting in SREBP-1c stimulation. The T0901317-induced cells were simultaneously treated with each fatty acid (Fig. 2-4A). After 24 h of 10 nM T0901317 induction, SREBP-1c mRNA expression in HepG2 cells was increased by more than 6-fold compared with vehicle control cells. Treatment with EPA significantly ameliorated the T0901317-induced effect by reducing the expression of SREBP-1c mRNA to 37% of T0901317-induced hepatic cells, while treatment with LA did not. Interestingly, most of oxygenated fatty acids fermented by *L. plantarum*, except 10-keto-18:0 (KetoB) and 9*Z*-12-keto-18:1 (KetoRA), significantly reduced T0901317-induced SREBP-1c mRNA expression. In addition, SREBP-1c mRNA levels of HYA, γ HYA, KetoA and γ KetoA treated groups were significantly different from LA. It was

noteworthy that all these four derivatives contain the same position of hydroxy or ketone group, which is located on 10th carbon from carboxyl end, and a double bond located on 12th carbon as well.

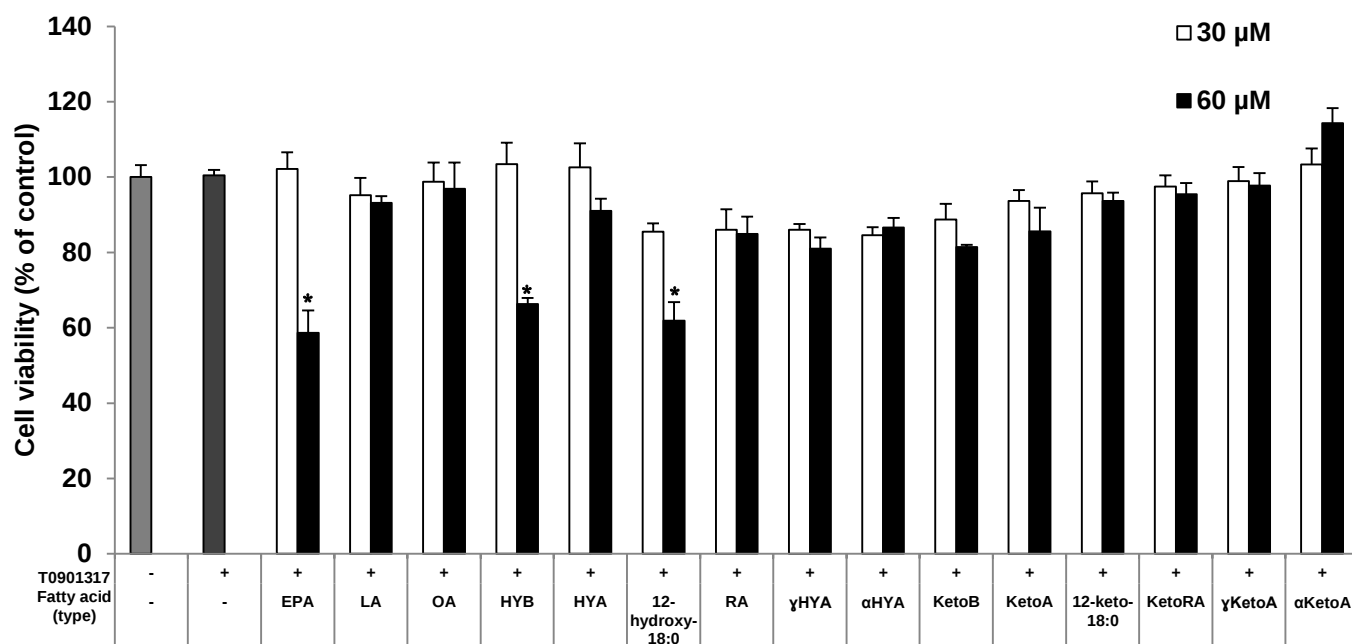


Figure 2-3. Effects of EPA, LA, OA, or oxygenated fatty acids fermented by *L. plantarum* on cell viability as determined by the water soluble tetrazolium (WST)-1 assay. Each fatty acid (30 or 60 μM) was added to HepG2 cells with T0901317 (10 nM) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.4%. After 24 h incubation, cells were washed with PBS and the WST-1 solution was added. The absorbance at 450 nm corresponds to cell viability. Expression levels are presented as relative percentage to vehicle control (ethanol). Data are reported as means ± SD (n = 4/group). A significant difference from the vehicle control group was shown as follows: * p < 0.05.

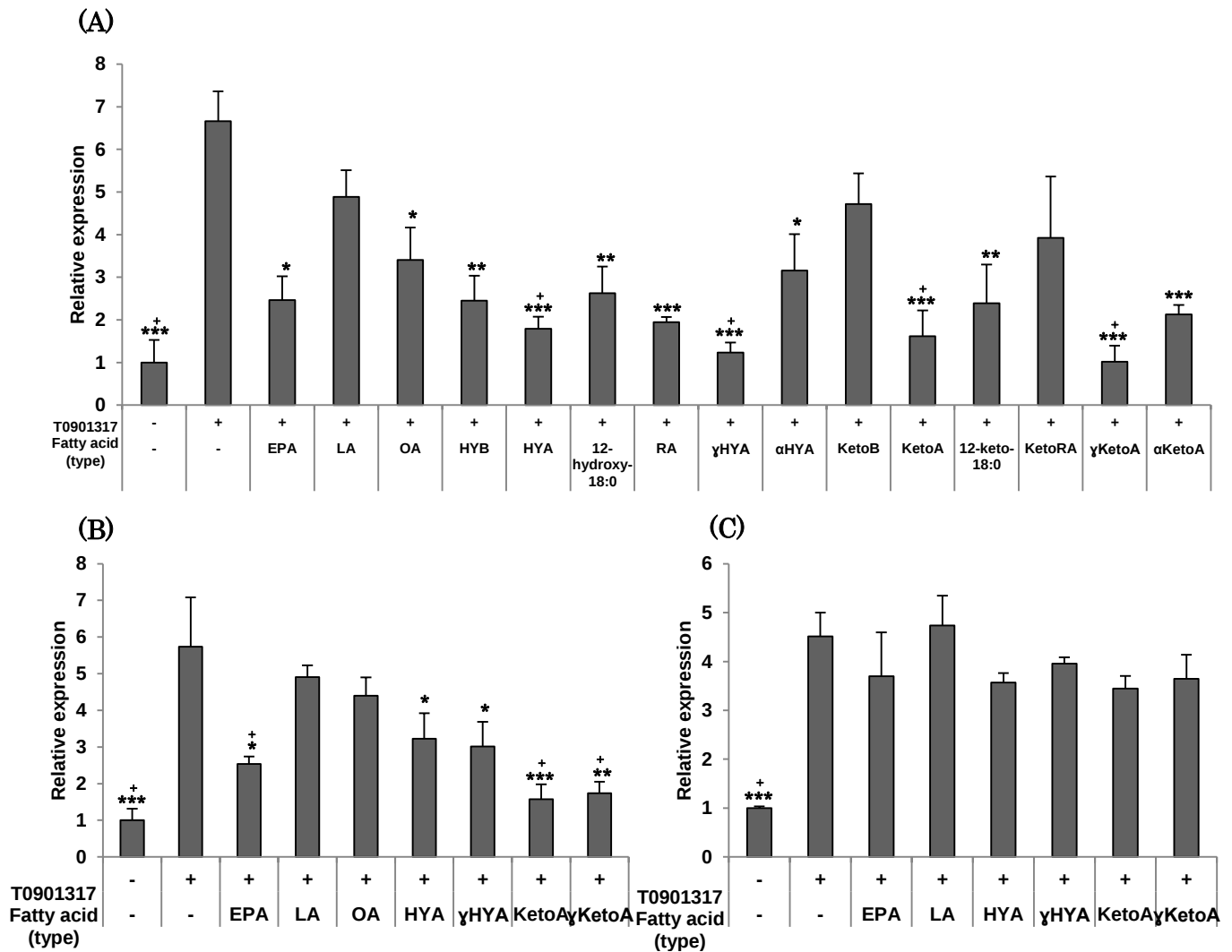


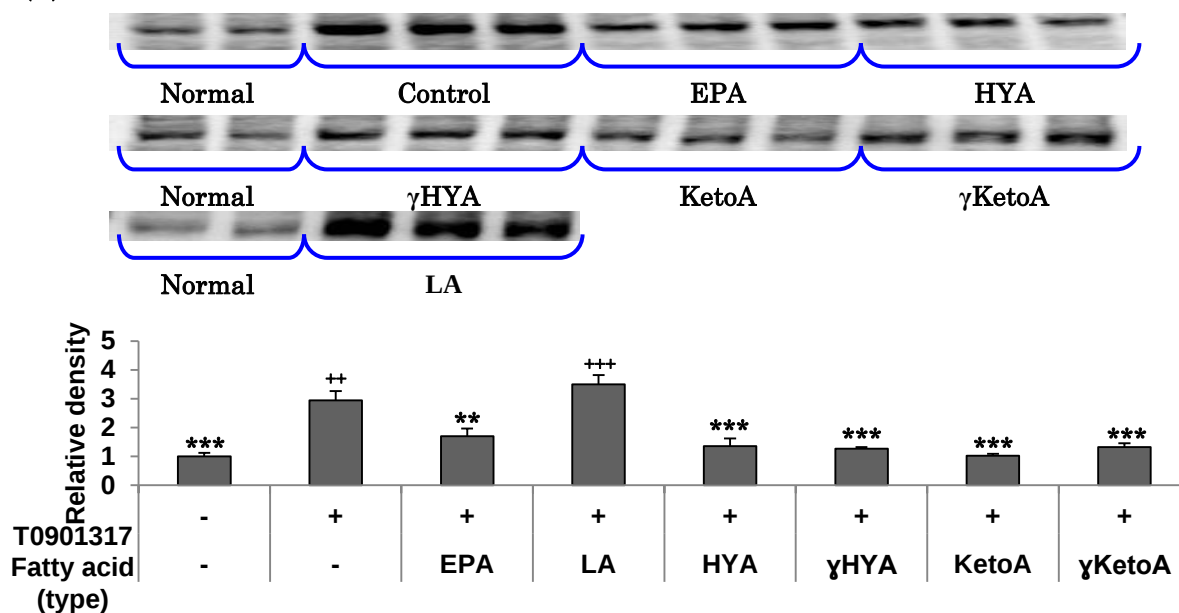
Figure 2-4. Effects of EPA, LA, or oxygenated fatty acids fermented by *L. plantarum* on the expression of sterol-regulatory element binding protein-1c (SREBP-1c, A and B) and -1a (SREBP-1a, C). Each fatty acid (60 μ M, A or 30 μ M, B) was added to HepG2 cells with T0901317 (10 nM) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.4%. After 24 h incubation, cells were harvested using sepsol reagent. SREBP-1a and -1c mRNA levels were quantified by real-time quantitative PCR. Each value of SREBP-1a and -1c mRNAs was adjusted by that of 18s rRNA (internal control). Expression levels are presented as -fold induction relative to the vehicle control (ethanol). Data are reported as means $\pm$ SD (n = 3/group). A significant difference from T0901317-induced control group was shown as follows: *p < 0.05; **p < 0.001; ***p < 0.0001. A significant difference from LA treated group was performed as +p < 0.05.

To further elucidate the dose-dependent manner of these fatty acids, the interesting oxygenated fatty acids, including HYA, γ HYA, KetoA and γ KetoA, were applied to HepG2 cells in a half concentration with 10 nM T0901317 (Fig. 2-4B). EPA, HYA, γ HYA, KetoA and γ KetoA significantly down-regulated SREBP-1c mRNA expression that aroused by T0901317 in a dose-dependent manner. Although EPA, HYA, γ HYA, KetoA and γ KetoA at 60 μ M concentration showed almost the same level of the suppressive effect (Fig. 2-4A), at lower concentration (30 μ M) KetoA and γ KetoA treatment tended to decrease SREBP-1c mRNA expression more potently than did HYA and γ HYA. On the other hand, these fatty acids did not significantly affect the expression of SREBP-1a (Fig. 2-4C).

III. HYA, γ HYA, KetoA and γ KetoA effectively decrease SREBP-1c maturation

To investigate the effect of these oxygenated fatty acids on SREBP-1 protein levels, the full-length precursor form in cell membranes (125 kDa) and the cleaved mature form (68 kDa) in nuclear extracts were estimated by immunoblotting. Because the antibody used cannot distinguish between the SREBP-1c and -1a isoforms, the general term SREBP-1 was used to refer to the results. T0901317 increased the levels of both the precursor and the mature forms of SREBP-1 (Fig. 2-5A, B). HYA, γ HYA, KetoA and γ KetoA as well as EPA significantly decreased the T0901317 induction of both precursor and mature forms of SREBP-1. It is coincident with the result of SREBP-1c mRNA expression, LA had no effects on both forms of SREBP-1. Interestingly, the mature form of SREBP-1 was down-regulated by HYA, γ HYA, KetoA, and γ KetoA more significantly than EPA.

(A) Precursor SREBP-1



(B) Mature SREBP-1

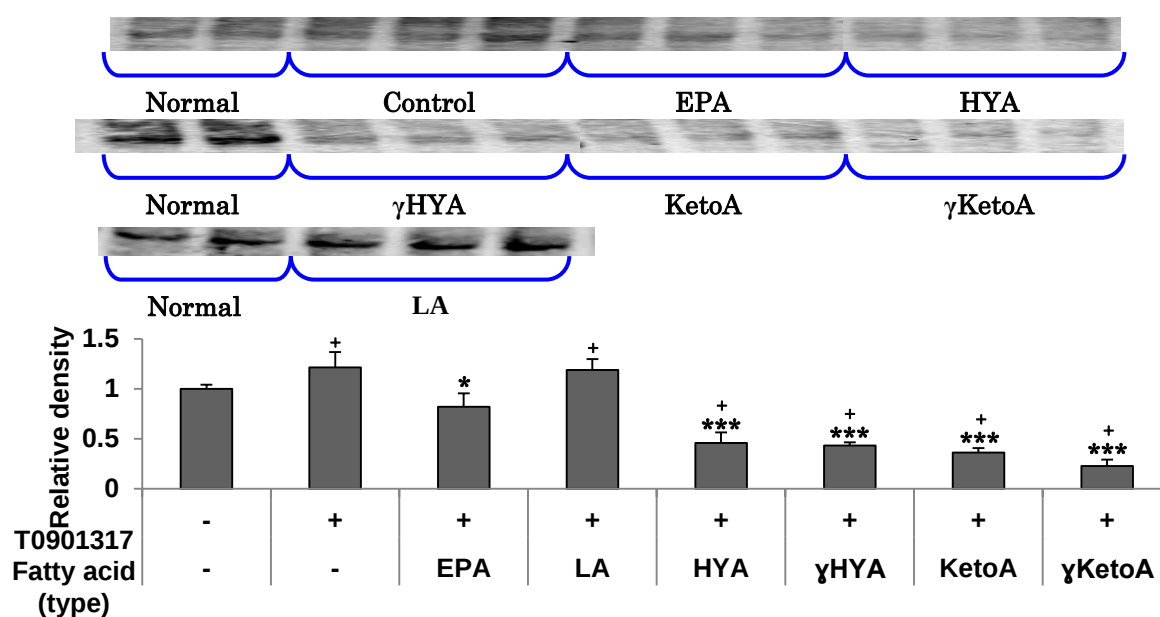


Figure 2-5. Effects of EPA, LA, HYA, γHYA, KetoA or γKetoA on the expressions of precursor (A) and mature (B) forms of sterol-regulatory element binding protein-1 (SREBP-1) protein. Each fatty acid was added to HepG2 cells with T0901317 (10 nM) in serum-free medium containing 0.1% BSA. The data represent the mean fold change of the precursor and the mature forms of SREBP-1 from the vehicle control (ethanol). Data are reported as means ± SD (n = 3-4/group). A significant difference from T0901317 induced control group was shown as follows: *p < 0.05; **p < 0.001; ***p < 0.0001. A significant difference from EPA treated group was performed as follows: +p < 0.05; ++p < 0.001; +++p < 0.0001.

IV. Regulation of LXR α in the SREBP-1c promoter by HYA, γ HYA, KetoA, and γ KetoA

Then the effects of these fatty acids on luciferase activity of LXR α ligands were determined to clarify whether these fatty acids suppress SREBP-1c via LXR α . In the presence of synthetic LXR agonist, the activity LXR α reporter in HepG2 cells was increased approximately 4-fold compared with the vehicle control. In luciferase reporter assay, the simultaneous addition of EPA, HYA, γ HYA, KetoA and γ KetoA at 30 μ M or 60 μ M concentrations dose-dependently decreased the LXR α reporter activity compared to T0901317-induced cells (Fig. 2-6). This result indicated that the suppressive effect of SREBP-1c by EPA, HYA, γ HYA, KetoA, and γ KetoA was regulated through the antagonism of LXR α .

V. Down-regulation of lipogenic genes by HYA, γ HYA, KetoA, and γ KetoA

To further elucidate the effects of HYA, γ HYA, KetoA, and γ KetoA on lipid metabolism, the expression of SREBP-1c target genes were examined. Treatment with all fatty acids, including EPA and LA, significantly decreased the expression of SCD-1 and FAS mRNAs (Fig. 2-7A, B). Moreover, EPA, HYA, γ HYA, KetoA and γ KetoA, but not LA, treatment significantly down-regulated the T0901317-induced expression of ACC1 and ACC2. Unexpectedly, HYA and KetoA significantly reduced ACC2 mRNA levels more than EPA (Fig. 2-7C, D).

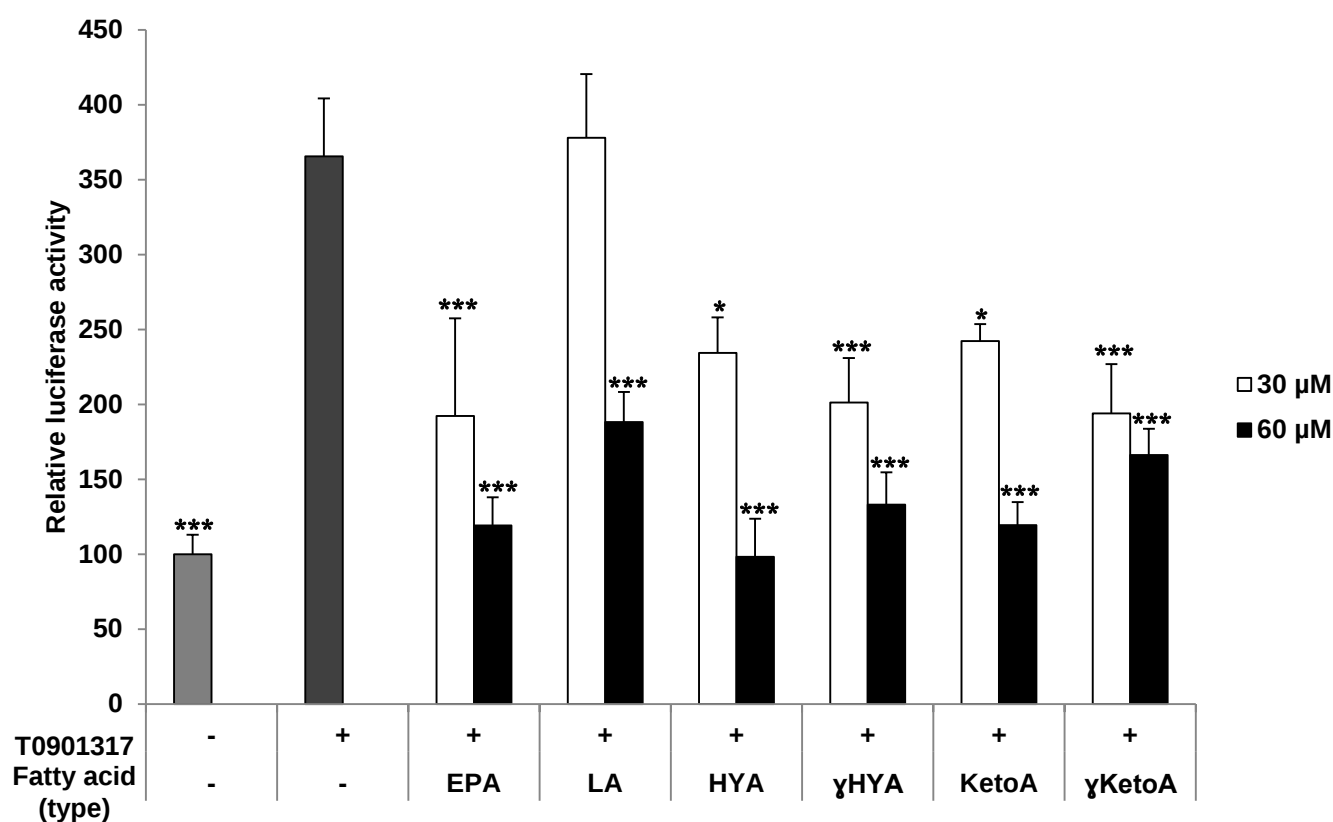


Figure 2-6. Suppression of luciferase activity of LXR α ligands by EPA, LA, HYA, γ HYA, KetoA or γ KetoA. Ethanol (vehicle control) or T0901317 (500 nM) was added to transfected HepG2 cells in serum-supplemented medium. The indicated concentration of each fatty acid was added to transfected cells with T0901317 (500 nM) in serum-supplemented medium as well. The final ethanol concentration was 0.2%. The relative luciferase activities compared with the control are shown. Data are reported as means $\pm$ SD (n = 3-5/group). A significant difference from T0901317-induced control group was shown as follows: *p < 0.05; **p < 0.001; ***p < 0.0001.

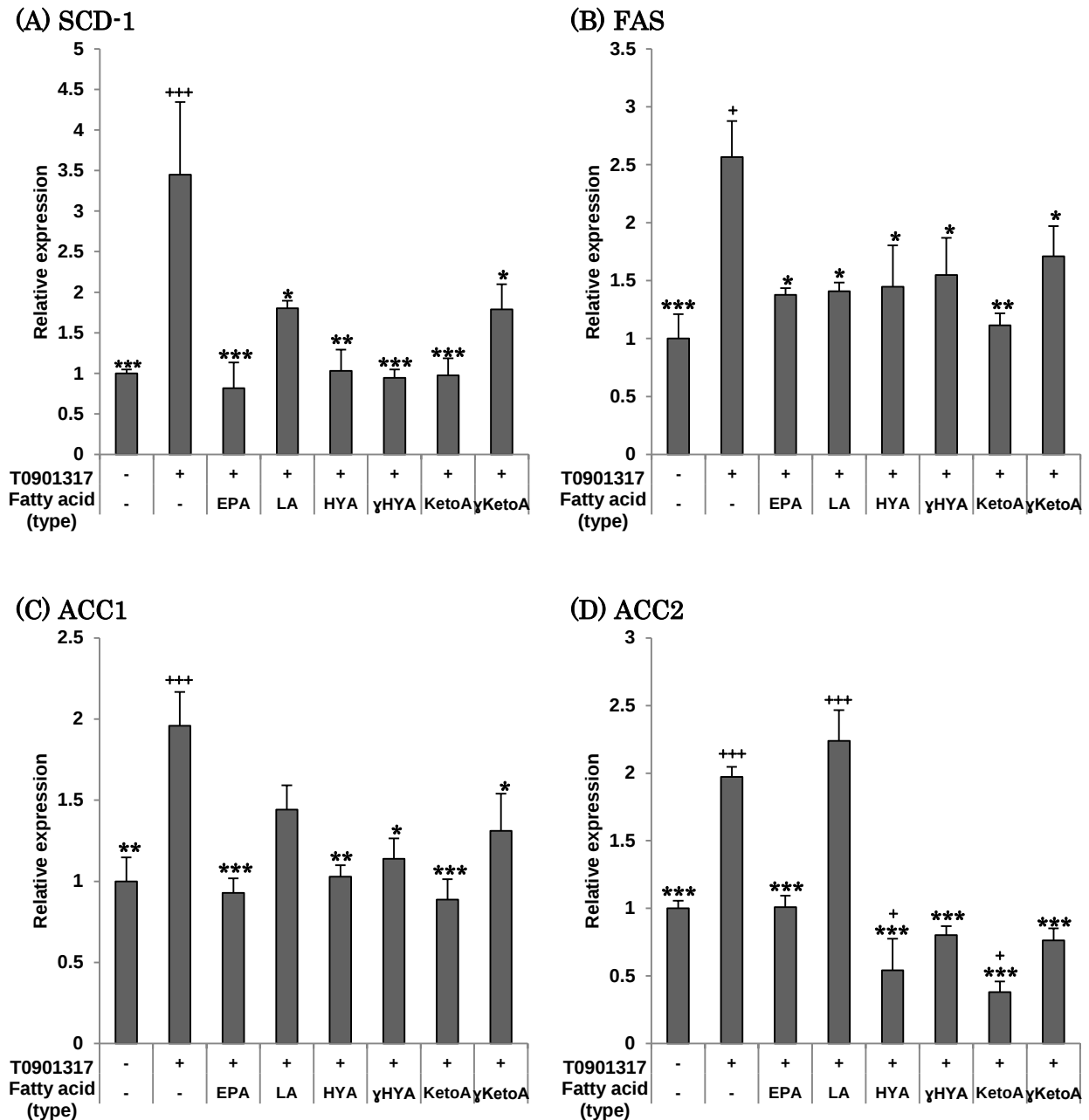


Figure 2-7. Effects of EPA, LA, HYA, γ HYA, KetoA or γ KetoA on the expression of stearoyl-coenzyme A desaturase-1 (SCD-1, A), fatty acid synthase (FAS, B), acetyl-CoA carboxylase-1 (ACC1, C), and acetyl-CoA carbokylase-2 (ACC2, D) mRNAs. Each fatty acid (60 μ M) was added to HepG2 cells with T0901317 (10 nM) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.2%. After 24 h incubation, cells were harvested using seposol reagent. SCD-1, FAS, and ACC mRNA levels were quantified by real-time quantitative polymerase chain reaction. Each Value of SCD-1,

FAS, and ACC mRNAs was adjusted by that of 18s rRNA (internal control). Expression levels are presented as fold induction relative to the vehicle control (ethanol). Data are reported as means $\pm$ SD (n = 3/group). A significant difference from T0901317 induced control group was shown as follows: *p < 0.05; **p < 0.001; ***p < 0.0001. A significant difference from EPA treated group was performed as follows: +p < 0.05; ++p < 0.001; +++p < 0.0001.

VI. HYA, γ HYA, KetoA, and γ KetoA prevent the development of cellular TG synthesis

From the results mentioned above, it is interesting to check the effects of EPA, LA, HYA, γ HYA, KetoA and γ KetoA on TG synthesis in T0901317-induced HepG2 liver cells. First of all, the preliminary experiment was done to test the effects of T0901317 and oxygenated fatty acids at different incubation time on TG accumulation of HepG2 cells (Fig. 2-8). It was revealed that T0901317 induced cells accumulated TG in almost the same level with vehicle control cells and KetoA treated cells at 24 h incubation. At 48 h incubation synthetic LXR agonist induced TG levels to 3-folds compared with vehicle control cells. Importantly, 60 μ M KetoA treatment prevented the effect of T0901317 resulting in unchanging of TG levels compared with vehicle control cells at the similar incubation time. Figure 2-9 illustrated that after 48 h incubation T0901317 significantly augmented the cellular levels of TG (about 2,500 μ g/mg protein). Consistent with a previous study (46), EPA significantly reduced TG synthesis of T0901317-treated HepG2 cells to around 1,700 μ g/mg protein. Although the cellular TG content in the LA-treated group was nearly equivalent to that in the T0901317-induced cells, hepatic TG accumulations in *L. plantarum* fermented products treated cells were significantly lower than did the T0901317-activated cells. As anticipated, HYA, KetoA and γ KetoA showed

significantly stronger hypolipidemic effects than EPA. Remarkably, treatment with KetoA, which contains a ketone group on the 10th carbon instead of a hydroxy group in HYA (Fig. 2-2), significantly inhibited TG levels more than that with HYA. Besides, KetoA, which is composed of a double bond on the 12th carbon (Fig. 2-2), also significantly inhibited the TG synthesis more than γ KetoA, which contains two double bonds located on the 6th and the 12th carbons (Fig. 2-9). The trend of the result of TG accumulations is similar to the result of ACC2 expression. These findings indicated that position and number of hydroxy group, ketone group, and double bond might play a crucial role in the effect of unsaturated or oxygenated fatty acids on the inhibition effect of cellular TG synthesis in liver cells.

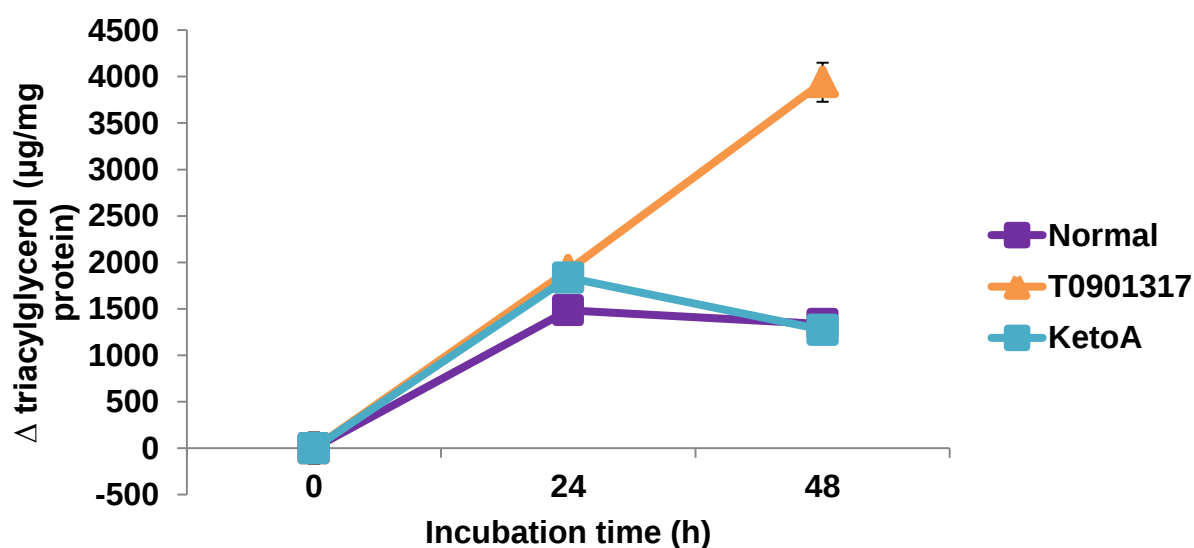


Figure 2-8. Effect of KetoA on TG synthesis in HepG2 cells at different incubation times. KetoA (60 μ M) was added to HepG2 cells with T0901317 (10 nM) in serum-free medium containing 0.1% BSA and incubated for 24 and 48 h. The final ethanol concentration was 0.3%. The increased TG levels per mg protein (TG levels after 24 and 48 h of incubation minus the levels of reference cells) are shown. Data are reported as means $\pm$ SD (n = 2/group).

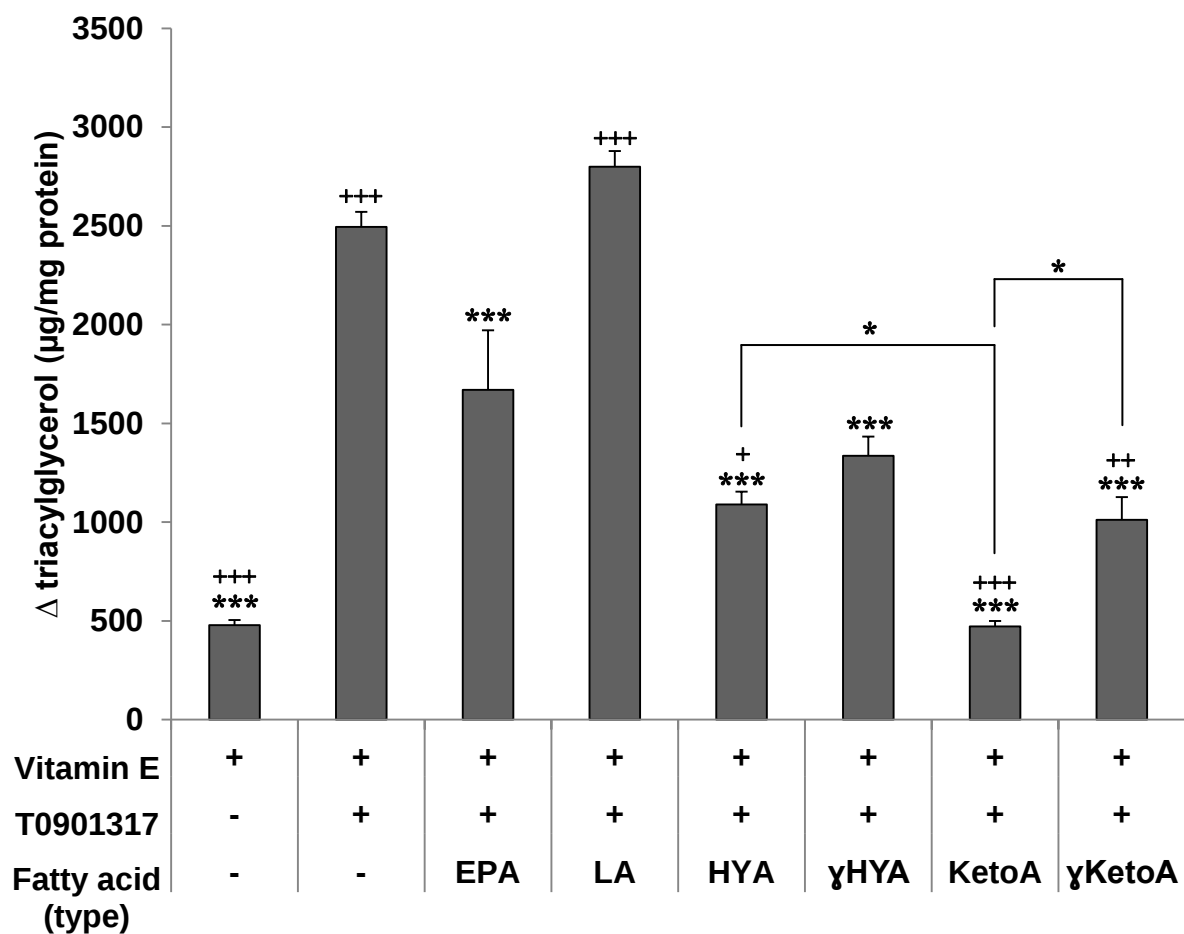


Figure 2-9. Effect of EPA, LA, HYA, γ HYA, KetoA or γ KetoA on TG synthesis in HepG2 cells. Each fatty acid (60 μ M) was added to HepG2 cells with T0901317 (10 nM) and vitamin E (10 μ M) in serum-free medium containing 0.1% BSA for 48 h. The final ethanol concentration was 0.3%. The increased TG levels per mg protein (TG levels after 48 h of incubation minus the levels of reference cells) are shown. Data are reported as means $\pm$ SD (n = 3/group). A significant difference from T0901317 induced control group was shown as follows: *p < 0.05; **p < 0.001; ***p < 0.0001. A significant difference from EPA treated group was performed as follows: +p < 0.05; ++p < 0.001; +++p < 0.0001.

VII. Effects of HYA and KetoA on SREBP-1c and ACCs mRNA expression and SREBP-1 maturation in animal models

In vitro experiments oxygenated fatty acids, especially HYA and KetoA, showed the strong ameliorating effects on TG reduction in liver cells, however *in vivo* study is necessary to elucidate and ensure their effects on lipid metabolism. Therefore, KK-Ay mice were fed with HFD containing HYA or KetoA at the concentrations of 0.05 or 0.10% and were sacrificed after 4 weeks treatment. The hepatic SREBP-1 protein expression and SREBP-1c and ACCs mRNA levels were measured by using western blotting and real time RT-PCR, respectively.

In accordance with *in vitro* results, the precursor forms of SREBP-1 were significantly down-regulated in mice treated with the diet containing high concentration (0.1%) of both HYA and KetoA compared with vehicle control allies. Consequently, the mature forms were significantly reduced in every group treated with the diet containing oxygenated fatty acids. This result indicates that these oxygenated fatty acids can ameliorate SREBP-1 protein expression in a diabetic subject which additionally stimulated by HFD (Fig. 2-10). HYA and KetoA at high concentration tended to be able to reduce SREBP-1c mRNA levels, however only the levels of 0.1% KetoA treated group significantly reached the difference with vehicle control group (Fig. 2-11A). Then the mRNA expression of ACC1 and ACC2, which are the target genes of SREBP-1c, was measured and found that the KK-Ay mice treated by 0.05% KetoA had no changes in both ACC1 and ACC2 mRNA levels from non-treated group (Fig. 2-11B, C). The mice fed by HFD containing 0.05% HYA also had no significant differences in ACC1 expression, yet ACC2 expression was statistically higher compared with vehicle control mice. However, at higher concentration, HYA and KetoA had the tendency to decrease both ACC1 and ACC2 expression, but only 0.1% HYA significantly reduced ACC1 mRNA levels, while just 0.1% KetoA statistically down-regulated ACC2 mRNA levels.

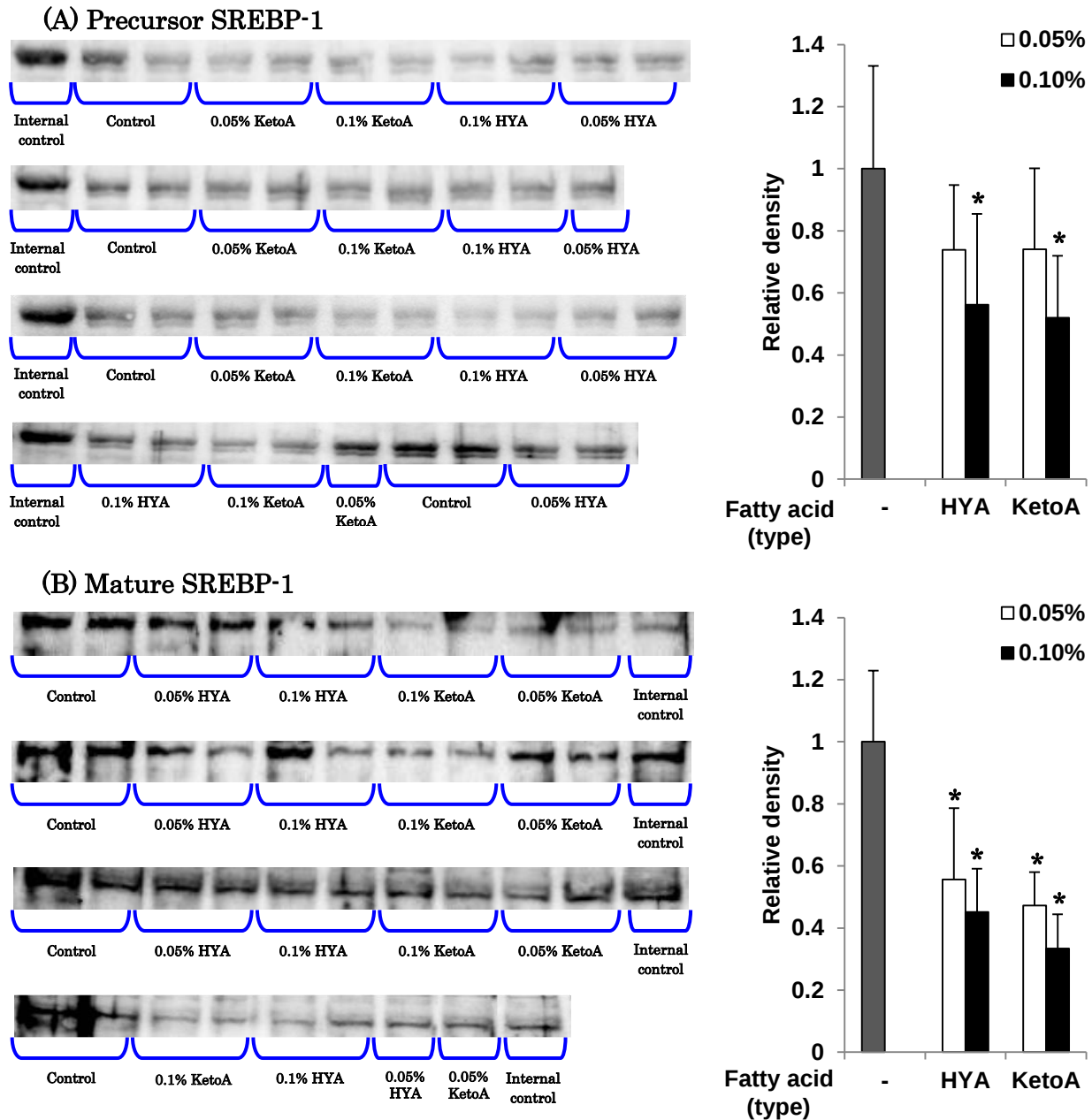


Figure 2-10. Effects of HYA or KetoA on precursor and mature SREBP-1 protein expression in the liver of KK-Ay mice. HYA and KetoA (0.05 and 0.10% concentrations) were mixed into the HFD and were fed to KK-Ay mice for 4 weeks. The livers of mice were collected and fractionated to measure the precursor and mature forms of SREBP-1 by western blotting. The data represent the mean -fold change of the precursor and the mature forms of SREBP-1 from vehicle control group. Data are reported as means $\pm$ SD (n = 7-8/group). A significant difference from vehicle control group was shown as follows: *p < 0.05.

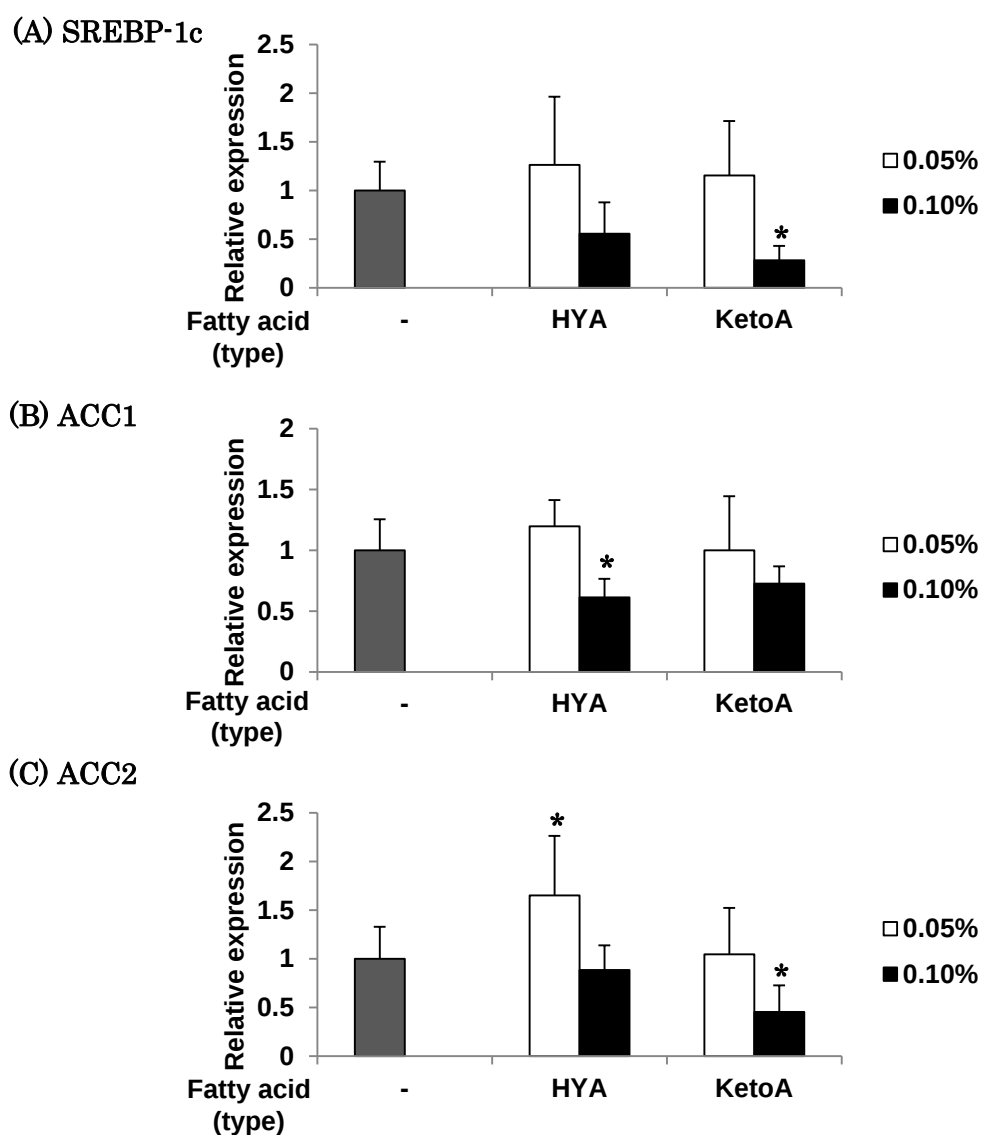


Figure 2-11. Effects of HYA or KetoA on the expression of sterol-regulatory element binding protein-1c (SREBP-1c; A), acetyl-CoA carboxylase-1 (ACC1, B) and acetyl-CoA carbokylase-2 (ACC2, C) mRNAs in the liver of KK-Ay mice. HYA and KetoA (0.05 and 0.10% concentrations) were mixed into the HFD and were fed to KK-Ay mice for 4 weeks. SREBP-1c, ACC1 and ACC2 mRNA levels in the livers were quantified by real-time RT-PCR. Each value of SREBP-1c, ACC1 and ACC2 mRNAs was adjusted by that of GADPH rRNA (internal control). Expression levels are presented as fold induction relative to the vehicle control group. Data are reported as means $\pm$ SD (n = 6-8/group). A significant difference from vehicle control group was shown as follows: *p < 0.05.

DISCUSSION

Nowadays, the prevalence of CVD has been increasing globally [64, 82, 83] and has caused around 30% of all deaths worldwide according to the World Health Organization (WHO). Many studies have investigated the connection between the malfunction of lipid metabolism and the risk of CVD [66, 67]. Hence, the amelioration of lipid metabolism has been more concerned as a therapeutic target of CVD prevention. The most popular fatty acids, which were utilized in the commercial supplements, are EPA and DHA owing to their eminent hypolipidemic effects [47, 83]. Thus, EPA was concerned as a positive control in this experiment. Recent studies illustrated that oxygenated fatty acids also showed many interesting benefits in our health. Notwithstanding, the processes and the effects of many species of oxygenated fatty acids were not clearly elucidated yet, especially new finding oxygenated fatty acids which are fermented by bacteria. In the present study, the aim is to investigate the effects of brand new oxygenated fatty acids, which were originated from the intermediates of the metabolic pathway of *L. plantarum*, on hypolipidemic effects in the hepatic cells, particularly via LXR α and SREBP-1c pathway in the liver, because this lipogenesis pathway is a potential target of atherosclerosis and other metabolic diseases treatments, which are the most common causes of CVD [84-86]. T0901317, a synthetic LXR agonist, were treated to the cells to imitate an immoderate lipid synthesis model by up-regulating the expressions of LXR α and SREBP-1c in the study using cultured cells. In addition, the KK-Ay mice which are well-known as diabetic models were fed by HFD with or without HYA or KetoA to study the effects of these oxygenated fatty acids on lipid metabolism *in vivo* as well.

In vitro study, the luciferase assay revealed that some oxo- and hydroxy-ODAs and ODDAs that produced from unsaturated fatty acids by *L. plantarum*, including HYA, γ HYA, KetoA and γ KetoA, dose-dependently decreased the activation of LXR α , as

similar to EPA. Consistently, HYA, γ HYA, KetoA and γ KetoA reduced SREBP-1c mRNA expression by competing with T0901317 in the activation of LXR α to almost the same levels as EPA. In parallel with previous study [50], EPA decreased both precursor and mature forms of T0901317-induced SREBP-1 protein. HYA, γ HYA, KetoA and γ KetoA repressed the precursor form of SREBP-1 to the same level with EPA, yet they significantly reduced the mature form of SREBP-1 more significantly than EPA. Corresponding to *in vitro* experiments, mice fed with HFD containing 0.1% KetoA also showed a significant reduce of SREBP-1c mRNA levels compared with their vehicle control allies. Furthermore, both HYA and KetoA at 0.1% concentration can abate the precursor and the mature forms of SREBP-1 protein. At 0.05% concentration neither HYA nor KetoA can decrease the precursor form of SREBP-1, but either of them at 0.05% concentration significantly down-regulated the mature form of SREBP-1. These results may be one of the important processes how these oxygenated fatty acids potentially reduce cellular TG synthesis induced by the LXR agonist more than EPA and LA.

Regarding the importance of SREBP-1 maturation, it was suggested that EPA improves hepatic steatosis independent of PPAR α activation through the inhibition of SREBP-1 maturation by decreasing SREBP cleavage-activating protein (SCAP) and site-1 protease (S1P). Moreover, SCAP itself interacts with insulin-induced gene (INSIG) protein which retains the complex SREBP-1c/SCAP in the endoplasmic reticulum. In the presence of an adequate signal (insulin or ER stress), SCAP dissociates from INSIG and the complex SCAP–SREBP-1c is transferred to the golgi apparatus in coat protein II (COPII) vesicles [87], thus the over-expression of INSIG was associated with a striking reduction in the elevated levels of nuclear SREBP-1 as mentioned in animal study [88]. Therefore, there are three possible hypotheses that might be able to explain the mechanism of HYA, γ HYA, KetoA and γ KetoA in potentially improving SREBP-1 maturation more than EPA. Firstly, SCAP and S1P are efficiently down-regulated.

Secondly, the dissociation of SCAP and INSIG were prevented by lessening sensitivity of the sterol sensing domain of SCA, or lowering binding of regulatory sterols directly [89, 90]. Thirdly, the production of INSIG in liver cells is promoted. This is an interesting mechanism which is needed to be elucidated to clarify the effect on SREBP-1c maturation of these new finding oxygenated fatty acids comparing with EPA (Fig. 2-12).

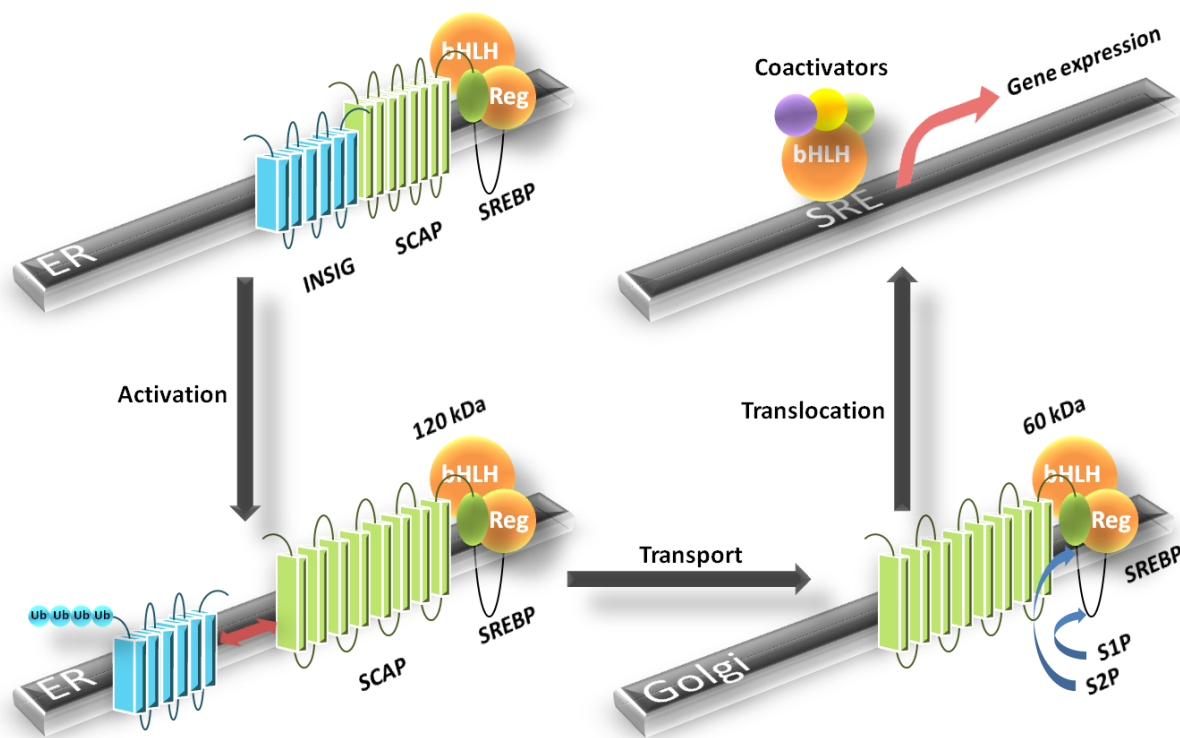


Figure 2-12. Maturation of SREBP-1 protein. In the inactive state, precursors of SREBP-1 in the endoplasmic reticulum (ER) are in a complex with the SREBP-cleavage activating protein (SCAP) and the insulin-induced gene (INSIG). Upon activation, SCAP undergoes a conformational change and is released from INSIG and SCAP assists the transport of the 120 kDa precursor SREBP to the Golgi apparatus, followed by a two-step proteolytic cleavage by S1P and S2P proteases, thus releasing a 60 kDa transcriptionally active domain, or mature form. The mature form is translocated to the nucleus and activates the expression of target lipogenic genes by binding to their sterol regulatory element.

HYA, γ HYA, KetoA and γ KetoA also ameliorated the mRNA expression of SREBP-1c target genes, involved in de novo fatty acid synthesis such as, FAS, SCD-1, and ACC1, at almost similar levels with EPA. Astonishingly, an increase of ACC2 mRNA expression of T0901317-induced cells was reduced by HYA and KetoA to the levels that are significantly lower than did EPA treated cells (Fig. 2-7D). In accordance with *in vitro* study, ACC1 and ACC2 mRNA levels were also significantly abated by HYA and KetoA *in vivo* study, respectively. This finding showed that ACC2 might be a crucial role of hypolipidemic effect of these oxygenated fatty acids. Figure 2-13 illustrated that ACC2 is a major player in energy homeostasis that turns acetyl-CoA to malonyl-CoA. The inhibition of CPT1 by malonyl-CoA is a considerable mechanism for the control of two opposing pathways: fatty acid synthesis and β -oxidation [4, 75]. ACC2 is located at the mitochondrial membrane and highly expressed in tissues with a high rate of fatty acid oxidation, such as adipose tissue, skeletal muscle and liver [75, 91]. Further support for this hypothesis was derived from studies of ACC2^{-/-} mutant mice. They continuously oxidized fatty acids, ate more food, and gained less weight than their wild type allies [92]. Hence, the suppressive effect on ACC2 is preferable for increasing fatty acid β -oxidation and decreasing lipid accumulation. However, ACC2 is not dominantly expressed only in the hepatic cells, but also in the adipose tissue and the skeletal muscle, thus the further studies in other cell lines and animal studies are necessary to clarify the effects of these oxo- and hydroxy-ODAs and ODDAs as well.

This study showed that HYA, KetoA and γ KetoA significantly suppressed T0901317-induced-TG accumulation in hepatic cells more than LA and EPA. The suppressive effect on lipid synthesis by HYA, KetoA, and γ KetoA might be due to the strong reduction of mRNA expression of SREBP-1c and ACC2 and the potent inhibition of the mature form of SREBP-1. Taken as whole, these findings proposed that HYA, γ HYA, KetoA and γ KetoA might be the new alternative choices to utilize in the therapy

of CVD or atherosclerosis or any other diseases that caused by SREBP-1c over-expression [83]. Notwithstanding, it is essential to examine the effects of dietary these oxygenated fatty acids on lipid metabolism in other tissues, such as muscle and adipose tissue to clarify their mechanism. In addition, it is interesting whether these oxygenated fatty acids can be produced by intestinal bacterial flora and absorbed from intestine.

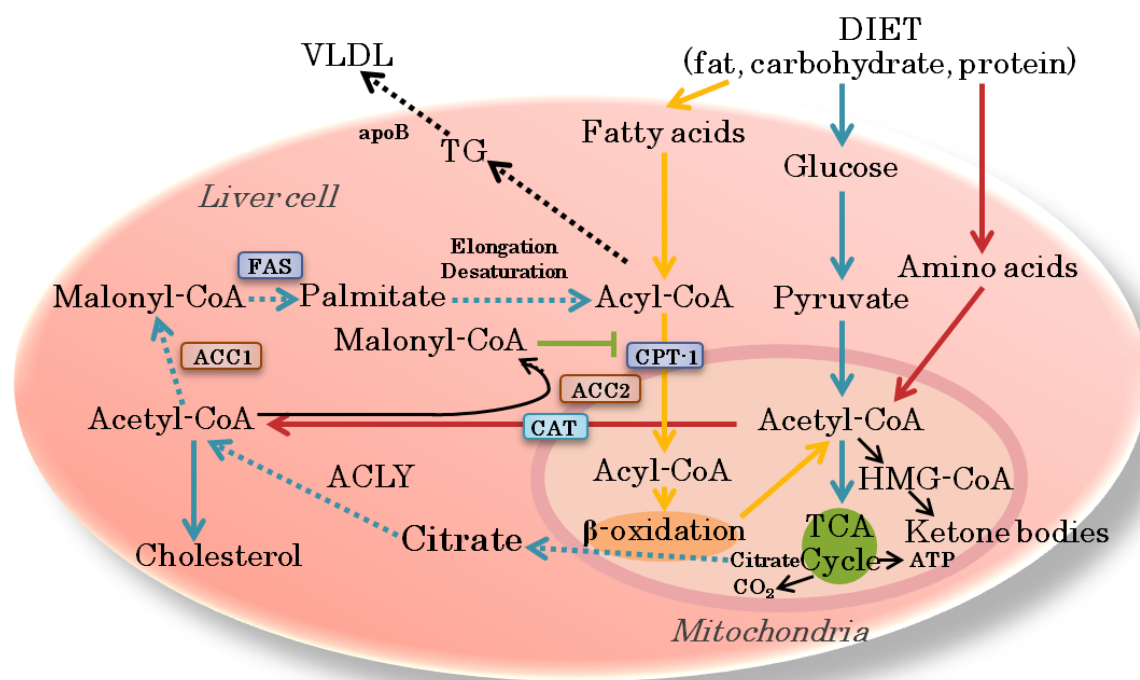


Figure 2-13. Acetyl-CoA carboxylase-1 (ACC1) and -2 (ACC2) play distinct roles in lipid metabolism in liver cells. HMG-CoA, hydroxymethylglutaryl-CoA; TCA, tricarboxylic acid; ACLY, ATP citrate lyase; TG, triacylglycerol; VLDL, very-low-density lipoprotein; CAT, carnitine/acetyl-CoA; CPT-1, carnitine/palmitoyl-transferase 1; FAS, fatty acid synthase.

Chapter 3: Oxygenated fatty acids fermented by *Lactobacillus*
plantarum reduce intracellular lipid accumulation in 3T3-L1 cells

INTRODUCTION

Dysregulation or imbalance of lipid metabolism in adipose tissue directly relates to obesity, of which the incidence has been dramatically increasing [93]. In 2008, the World Health Organization estimated that over 1.4 billion adults worldwide were overweight and around 500 million adults were obese [94]. This is majorly due to the changes of lifestyle, especially consuming high-fat and high-carbohydrate diet and lacking of exercise that contribute to the accumulation of adipose tissue. Moreover, the obese have high opportunity to develop many other diseases, such as, atherosclerosis and cardiovascular disease (CVD) which is the leading cause of mortality worldwide. Therefore, it cannot deny that the amelioration of lipid metabolism in adipose tissue is crucial to forestall many life-style related diseases. One of the most important factors that are concerned as the therapeutic targets of lipid metabolism disorders in adipocytes is over-production of triacylglycerol (TG), because adipocyte hypertrophy that occurs when TG synthesis (esterification) exceeds TG breakdown (lipolysis), resulting in elevated TG storage, primarily contributes to the development of obesity [3-5].

Novel intermediate fatty acids from metabolic pathway of *L. plantarum* were recently discovered by Kishino et al. The intermediates contain diverse species of hydroxy-fatty acids and oxo-fatty acids [23-27]. In chapter 2, it is shown that the effects of oxygenated fatty acids on TG accumulation in hepatocytes and uncovered that 10-hydroxy-12Z-18:1 (HYA), 6Z-10-hydroxy-12Z-18:2 (γ HYA), 10-keto-12Z-18:1 (KetoA), and 6Z-10-keto-12Z-18:2 (γ KetoA) outstandingly reduced TG accumulation in HepG2 cells through inhibition of SREBP-1c and ACCs expression. Moreover, it recently found that *L. plantarum* LG42 isolated from Gajami Sik-Hae, the Korean traditional fermented seafood, has efficiency to suppress adipogenesis in adipocytes by a decrease of glycerol-3-phosphate dehydrogenase (GPDH) activity and a reduction of intracellular TG storage

[95]. These beneficial effects might be due to γ -aminobutyric acid (GABA) which is also the product of fermentation from *L. plantarum*, however the intermediate fatty acids from its metabolic pathway might involve in its benefits as well. Hence, these oxygenated fatty acids fermented from *L. plantarum* might be able to improve lipid accumulation in adipocytes.

Mouse embryonic fibroblast-adipose like cell line (3T3-L1) was applied in this study because it is one of the most well-characterized and reliable models for studying adipogenesis. On reaching confluence, the treatment with adipogenic agents induces mitotic clonal expansion [96, 97]. The adipocyte differentiation (adipogenesis) pathway was described in Figure 3-1. Briefly, CCAAT/enhancer binding protein (C/EBP) β is highly expressed after the treatment with adipogenic agents [98]. C/EBP β consequently stimulates the expression of peroxisome proliferator-activated receptor (PPAR) γ and C/EBP α mRNA levels that coordinately activate the transcription of adipocyte-specific genes resulting in up-regulation of lipid synthesis in adipocytes [99].

The purpose of this chapter is to investigate the potential of oxygenated fatty acids obtained from the intermediates of metabolic pathway of *L. plantarum* to function as an antiobesity agent via modulation of adipocyte lipid storage in 3T3-L1 adipocytes via the adipogenic pathway.

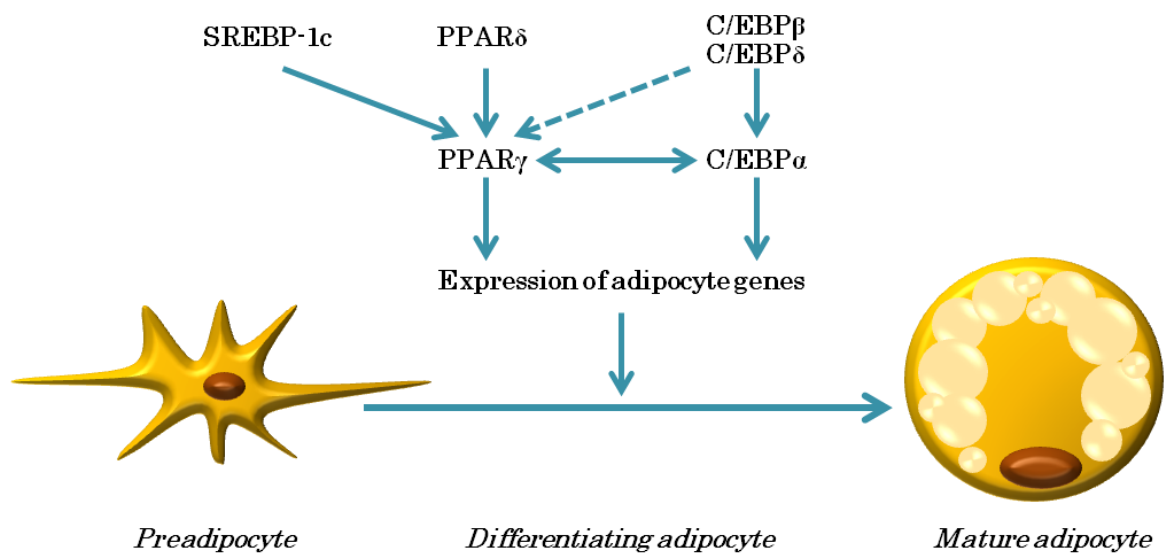


Figure 3-1. Adipocyte differentiation (adipogenesis) and transcriptional events in adipogenesis. The preadipocyte enters the adipogenesis stage via environmental and gene expression signals. In an early stage of adipogenesis, major transcriptional factors such as PPAR γ and C/EBP α are expressed, and these factors strongly regulate the expressions of adipogenesis-related genes. The adipocyte secretes various factors, including adipokines, and the secreted factors play an important role in glucose and lipid metabolism, immune system, appetite regulation, and vascular disease.

MATERIALS AND METHODS

I. Chemicals and reagents

Various kinds of oxygenated fatty acids fermented from fatty acids like LA by *Lactobacillus plantarum* were kindly provided from Prof. Dr. Jun Ogawa et al., Laboratory of Fermentation Physiology and Applied Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University. The chemical structures of these oxygenated fatty acids are demonstrated on Figure 2-3. Eicosapentaenoic acid, linoleic acid, DL- α -tocopherol (vitamin E) and formaldehyde were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Methyl-isobutyl-xanthine and Oil red O dye were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, 2-propanol, phosphate-buffered saline (PBS), dexamethasone and insulin were from Wako Pure Chemical Industries (Osaka, Japan).

II. Cell culture and induction of differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes (Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and antibiotics (100 unit/ml penicillin and 100 μ g/ml streptomycin; Gibco Life Technologies Corp, Grand Island, NY, USA) at 37°C in a humidified atmosphere in the presence of 5% CO₂. In this study the use of 3T3-L1 cells were limited only passages 3-10. The cultured medium was changed every 2 days until the confluence reaches 100%. The preadipocytes were induced to differentiate 2 days post-confluence by treated DMEM containing 1.0 μ M dexamethasone, 0.5 mM methyl-isobutyl-xanthine, 1.0 μ g/mL insulin, 10% FBS and antibiotics as mentioned above. After that the medium containing

1.0 µg/mL insulin, 10% FBS and antibiotics with or without fatty acids was replaced every other day until each experiment completed.

III. Cell viability analysis

Cell viability was assessed by the WST-1 method. 3T3-L1 cells were plated in 96-well culture plates at a density of 4.0×10^3 cells/well in 200 µl DMEM containing 10% FBS and antibiotics as detailed above, and were incubated at 37°C for 6 days. Among this period, the medium was changed every 2 days. Each fatty acid (30 or 60 µM) was then added to 3T3-L1 cells and/or vitamin E (10 µM) in DMEM containing 1.0 µM dexamethasone, 0.5 mM methyl-isobutyl-xanthine, 1.0 µg/ml insulin, 10% FBS and antibiotics. The final ethanol concentration was 0.2%. After incubation for 72 h at 37°C, 10 µl WST-1 solution (Dojindo Laboratories, Co., Kumamoto, Japan) was added to each well to evaluate cell viability. After incubation for 90 min at 37°C, cell viability was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA) at a wavelength of 450 nm.

IV. Oil red O staining

3T3-L1 preadipocytes were seeded in 24-well plates at 5.0×10^4 cells/ml in DMEM supplemented with 10% FBS and antibiotics as stated above. Two days post-confluence, the cells, defined as day 0, were induced to differentiate by replacing the medium with DMEM containing 1.0 µM dexamethasone, 0.5 mM methyl-isobutyl-xanthine, 1.0 µg/ml insulin, 10% fetal bovine serum and antibiotics as noted. Each fatty acid was simultaneously added to the cells. The final ethanol concentration was 0.1%. The adipocytes were cultured with DMEM containing 1.0 µg/ml insulin, 10% FBS,

antibiotics and each fatty acid every other day. At day 8 of differentiation, 3T3-L1 cells were stained with oil red O to analyze intracellular lipid accumulation. Briefly, the cells were washed twice by PBS, fixed with 10% formaldehyde in PBS for 20 min and repeatedly washed with PBS once. Then the cells were rinsed with 60% 2-propanol, incubated for 1 min and removed. Oil red O solution (6 parts of 0.3% saturated Oil red O dye in isopropanol diluted with 4 parts of distilled water) was added to stain the cells. After 10 min incubation, excessive stain was removed by rinsing the cells with 60% 2-propanol and washing with PBS. The stained lipid droplets within the cells were photographed by fluorescence microscope at 20X magnification and a digital camera (model BZ-9000; Keyence, Osaka, Japan). Stained oil red O was eluted with 100% 2-propanol and quantified by measuring absorbance at 490 nm using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA).

V. Determination of mRNA expression levels by real-time RT-PCR

3T3-L1 preadipocytes were seeded in 24-well plates at the similar concentration as described above. Two days post-confluence, the cells, defined as day 0, were induced to differentiate by replacing with the differentiating medium as shown above. Each fatty acid was simultaneously added to the cells in this stage. The final ethanol concentration was 0.1%. The adipocytes were cultured with DMEM containing 1.0 µg/ml insulin, 10% FBS, antibiotics and each fatty acid every other day. After 4 h of incubation, the cells were harvested to extract total RNA to measure the expression of C/EBPβ. To examine the expression of C/EBPα, PPARγ and SREBP-1c, total RNA was extracted from the cells after 8 day incubation by using Sepasol reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. RNAs were treated with RNase-free DNase (Promega, Madison, WI, USA) to remove contaminating genomic DNA. After

inactivating DNase by adding DNase stop solution (Promega, Madison, WI, USA) and heating at 65°C for 10 min, each RNA was transcribed to cDNA using SuperScript RNase II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamers at 25°C for 10 min and then at 42°C for 50 min. The reactions were stopped by incubation at 70°C for 15 min. To quantify the mRNA expression levels, real-time quantitative RT-PCR was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primers used for the quantification of each gene are listed in Table 3-1. Primer pairs were selected to yield gene-specific single amplicons based on analyses by melting curves and by agarose gel electrophoresis. The thermal cycling conditions were as follows: 15 min at 95°C for one cycle, followed by amplification of the cDNA for 43 cycles with melting for 15 s at 95°C and with annealing and extension for 30 s at 60°C. Values were normalized against GADPH rRNA as an endogenous internal standard.

Table 3-1. Real-time RT-PCR primers used for the quantification of mouse mRNAs

Gene name	Reference Accession Number	or	Forward (from 5' to 3')	Reverse (from 5' to 3')
C/EBP β	[100]		GCAAGAGCCGCGACAAG	GGCTCGGGCAGCTGCTT
C/EBP α			CAGGGCAGGAGGAAGATACA	GGAAACCTGGCCTGTTGTAA
PPAR γ			GCCCTTTGGTGACTTTATGG	GGCGGTCTCCACTGAGAATA
SREBP-1c	NM_011480		GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGCCCAG
GADPH	NM_008084.2		CGTCCCGTAGACAAAATGGT	TGCCGTGAGTGGAGTCATAC

VI. Western blot analysis

3T3-L1 cells were plated in 6-well plates at 6.0×10^4 cells/ml in DMEM supplemented with 10% fetal bovine serum and antibiotics as detailed above for 6 days. At this stage, the new medium was changed every two days. The cells were then treated with each fatty acid at the concentration of 60 μ M in differentiating medium. After incubation for 30, 60, 90 and 120 min, cells were harvested by washing with ice-cold PBS twice and scraping in the lysis buffer (20 mM Tris-HCl, pH 8, 150mM NaCl, 1% triton-X 100) containing protease inhibitors (Complete Mini Protease Inhibitor tablet, Roche, Mannheim, Germany). The scraped cells were then incubated on ice for 30 min and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and the concentration of soluble proteins in the supernatant was quantified using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For western blot analysis 10 μ g of protein was separated by 10% SDS-PAGE. Protein bands were transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). The filters were individually probed with a rabbit monoclonal anti-pAkt (T308) antibody (C31E5E, 1:2,000 dilution), a rabbit monoclonal anti-Akt antibody (C67E7, 1:1,000 dilution) and a rabbit monoclonal anti-Beta-actin antibody (13E5, 1:1,000 dilution; Cell Signaling Technology, Danvers, MA). Bound antibodies were labelled with alkaline phosphatase-conjugated anti-rabbit IgG (1:500 dilution; Cell Signaling Technology, Danvers, MA). The bands were visualized with the substrate, chemi-lumi one L (Nacalai Tesque, Kyoto, Japan) using a FUJIFILM visualizer (LAS-3000, Fujifilm Corporation, Tokyo, Japan).

VII. Statistical analysis

Data are reported as means $\pm$ SD. *In vitro* study statistical analyses were carried out by one-way ANOVA with Tukey-Kramer post-hoc multiple comparison test to identify significant difference using Stat View software (SAS Institute, Cary, NC, USA). Statistically difference was set at $p < 0.05$.

RESULTS

I. Effects of each fatty acid on 3T3-L1 cell viability

To study the effects of each fatty acid on cell viability, various concentrations of fatty acids were added to 3T3-L1 preadipocytes in culture simultaneously with the induction of cell differentiation. Figure 3-2 illustrates the results of the viability assay after treatment for 72 h. There are no significant differences between any groups treated by each fatty acid compared with insulin-induced control group. The results indicate that all fatty acids in this study have no cytotoxic effects on 3T3-L1 cells under these experimental conditions.

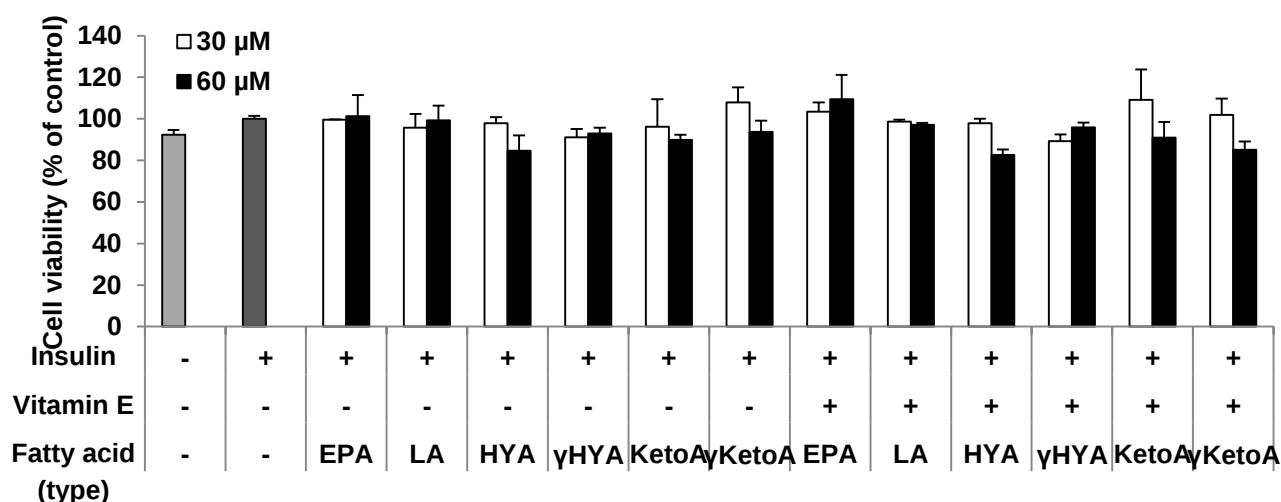


Figure 3-2. Effects of EPA, LA, HYA, γ HYA, KetoA and γ KetoA on cell viability as determined by the water soluble tetrazolium (WST)-1 assay. Each fatty acid (30 or 60 μ M) was added to 3T3-L1 cells with or without vitamin E (10 μ M) in differentiating medium. The final ethanol concentration was 0.2%. After 72 h incubation, the cells were added with WST-1 solution. The absorbance at 450 nm corresponds to cell viability. Expression levels are presented as relative percentage to vehicle control (ethanol). Data are reported as means $\pm$ SD (n = 3/group). * A significant difference from insulin-induced group (p < 0.05).

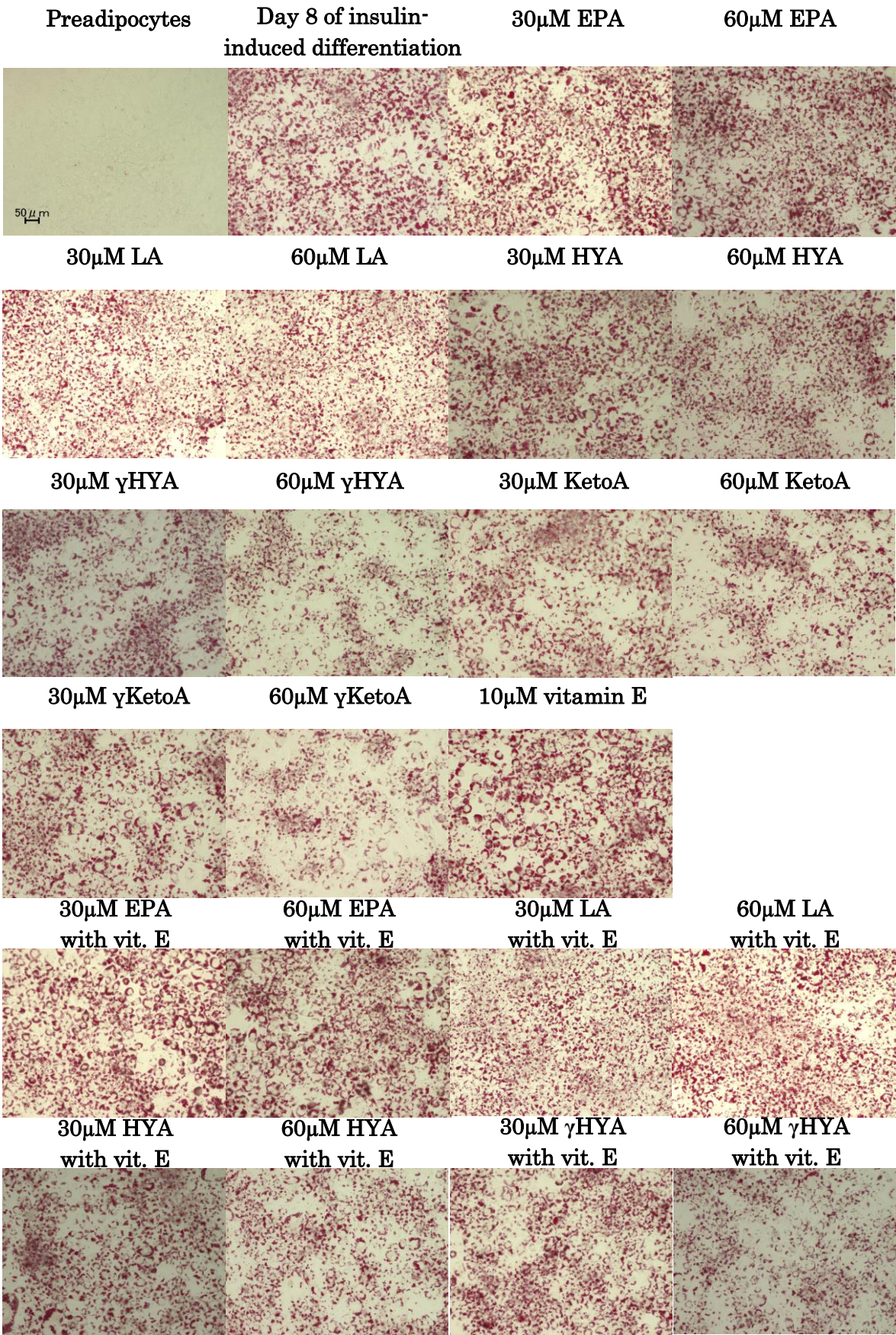
II. HYA, γ HYA, KetoA and γ KetoA prevent the development of intracellular TG accumulation

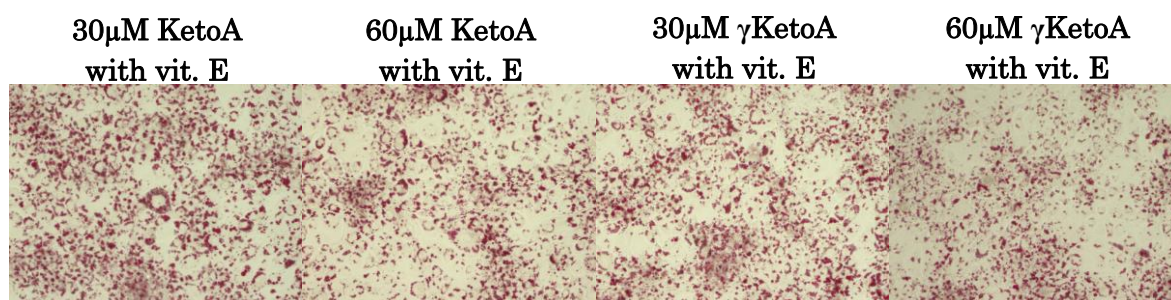
3T3-L1 preadipocytes were similar to fibroblasts in regard to appearance and contained no fat droplets in cytoplasm (Fig. 3-3A). On day 8 of differentiation, insulin-induced 3T3-L1 preadipocytes became bigger and rounder, and differentiated into mature adipocytes with lots of fat droplets found in the cytoplasm.

According to the experimental groups without vitamin E treatment, EPA or LA treated adipocytes did not show any significant differences at both concentrations. As expected, HYA, γ HYA, KetoA and γ KetoA treatment suppressed cell differentiation and significantly decreased lipid accumulation compared to insulin-induced control adipocytes, particularly γ KetoA treated group whose lipid accumulation reached the statistical difference from insulin-induced control group at both concentrations (Fig. 3-3A, B).

In the presence of vitamin E, addition of 30 or 60 μ M EPA or 30 μ M LA into the medium also has no changes compared to control group, however 60 μ M LA treatment significantly increased lipid synthesis of 3T3-L1 cells. Corresponding to the absence of vitamin E results, HYA and KetoA at high concentration and γ KetoA at both concentrations significantly reduced lipid accumulation in 3T3-L1 cells compared with insulin-induced differentiated cells. Notwithstanding, it seem that the ameliorating effect of γ HYA was abated by co-treatment with vitamin E. Vitamin E solely has no effects on inhibition of cell differentiation and lipid accumulation (Fig. 3-3A, B).

(A) Intracellular lipid droplets stained by Oil red O





(B) Quantification of lipid accumulation from destained Oil red O extracted from the cells

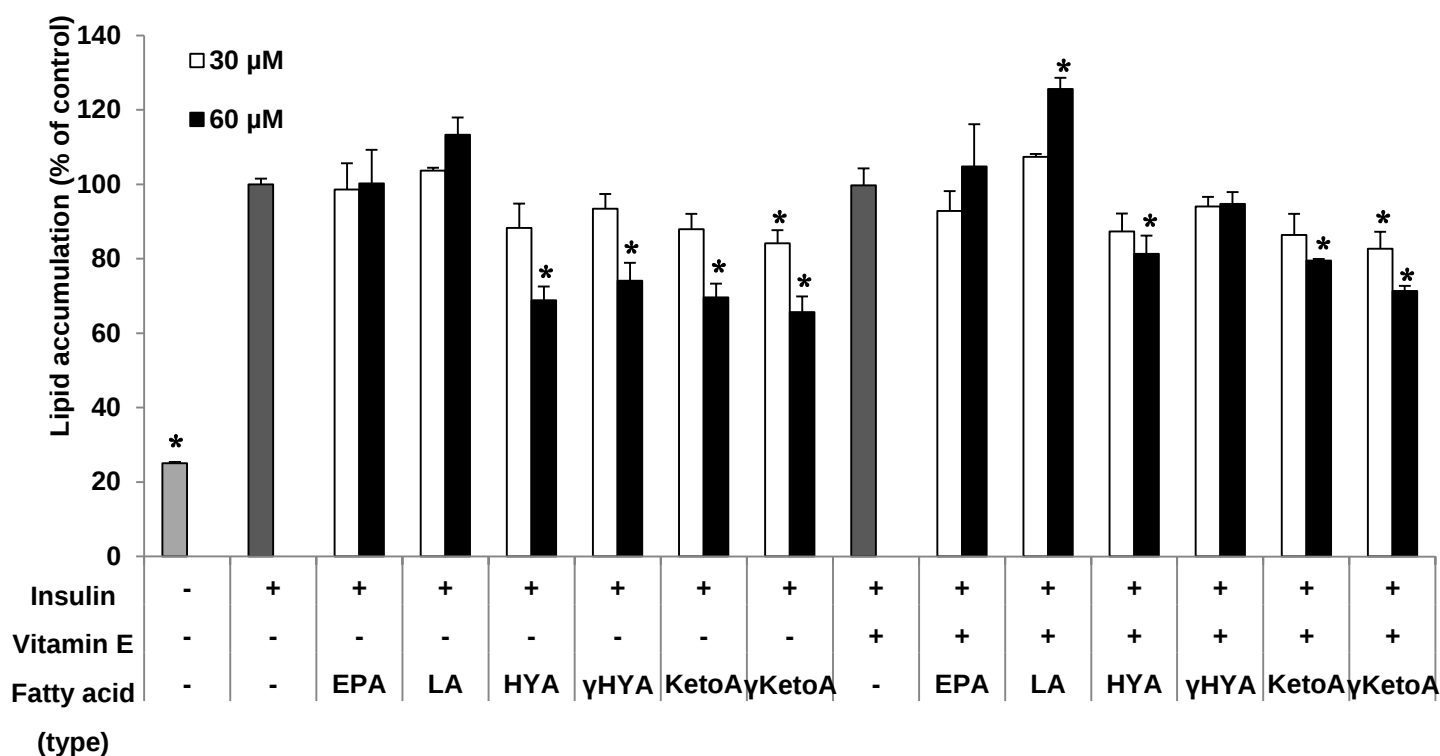


Figure 3-3. Effects of EPA, LA, HYA, γ HYA, KetoA and γ KetoA on intracellular lipid accumulation in 3T3-L1 preadipocytes. Each fatty acid (30 or 60 μ M) was added to 3T3-L1 cells with or without vitamin E (10 μ M) in differentiating medium. The final ethanol concentration was 0.2%. After treatment for 8 days, the cells were stained with oil red O solution (A). Quantification of lipid accumulation was based on the optical density values (at 490 nm) of destained oil red O extracted from the adipocytes (B). Expression levels are presented as relative percentage to insulin-induced differentiated control (ethanol). Data are reported as means $\pm$ SD ($n = 3/\text{group}$). * A significant difference from insulin-induced group ($p < 0.05$).

III. Expression of C/EBPs, PPAR γ and SREBP-1c genes after oxygenated fatty acids treatment in differentiated adipocytes

The alteration in expression of the transcription factors involved in adipocyte differentiation, C/EBP β , C/EBP α and PPAR γ , was further examined (Fig. 3-1). Additionally, SREBP-1c was investigated because it plays the major role in lipogenesis. As shown in Figure 3-4A, after 4 h treatment of differentiating medium, C/EBP β expression increased around 3-folds compared with undifferentiated cells. Simultaneously the low concentration of EPA treated cells did not show significant change in the expression of C/EBP β , but at high concentration, EPA treatment significantly decreased C/EBP β mRNA levels compared with insulin-induced control cells. As anticipated, treatment with HYA, KetoA or γ KetoA significantly inhibited the expression of C/EBP β compared to insulin-induced cells at both 30 and 60 μ M. However, γ HYA at both concentrations cannot significantly decrease C/EBP β expression. The Figure 3-4B shows that only KetoA and γ KetoA significantly reduced the expression of C/EBP α , meanwhile 30 μ M EPA significantly increased the expression of this gene. All oxygenated fatty acids fermented by *L. plantarum* in this experiment, except for γ KetoA, significantly decreased PPAR γ mRNA expression compared to differentiated control cells (Fig. 3-4C). EPA treatment statistically down-regulated the expression of SREBP-1c compared to insulin-differentiated adipocytes only at 60 μ M concentration, meanwhile there are no changes among oxygenated fatty acids treated groups (Fig. 3-4D). Regarding LA, Figure 3-5A and B illustrate that LA did not significantly differentiate the expression of both C/EBP α and PPAR γ , respectively.

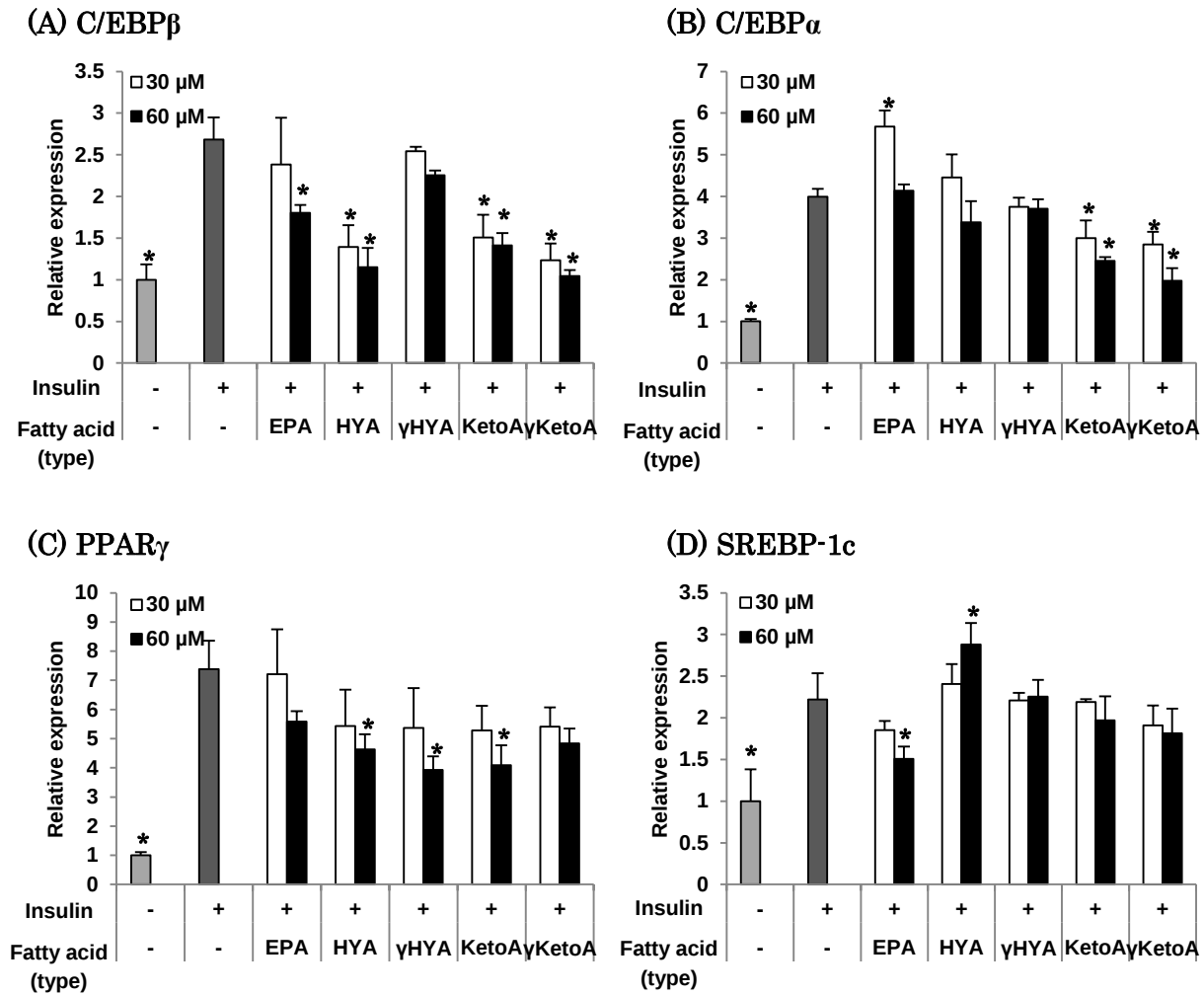


Figure 3-4. Effects of EPA, HYA, γ HYA, KetoA or γ KetoA on the expression of CCAAT/enhancer binding protein β (C/EBP β , A), C/EBP α (B), peroxisome proliferator-activated receptor γ (PPAR γ , C), and sterol responsive element binding protein-1c (SREBP-1c, D) mRNAs. Each fatty acid (30 or 60 μ M) was added to HepG2 cells in differentiating medium. The final ethanol concentration was 0.1%. After 4 h (C/EBP β) or 8 d (C/EBP α , PPAR γ and SREBP-1c) incubation, cells were harvested using sepsol reagent. C/EBP β , C/EBP α , PPAR γ and SREBP-1c mRNA levels were quantified by real-time quantitative PCR. Each value of C/EBP β , C/EBP α , PPAR γ and SREBP-1c mRNAs was adjusted by that of GAPDH rRNA (internal control). Expression levels are presented as fold induction relative to the insulin-induced control (ethanol). Data are reported as means $\pm$ SD (n = 3/group). * A significant difference from insulin-induced group (p < 0.05).

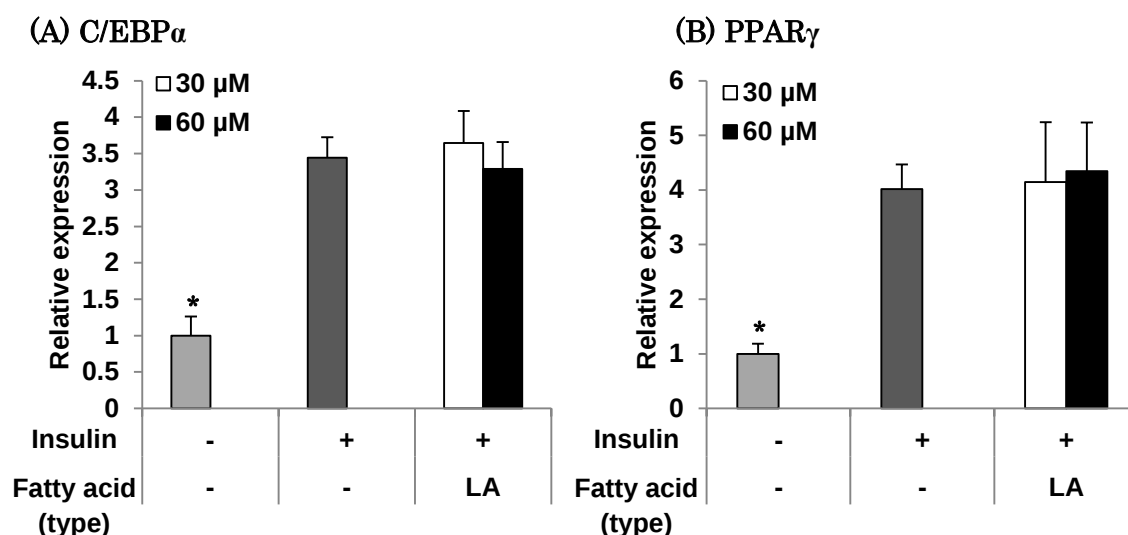


Figure 3-5. Effects of LA on the expression of CCAAT/enhancer binding protein α (C/EBP α , A), and peroxisome proliferator-activated receptor γ (PPAR γ , B) mRNAs. LA (30 or 60 μ M) was added to HepG2 cells in differentiating medium. The final ethanol concentration was 0.1%. After 8 d incubation, cells were harvested using sepaol reagent. C/EBP α and PPAR γ mRNA levels were quantified by real-time quantitative PCR. Each value of C/EBP α and PPAR γ mRNAs was adjusted by that of GADPH rRNA (internal control). Expression levels are presented as fold induction relative to the insulin-induced control (ethanol). Data are reported as means $\pm$ SD ($n = 3$ /group). * A significant difference from insulin-induced group ($p < 0.05$).

IV. Effects of each fatty acid on phosphorylation of Akt

In order to test whether the effects of these fatty acids on cellular lipid filling are involved by the Akt pathway, downstream of insulin signal, the effects of individual fatty acids on phosphorylation of Akt were also studied by using western blotting [101]. Protein expression of beta-actin was also examined as internal control. Phosphorylation of Akt was stimulated after adding differentiating medium to the cells from 30 min and continuously through 120 min incubation compared with preadipocytes. Nevertheless, the results indicated that EPA, LA, KetoA and γ KetoA at high concentration (60 μ M) cannot suppress phosphorylation of Akt at every incubation time point done in this study (Fig. 3-6).

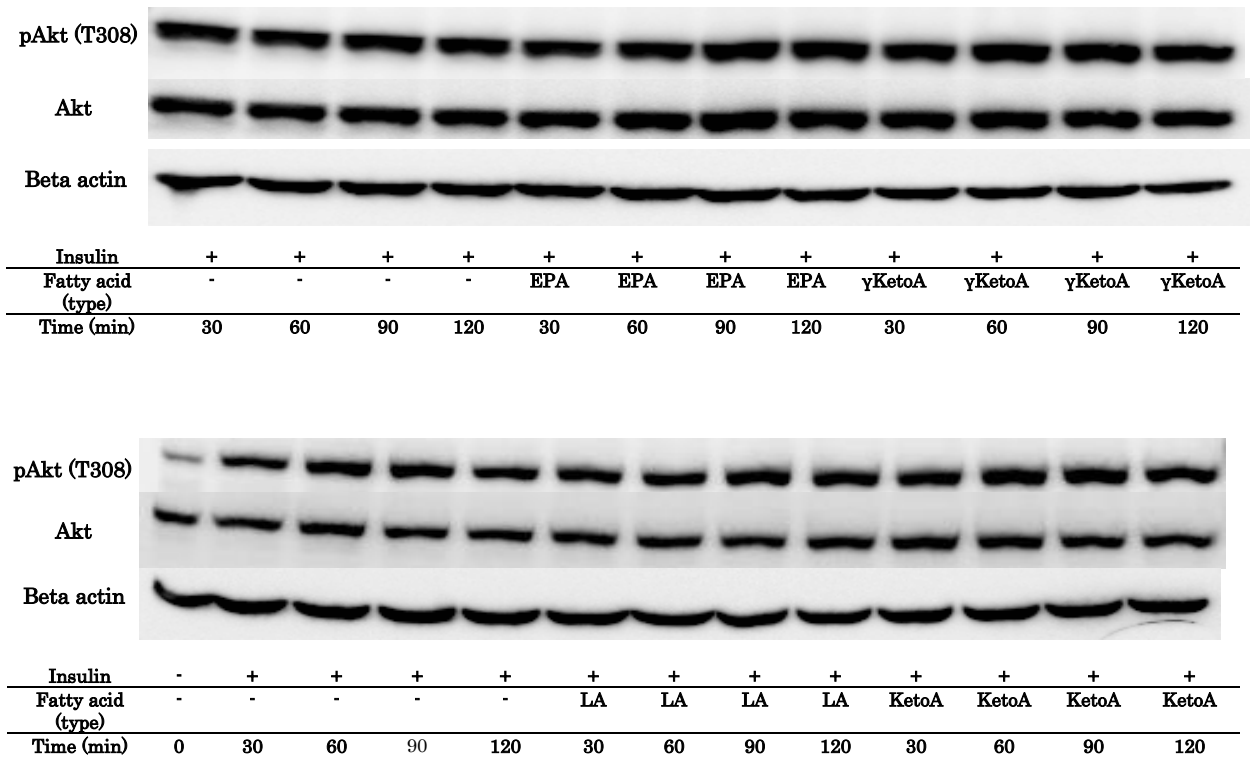


Figure 3-6. Effects of EPA, LA, KetoA or γ KetoA on the expression of pAkt, Akt and Beta-actin. Each fatty acid was added to 3T3-L1 preadipocytes with differentiating medium for 30, 60, 90 and 120 min. The 3T3-L1 cells were harvested in lysis buffer and the protein expression was measured by western blotting.

DISCUSSION

Obesity is well known as the main risk factor of many diseases. Concernedly, the incidence of obesity has been continuously rising in our society [93]. Excessive TG synthesis in adipocytes contributes to adipocyte hypertrophy and resulting in obesity [5]. Therefore, the alleviation of lipid metabolism in adipocytes is necessary to prevent and relieve obesity. In chapter 2, the data illustrated that hydroxy- and oxo-fatty acids extracted from intermediates of metabolic pathway of *L. plantarum* suppressed TG synthesis in hepatocytes induced by T0901317, synthetic LXR agonist. Furthermore, Itoh et al. described that hydroxy- and oxo-fatty acids act as ligands for PPAR γ [21]. From all reasons mentioned above, it can be predicted that these oxygenated fatty acids, including HYA, HYA, KetoA and KetoA, might be able to prevent the differentiation of adipocytes. EPA is one of the most famous PUFAs utilized in the supplement market due to its diverse benefits for health [11, 12]. Many studies showed that it has ameliorating effects in the reduction of TG in both liver and serum [13, 14, 41, 49].

The result from Oil red O staining showed that HYA, γ HYA, KetoA and γ KetoA at 60 μ M concentration in the absence of vitamin E significantly decreased lipid synthesis in 3T3-L1 cells. It has been proposed that *L. plantarum* LG42 isolated from Gajami Sik-Hae, the Korean traditional fermented seafood, also inhibited adipogenesis in 3T3-L1 adipocytes through the suppression of PPAR γ and C/EBP α . Furthermore, the treatment of *L. plantarum* LG42 also showed the suppressive effects on glycerol-3-phosphate dehydrogenase (GPDH) activity and intracellular TG storage [95]. Thus, the effects of *L. plantarum* LG42 might also be explained by the intermediate fatty acids from its metabolic pathway as examined in this study. On the contrary, both EPA and LA were unable to inhibit the effect of differentiating medium containing dexamethasone, methyl-isobutyl-xanthine and insulin in this experimental condition.

In the presence of vitamin E, LA and EPA also cannot reduce TG accumulation in 3T3-L1 cells compared to insulin-induced control. Besides, 60 μ M LA with vitamin E significantly increased lipid synthesis of adipocytes. This result corresponds with the study of Evens et al showing that after treatment 3T3-L1 preadipocytes with LA and vitamin E simultaneously, TG content was significantly augmented comparative to individually LA treated cells [20]. Furthermore, it was revealed that treatment of LA either with or without insulin increased the lipid accumulation in 3T3-L1 cells [102]. Not only LA, but vitamin E seem to abate the alleviative effects of both LA and conjugated linoleic acid (CLA) and 10,12-CLA on TG content in 3T3-L1 cells as well. The effect of vitamin E is also noticeable in oxygenated fatty acids treated groups that showed the increasing trend of lipid accumulation compared with the groups untreated with vitamin E [20]. Interestingly, only γ HYA treatment groups which vitamin E completely terminated the capability of reducing adipogenesis.

Coincidentally, HYA, KetoA and γ KetoA at both concentrations significantly down-regulated the expression of C/EBP β , which is transiently induced during the early stages of adipocyte differentiation, while EPA significantly reduced the expression just at high concentration, and γ HYA has no effects at both concentrations [103]. The expression of PPAR γ and C/EBP α , which are the target genes of C/EBP β , was then examined. The result illustrated that all hydroxy- and oxo-fatty acids used in this experiment had the tendency to suppress PPAR γ mRNA levels, but only the treatment of γ KetoA did not reach the significant difference. On the other hand, EPA and LA, which do not contain any hydroxy or ketone groups on their carbon backbone, cannot significantly reduce PPAR γ expression at both concentrations. The study of Itoh et al. revealed that some types of hydroxy- and oxo-fatty acids act as ligands for PPAR γ and thus are effective activators of PPAR γ [21]. These different functions of oxygenated fatty acids can be described that the location and number of functional group and double bond

might be crucial and resulting in an individual ability of each oxygenated fatty acid. Furthermore, oxygenated derivatives of cholesterol, as known as oxysterols, are natural ligands of LXR α , yet oxidized EPAs (OEPAs) or hydroxy-EPAs (HEPEs) which also contain hydroxy group in their structure decreased the expression of SREBP-1c, the target gene of LXR α , more efficiently than their intact form, EPA (Chapter 1). Therefore, it is speculated that oxygenated fatty acids from metabolic pathway of *L. plantarum* might be able to antagonize PPAR γ as well. PPAR γ is expressed predominantly in adipose tissue and macrophages, is closely related to the regulation of lipid and glucose metabolisms, and is associated with the control of obesity and related diseases [104-106]. Hence, a decrease of PPAR γ by these intermediate oxygenated fatty acids from metabolic pathway of *L. plantarum* also additionally explains the mechanism of *L. plantarum* isolated from food in the study of Park et al. [95]. Notwithstanding, only oxo-fatty acids, including KetoA and γ KetoA, significantly decreased the expression of C/EBP α . This study shows that in differentiated 3T3-L1 cells only EPA at high concentration significantly reduced the expression of SREBP-1c, while the others did not. It has been described that HYA, γ HYA, KetoA and γ KetoA down-regulated the transcription of SREBP-1c to almost the similar level as EPA did in hepatocytes (Chapter2). This might be the cell-specific effect of these oxygenated fatty acids which need further study to elucidate that.

Akt, a serine/threonine protein kinase, also known as protein kinase B, regulates a variety of cellular processes, including cell proliferation [104]. Once in correct position in the plasma membrane, Akt can be phosphorylated by 3-phosphoinositide dependent protein kinase 1 (PDK1) at threonine 308 (Thr308) residue [105, 106]. However, the result shows that all fatty acids in this study have no effects on the progression of phosphorylation of Akt, especially at Thr308 residue, during adipocyte differentiation.

This study suggests that the alleviative effect of *L. plantarum* on obesity does not cause by only GABA, but also the intermediate fatty acids that also suppress the adipogenesis, which is a main factor of development of obesity [20, 95, 107]. Taken together, these oxygenated acids not only showed the ameliorating effects on TG synthesis in the hepatic cells, but also exerted to suppress adipocyte differentiation via C/EBP β and PPAR γ pathway independently of Akt phosphorylation. Furthermore, it was discovered that only KetoA and γ KetoA, which are oxo-fatty acids, can down-regulate the expression of C/EBP α , while HYA and HYA, which are hydroxy-fatty acids, showed no significant effects. This indicates that these oxygenated fatty acids might be able to utilize as anti-obesity agent. Nevertheless, further study *in vivo* is necessary to confirm their benefits before practical use.

SUMMARY AND CONCLUSION

The present study demonstrated the beneficial functions of diverse kinds of oxygenated fatty acids, particularly on lipid metabolism. The 4-24 h oxidized eicosapentaenoic acids (OEPAs) were analyzed by LC/MS technique to investigate the oxidation products, and then their effects on lipogenesis in hepatocytes were examined. To elucidate and ensure the advantageous effects of oxygenated fatty acids, many species of intermediate hydroxy- and oxo-fatty acids from metabolic pathway of *L. plantarum* were screened and clarified the mechanism in lipid metabolism in both hepatocytes and adipocytes.

In chapter 1, the effects of EPA and OEPAs on lipogenesis pathway in the HepG2 cells were evaluated. OEPAs, especially 4h OEPAs, significantly decreased triacylglycerol (TG) accumulation, and SREBP-1c transcription and maturation more than intact EPA. Four h OEPA, which showed the best capability of suppression of TG synthesis pathway among all fatty acids in this experiment, contains outstanding amount of hydroxy-EPA (HEPE). Moreover, the trend of inhibitory activities of TG accumulation and the SREBP-1c expression of each OEPA also directly associated with the change of HEPE. Interestingly, PGC-1 β and GPA mRNA levels were reduced by 4 h OEPA, while EPA did not. Moreover, all HEPEs standards applied in this study decreased the expression of SREBP-1c and other lipogenic genes more efficiently than did EPA. Notwithstanding, 18-HEPE showed the best potential in the suppression of lipogenic gene transcription among 3 types of HEPEs standards. These results evidence that the location of hydroxy group on HEPEs might relate to the preferable ability of ameliorating effects on lipid metabolism of OEPAs. Taken as whole, OEPAs reduced the TG accumulation through SREBP-1c, GPA and PGC-1 β pathway more efficiently than EPA due to their specific oxygenated metabolites, particularly HEPE.

In chapter 2, the effects of oxygenated fatty acids fermented by *Lactobacillus plantarum* on lipogenesis pathway in hepatocytes were evaluated. Diverse species of oxygenated fatty acids provided were screened and found that HYA, γ HYA, KetoA and γ KetoA prominently decreased SREBP-1c expression. Interestingly, these fatty acids contain hydroxy or ketone group and double bond on the same location. The results illustrated that these oxygenated fatty acids significantly suppressed cellular TG accumulation more than EPA due to potent inhibition of SREBP-1c maturation and strong reduction of the expression of SREBP-1c and ACC2, which can regulate not only de novo lipogenesis but also β -oxidation. Moreover, KetoA, which contains ketone group on 10th carbon instead of hydroxy group in HYA, potently diminished TG levels more than HYA. Besides, KetoA, which is composed of a double bond only on 12th carbon, also significantly inhibited TG synthesis more than γ KetoA, which has two double bonds located on 6th and 12th carbons. Therefore, position, number and species of functional group and double bond in fatty acids might play a crucial role in the effect of unsaturated or oxygenated fatty acids on the inhibition of cellular TG synthesis in liver cells.

In chapter 3, four types of interesting fatty acids from last chapter were elucidated their effects on adipocyte differentiation in 3T3-L1 preadipocytes, because hypertrophy of adipose tissue is also the main cause of the development of obesity. HYA, γ HYA, KetoA and γ KetoA significantly lowered lipid filling of differentiated 3T3-L1 cells, while EPA and LA did not. As expected, C/EBP β transcription was significantly down-regulated by HYA, KetoA and γ KetoA. Besides, PPAR γ transcription was decreased by all four oxygenated fatty acids. On the other hand, only KetoA and γ KetoA, which are oxo-fatty acids, can reduce the expression of C/EBP α . This affirms that the type of functional group contained in oxygenated fatty acids involves in the ability of each oxygenated fatty acid. Taken together, this chapter proposes that these oxygenated fatty

acids suppressed the adipogenesis via the down-regulation of C/EBP β and PPAR γ pathway, but not Akt phosphorylation.

It has been concerned that the stability of oxygenated fatty acids from autoxidation may be difficult to conserve. Moreover, the oxidized derivatives of EPA obtained from autoxidation contain numerous types and are hard to identify and purify for future use. In contrast, the fatty acids fermented by the enzymes of *L. plantarum* from fatty acids like linoleic acid, are more stable. In addition, these enzymes change the chemical structure at the specific location and function resulting in convenient identification and purification. Hence, it is interesting to oxygenate EPA with the enzyme of *L. plantarum* to get more stable and specific types of OEPA.

In conclusion, these data suggest that dietary some specific species of oxygenated fatty acids could be potentially useful as hypolipidemic and anti-obesity agents. The advantages of the oxygenated fatty acids in this study are also possible to connect with the prevention of many other metabolic and cardiovascular diseases. The author believes that this study would provide a significant opportunity to develop and utilize oxygenated fatty acids from marine sources for supplement or medicinal use. Nevertheless, the oxidation products always develop the worse smell than their substrates and it is difficult to relieve this drawback in the real food. However, their benefits are also very attractive. Therefore, to utilize them as supplement, the technology of capsule can prevent the bad smell to a consumer. In addition, using EPA extracted from algae or other sources, instead of fish, may reduce the fishy smell even though after oxidation.

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