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Gut microbial fatty acid metabolites reduce triacylglycerol levels in hepatocytes

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Abstract

Hydroxy and oxo fatty acids were recently found to be produced as intermediates during gut microbial fatty acid metabolism. *Lactobacillus plantarum* produces these fatty acids from unsaturated fatty acids such as linoleic acid. In this study, we investigated the effects of these gut microbial fatty acid metabolites on the lipogenesis in liver cells. We screened their effect on sterol regulatory element-binding protein-1c (SREBP-1c) expression in synthetic liver X receptor α (LXRα) agonist (T0901317)-treated HepG2 cells. The results showed that 10-hydroxy-12(*Z*)-octadecenoic acid (18:1) (HYA), 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid (18:2) (γHYA), 10-oxo-12(*Z*)-18:1 (KetoA), and 10-oxo-6(*Z*),12(*Z*)-18:2 (γKetoA) significantly decreased *SREBP-1c* mRNA expression induced by T0901317. These fatty acids also downregulated the mRNA expression of lipogenic genes by suppressing LXRα activity and inhibiting SREBP-1 maturation. Oral administration of KetoA, which effectively reduced triacylglycerol accumulation and Acetyl-CoA carboxylase 2 (*ACC2*) expression in HepG2 cells, for 2 weeks significantly decreased *Srebp-1c*, *Scd-1,* and *Acc2* expression in the liver of high sucrose diet-fed mice. Our findings suggest that hypolipidemic effect of the fatty acid metabolites produced by *L. plantarum* can be taken advantage of in the treatment of cardiovascular diseases or dyslipidemia.

Keywords: Acetyl-CoA carboxylase, hydroxy fatty acid, oxo fatty acid, lipogenesis, liver, sterol regulatory element-binding protein-1c

Abbreviations

CLA	Conjugated linoleic acids
CVD	Cardiovascular disease
DMEM	Dulbecco's modified essential medium
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
LA	Linoleic acid
α-LNA	α-Linolenic acid
LXRα	Liver X receptor α
OA	Oleic acid
PUFA	Polyunsaturated fatty acids
RA	Ricinoleic acid
SREBP-1c	Sterol regulatory element-binding protein-1c

Introduction

Lactobacillus plantarum is a non-pathogenic gram-positive bacterium that naturally exists in the saliva and gastrointestinal tracts of humans and other mammals. It belongs to the lactic acid bacteria family and is commonly used in the fermentation of foods such as vegetables, fish, and dairy products [1–3]. Owing to its use as a probiotic, its biotherapeutic applications, which include reduction of the incidence of diarrhea, pain, and constipation associated with irritable bowel syndrome, bloating, and flatulence, have been increasingly acknowledged [4, 5]. We previously demonstrated that *L. plantarum* produces many specific types of conjugated linoleic acids (CLAs), which may be responsible for its advantageous effects [6–9]. Furthermore, it was found that CLA synthesis is part of the process of polyunsaturated fatty acids (PUFA) saturation in this strain and that a number of hydroxy and oxo fatty acids with diverse chemical structures are produced during the process of PUFA saturation. Moreover, the levels of hydroxy fatty acids were found to be much higher in specific pathogen-free mice than in germ-free mice, indicating that these fatty acids were generated through the metabolism of gastrointestinal microorganisms [10]. These findings suggest that lipid metabolism by gastrointestinal microbes affects the health of the host by modifying fatty acid profiles.

Triacylglycerol (TAG), in either the serum or the liver, is a major risk factor for cardiovascular disease (CVD). With respect to the importance of hepatic TAG level, nonalcoholic fatty liver disease is highly associated with CVD [11]. Omega-3 (ω-3) PUFAs, especially eicosapentaenoic acid (EPA), have been commercially developed as dietary supplements due to their various health benefits, particularly their TAG-reducing effects. We have previously established that structurally modified EPA such as *trans* isomers and oxidized products resulted in significantly lower lipogenic gene expression than EPA; this

downregulation resulted in a higher suppression of hepatocellular TAG levels [12, 13]. Therefore, the effect of the hydroxy and oxo fatty acids generated by gut microorganisms on the lipid metabolism in the host liver is of particular interest. In this study, we investigated the effects of fatty acid metabolites produced by *L. plantarum* on TAG synthesis and compared them to the effects of EPA. We also attempted to elucidate the structure-related functions of hydroxy and oxo fatty acids in the LXRα and SREBP-1c pathway, which plays important roles in lipid metabolism in liver cells.

Materials and Methods

Materials

Various types of hydroxy and oxo fatty acids produced from dietary fatty acids such as linoleic acid (LA) by *L. plantarum* were prepared using previously described methods [6–10] (Fig. 1). RA (12-hydroxy-9*Z*-18:1) and 12-hydroxy-octadecanoic acid (18:0) were obtained from Nu-Chek Prep Inc. (Elysian, MN, USA) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. 12-Oxo-9(*Z*)-18:1 (ketoRA) and 12-oxo-18:0 were prepared from RA and 12-hydroxy-18:0, respectively, using Jones oxidation, which is the oxidation of a hydroxy group with CrO3 [14] and by further purification using a silica column. The purities of the fatty acids were evaluated by gas-liquid chromatography and each fatty acid was quantified based on their weights. EPA, LA, oleic acid (OA), and DL-α-tocopherol (vitamin E) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). T0901317 was obtained from Cayman Chemicals (Ann Arbor, MI).

Cell Culture

HepG2 cells (JCRB 1054; Health Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco's modified essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen) and antibiotics (100 unit/mL penicillin and 100 µg/mL streptomycin; Life Technologies Corporation, Grand Island, NY, USA) at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Cell Viability Analysis

Cell viability was assessed using 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 µL DMEM containing 10% FBS and antibiotics as detailed above, and were incubated at 37°C for 24 h. Each fatty acid was then added to the HepG2 cell culture medium with T0901317 (10 nM) in serum-free medium containing 0.1% BSA (Sigma-Aldrich, Co., St. Louis, MO, USA). The final ethanol concentration was 0.4%. After incubation for 24 h at 37°C, 10 µL of the 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, the cell viability was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA) at a wavelength of 450 nm.

Determination of mRNA Expression by Real-time RT-PCR in HepG2 Cells

HepG2 cells were seeded on 12-well plates at 2×10^5 cells/mL in DMEM supplemented with 10% FBS and antibiotics. After 24 h of incubation, each fatty acid was added to the HepG2 cell culture medium in the presence of T0901317 (10 nM) in serum-free medium containing

0.1% BSA. 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 Inc.) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Promega, Madison, WI, USA) to remove any contaminating genomic DNA. After inactivating the DNase by adding DNase stop solution (Promega) and heating at 65°C for 10 min, the RNA was transcribed to cDNA using SuperScript RNase II reverse transcriptase (Invitrogen) with random hexamers. To quantify mRNA expression, 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). Primers used for the quantification of each gene are listed in Table 1. All data were normalized to 18S mRNA levels as an endogenous internal standard.

Luciferase Reporter Assay

HepG2 cells were grown in DMEM supplemented with 10% FBS and antibiotics at 37°C. A luciferase ligand assay was performed using the dual luciferase system (Promega) as previously described [15]. For the LXRα activity assay, p3xIR1-tk-Luc, pCMX-hLXRα, and pRL-CMV were transfected into HepG2 cells. Transfections into HepG2 cells cultured on 10-cm dishes were performed using Lipofectamine (Invitrogen) according to the manufacturer's protocol. After transfection for 4 h, the transfected cells were seeded into 96-well plates in media containing EPA, LA, 10-hydroxy-12(*Z*)-octadecenoic acid (18:1) (HYA), 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid (18:2) (γHYA), 10-oxo-12(*Z*)-18:1 (KetoA), 10-oxo-6(*Z*),12(*Z*)-18:2 (γKetoA), and/or T0901317 (500 nM). The luciferase activity was measured after 24 h of incubation.

Lipid extraction and TAG Quantification

HepG2 cells were plated in 6-well plates at 5 × 10^5 cells/mL for 24 h in DMEM supplemented with 10% FBS and antibiotics (as mentioned earlier). The cells were then treated with each fatty acid and/or T0901317 (10 nM) in the presence of vitamin E (10 μM) in the serum-supplemented medium. Fatty acids, vitamin E, and T0901317 were dissolved in ethanol. After incubation for 48 h, lipids were extracted from the cells using chloroform-methanol (2:1, v/v). Reference control cells were extracted before incubation (zero time control). The collected supernatants were gently evaporated under an N_2 stream, and TAG was quantified using a triglyceride E-test kit (Wako Pure Chemical Industries, Osaka, Japan). TAG accumulation during 48 h of incubation with each fatty acid was calculated using a minus zero time control.

Cell Fractionation and Immunoblotting

HepG2 cells were plated on 6-well plates at 5.0 × 10^5 cells/mL for 24 h in DMEM supplemented with 10% FBS and antibiotics. The cells were then treated with HYA, γHYA, KetoA, γKetoA, and/or T0901317 (10 nM) in serum-free medium containing 0.1% BSA. After incubation for 24 h, the membrane fractions and nuclear extracts from the cells were prepared by the method reported in the study by Hannah et al. (16). In brief, the cells were harvested by scraping, and the cell suspension was centrifuged at 1,000 × *g* for 5 min at 4°C. The cell pellet was then 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 a protease inhibitor tablet (Complete Mini Protease Inhibitor tablet, Roche, Mannheim, Germany). The cell suspension was passed 20 times through a 23-gauge needle and centrifuged at 1,000 × *g* for 5 min at 4°C. The pellet thus obtained was resuspended in 40

μL of 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 inhibitor tablet). This suspension was rotated at 4°C for 1 h and centrifuged at $10^5 \times g$ for 15 min at 4°C. The resulting supernatant was designated as the nuclear extract fraction. The supernatant obtained after the first 1,000 × *g* spin was re-centrifuged at $10^4 \times g$ for 15 min at 4°C, and then the pellet was dissolved in 25 μL of 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 a protease inhibitor tablet) and designated as the membrane fraction. The concentration of soluble proteins in the supernatant was quantified using a DC protein assay kit (Bio-Rad Laboratories). For the immunoblot analysis, given amounts of membrane fractions (25 μg) and nuclear extracts (20 μg) were separated by 7% and 10% SDS-PAGE, respectively. Protein bands were transferred to polyvinylidene difluoride membranes (Millipore Corporation, MA). The filters were probed with rabbit polyclonal anti-SREBP-1 antibody (H-160, 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were visualized using alkaline phosphatase-conjugated anti-rabbit IgG (1:500 dilution; Cell Signaling Technology, Danvers, MA), and the bands were visualized using the substrate, chemi-lumione L (Nacalai Tesque), and a FUJIFILM visualizer (LAS-3000, Fujifilm Corporation, Japan).

Animal Experiments

All experimental animal protocols were approved by the Animal Experimentation Committee of Kyoto University for the care and use of experimental animals. Six-week-old male ICR mice (Japan SLC, Inc) were kept in individual cages in a temperature-controlled facility with a constant 12-h light/dark cycle. The mice were divided into 2 groups and maintained on 40% high-sucrose diet (Table 2). KetoA (5 mg/mouse/day) in PBS containing 5% DMSO and

0.5% polysorbate 20 was administrated to each mouse in the experimental group by oral gavage for 2 weeks. After fasting for 16 h, the mice were euthanized under isoflurane anesthesia. The liver was excised, and total RNA and total lipids were extracted. To quantify mRNA expression, real-time quantitative RT-PCR was performed. Primers used for the quantification of each gene are listed in Table 3. The values were normalized against those for *Gapdh* that was used as an endogenous internal standard. TAG was quantified using a triglyceride E-test kit.

Statistical Analysis

Data are reported as means ± standard deviation (SD). Statistical analyses were conducted using Student's *t*-test or one-way ANOVA with Scheffe's F-test to identify significant differences using the Stat View software (SAS Institute, Cary, NC, USA).

Results

L. plantarum converted unsaturated fatty acids such as LA through saturation into diverse hydroxy and oxo fatty acids that have a hydroxy or oxo group at the C10 position and double bonds at different locations (Fig. 1). The effects of these C10-hydroxy and -oxo fatty acids were investigated in comparison with those of dietary fatty acids such as OA, LA, and EPA and structurally related C12-hydroxy and -oxo fatty acids such as RA (which is abundant in castor oil), KetoRA, 12-hydroxy-18:0, and 12-oxo-18:0.

To determine the effects of each compound on cell viability, various concentrations of fatty acids and T0901317 were added to HepG2 cells. Fig. 2 shows the result of the cell viability assay after 24 h of treatment. None of the fatty acids, excluding 60 μM EPA, HYB

and 12-hydroxy-18:0, had significant cytotoxic effects). In agreement with the results of this experiment, EPA, which is famous for its hypolipidemic effect, is well known for its high cytotoxic effect [17].

After 24 h of induction with 10 nM T0901317, *SREBP-1c* mRNA expression in HepG2 cells was more than 6-fold higher than that in the vehicle-treated cells. Most of the C10-hydroxy and -oxo fatty acids produced by *L. plantarum*, except 10-oxo-18:0 (KetoB), significantly reduced the T0901317-induced *SREBP-1c* mRNA expression; this effect was also observed with EPA, but not with LA. *SREBP-1c* mRNA levels in the HYA, γHYA, KetoA, and γKetoA treatment groups were significantly different from those of the LA treatment group. These four derivatives contained hydroxy or oxo groups at the same position (C10) and the double bond was located at Δ12. 10-Hydroxy-12(*Z*),15(*Z*)-18:2 (αHYA) and 10-oxo-12(*Z*),15(*Z*)-18:2 (αKetoA), which were derived from an omega-3 (ω3) PUFA, α-LnA, also demonstrated the same activity, albeit at a slightly lower level than HYA, KetoA, γHYA, and γKetoA, which were derived from an omega-6 (ω6) PUFA, LA or γ-LnA. Structurally related RA, 12-hydroxy-18:0, and 12-oxo-18:0 with hydroxy or oxo groups at C12, but not KetoRA, also showed slightly weaker effects on *SREBP-1c* mRNA expression in HepG2 cells (Fig. 3).

After 48 h of incubation, T0901317 significantly increased cellular TAG levels to approximately 14 μg/mg of protein in HepG2 cells. TAG accumulation in *L. plantarum* fatty acid metabolite-treated cells was significantly lower than that of the T0901317-activated cells. KetoA, which contains an oxo group at C10, most effectively reduced TAG levels. HYA, KetoA, and γKetoA showed significantly stronger hypolipidemic effects than EPA (Fig. 4).

In the presence of T0901317, LXRα reporter activity in HepG2 cells was approximately 4-fold higher than that in the vehicle-treated cells. In our luciferase reporter assay, EPA, HYA,

γHYA, KetoA, and γKetoA significantly decreased the LXRα reporter activity induced by T0901317 in a dose-dependent manner (Fig. 5A). These results indicated that the suppressive effect of these fatty acids on *SREBP-1c* mRNA expression was regulated through LXRα.

Next, the effect of these fatty acid metabolites on the mRNA levels of lipogenic gene was evaluated. HYA, γHYA, KetoA, and γKetoA significantly decreased stearoyl-CoA desaturase-1 (*SCD-1*) and *FAS* mRNA expression, as did EPA and LA (Fig. 5B and C). In addition, HYA, γHYA, KetoA, and γKetoA significantly decreased the T0901317-induced expression of *ACC1* and *ACC2* in a similar fashion as did EPA, but not as LA did (Fig. 5D and E). The decrease in *ACC2* mRNA levels caused due to HYA and KetoA was significantly more than that observed with EPA.

To investigate the effect of these fatty acids on SREBP-1 protein levels, the full-length precursor form of the protein in the cell membrane (125 kDa) and the cleaved mature form (68 kDa) in the nuclear extract were estimated by immunoblotting. Because the used antibody cannot distinguish between the SREBP-1c and -1a isoforms, we use the general term SREBP-1 to refer to the results. T0901317 increased the levels of both the precursor and the mature forms of SREBP-1 (Fig. 6A and B). HYA, γHYA, KetoA and γKetoA, as well as EPA, decreased the T0901317-mediated induction of the expression of both the precursor and mature forms of SREBP-1. Interestingly, the expression of the mature form of SREBP-1 was more significantly downregulated by HYA, γHYA, KetoA, and γKetoA than by EPA.

In this study, the dietary effects of fatty acid metabolites produced by *L. plantarum* were examined *in vivo*. KetoA, which was the most efficient in reducing TAG accumulation and *ACC2* expression in HepG2 cells, was administrated to high-sucrose diet fed-mice for 2 weeks. The mRNA expression of *Srebp-1c*, *Scd-1,* and *Acc2* was significantly lower in the KetoA group than in the control group, but the liver TAG content was not different from that in the control group (Fig 7).

Discussion

In the present study, the biological effects of hydroxy and oxo fatty acids, which can be produced by gut microorganisms from dietary fatty acids, were evaluated. We decided to study the effects of these hydroxy and oxo fatty acids on the LXRα and SREBP-1c lipogenic pathway in the liver, because this pathway may be a potential target for the treatment of atherosclerosis and other metabolic diseases. We used T0901317 to develop an immoderate lipid synthesis model by upregulating LXRα and SREBP-1c expression. In this study, we focused on the hypolipidemic effect of the hydroxy and oxo fatty acids on hepatic cells. The hypolipidemic effect of PUFAs is attributable both to a decrease in lipogenesis and to an increase in fatty acid catabolism through the regulation of SREBP-1 and PPARα, respectively [18]. Nevertheless, it is well known that PPARα is weakly expressed and has very low function in HepG2 cells, because these cells originate from hepatomas and some kinds of genes, such as *PPARα*, are mutated [19]. Thus, we focused on the regulation of lipogenesis via SREBP-1c.

In our results, most of C10-hydroxy and -oxo fatty acids produced by *L. plantarum*, except KetoB and the plant fatty acid-derived KetoRA, significantly reduced T0901317-induced *SREBP-1c* mRNA expression. In addition, HYA, γHYA, KetoA, and γKetoA caused a more significant decrease in *SREBP-1c* mRNA expression than did LA; this effect, however, was not observed with RA. Thus, the effects of C10-hydroxy and -oxo fatty acids seem to be relatively more potent than those of C12-hydroxy and -oxo fatty acids. The luciferase assay revealed that, similar to EPA, some oxo- and hydroxy-octadecenoic and octadecadienoic acids produced from unsaturated fatty acids by *L. plantarum*, including HYA, γHYA, KetoA,

and γKetoA, dose-dependently decrease LXRα activation [12]. This result indicated that HYA, γHYA, KetoA, and γKetoA reduced *SREBP-1c* mRNA expression by competing with T0901317 for LXRα activation, similar to EPA. This important mechanism may explain how these fatty acids can potently reduce cellular TAG synthesis that was induced by an LXRα agonist. In parallel with a previous study (20), we found that EPA decreased SREBP-1 maturation by reducing the T0901317-induced expression of both the precursor and mature forms of the SREBP-1 protein. HYA, γHYA, KetoA and γKetoA repressed the expression of precursor SREBP-1 to the same level as did EPA, but the decrease in the expression of mature SREBP-1 caused by these metabolites was significantly higher than that observed with EPA. Elucidation of the regulation of factors related to the maturation of SREBP-1, such as insulin-induced gene (INSIG), SREBP cleavage-activating protein (SCAP), and site-1 protease (S1P), might help explain the reason for this difference in the effects of these metabolites with regard to suppression of mature SREBP-1 expression.

HYA, γHYA, KetoA, and γKetoA also reduced the mRNA expression of the target genes of SREBP-1c, such as *FAS, SCD-1*, and *ACC1*, which were involved in *de novo* fatty acid synthesis. The increase in *ACC2* mRNA expression and TAG accumulation in T0901317-induced cells was reduced by HYA and KetoA to a level that was significantly lower than that observed in EPA-treated cells. This finding indicated that ACC2 is a crucial factor influencing the effects of hydroxy and oxo fatty acids on lipid metabolism. ACC2 plays a major role in energy homeostasis by converting acetyl-CoA to malonyl-CoA. Malonyl-CoA inhibition of CPT1 is a mechanism that controls the two opposing pathways: fatty acid synthesis and β-oxidation [21, 22]. ACC2 is located on the mitochondrial membrane and is highly expressed in tissues with a high rate of fatty acid oxidation, such as the adipose tissue, skeletal muscles, and the liver [22, 23]. This hypothesis was further supported by the results of a study on $Acc2^{-/-}$ mutant mice. These mice continuously oxidized fatty acids, ate more

food, and gained less weight than the wild-type mice [24]. Therefore, ACC2-suppressive effect may be preferable to increase fatty acid β-oxidation and decrease lipid accumulation.

The lipogenesis-suppressive effect of fatty acid metabolites produced by *L. plantarum* might be due to a significant reduction in SREBP-1c and ACC2 levels via a decrease in their mRNA expression. These findings suggest that HYA, γHYA, KetoA, and γKetoA might be novel alternatives that can be utilized in the treatment of CVD, atherosclerosis, or any other diseases that are caused by SREBP-1c overexpression, particularly through a hepatic insulin signaling-dependent pathway, which is required for SREBP-1c processing, maximal lipogenic gene expression, and steatosis induction [25]. In our animal experiment, oral administration of KetoA, the most efficient fatty acid to reduce TAG accumulation and *ACC2* expression in HepG2 cells, significantly decreased the hepatic gene expression of *Srebp-1c*, *Scd-1* and *Acc2*, but did not affect the TAG content in the liver. Further studies are required to investigate the potential effects of these hydroxy and oxo fatty acids on lipid metabolism and their bioavailability and metabolic fate *in vivo*.

In addition, it will be interesting to investigate the probiotic effects of *L. plantarum* on the health of a host fed a PUFA-rich diet. The results of the present study also show that gut microbial metabolites from PUFA are more effective against hepatic cellular TAG accumulation than those from monounsaturated fatty acids such as OA. The metabolites derived from ω6 PUFA showed higher activity than those derived from ω3 PUFA. These observations indicate that the ω6 and ω3 PUFA content of the food together with the PUFA-saturation activity of gut microbiota have an effect on specific health conditions.

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Figure Legends

Fig. 1 Chemical structure of hydroxy and oxo fatty acids produced by *Lactobacillus plantarum* and structurally related hydroxy and oxo fatty acids.

Fig. 2 Effects of EPA, LA, OA, or hydroxy and oxo fatty acids on cell viability as determined by the water-soluble tetrazolium (WST)-1 assay. Data are reported as means ± SD (n = 4/group). A significant difference as compared to the vehicle control group was shown as follows: * $p < 0.05$.

Fig. 3 Effects of EPA, LA, OA, or hydroxy and oxo fatty acids on SREBP-1c mRNA expression in HepG2 cells. Data are reported as means ± SD (n = 3). *Significantly different from the T0901317-induced control ($p < 0.05$).

Fig. 4 Effects of EPA, LA, 10-hydroxy-12Z-octadecenoic acid (18:1) (HYA), 10-hydroxy-6Z,12Z-octadecadienoic acid (18:2) (γHYA), 10-oxo-12Z-18:1 (KetoA), and 10-oxo-6Z,12Z-18:2 (γKetoA) on triacylglycerol synthesis in HepG2 cells. The increased TAG levels (TAG levels after 48 h of incubation minus the levels in reference cells before incubation) are

shown. Data are reported as means ± SD (n = 4). Means indicated with different letters showed statistically significant differences, as determined by Scheffe's F-test ($p < 0.05$).

Fig. 5 Suppression of pLXREs-Luc expression by EPA, LA, HYA, γHYA, KetoA, and γKetoA (a). Data are reported as means ± standard deviation (SD, n = 5). *Significantly different from the T0901317-induced control ($p < 0.05$). Effects of these fatty acids on the mRNA expression of stearoyl-CoA desaturase-1 (SCD-1, b), fatty acid synthase (FAS, c), acetyl-CoA carboxylase-1 (ACC1, d), and acetyl-CoA carboxylase-2 (ACC2, e). Data are reported as means ± SD (n = 3). Means indicated with different letters showed statistically significant differences, as determined by Scheffe's F-test ($p < 0.05$).

Fig. 6 Effects of HYA, γHYA, KetoA, or γKetoA on the expressions of the precursor (A) and mature (B) forms of sterol-regulatory element binding protein-1 (SREBP-1) protein. Data are reported as means ± SD (n = 3-4/group).
Means indicated with different letters showed statistically significant differences, as determined by Scheffe's F-test ($p < 0.05$).

Fig. 7 Effect of dietary KetoA on triacylglycerol and mRNA expression in the liver of high-sucrose diet-fed mice. Data are reported as means ± SD (n = 5). *Significantly different from the control group, as determined by Student's *t*-test ($p < 0.05$).

Table 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	[1]	GGAGGGGTAGGGCCAACGGCCT	CATGTCTTCGAAAGTGCAATCC
SCD-1	NM_005063	TGGTTTCACTTGGAGCTGTG	GGCCTTGGAGACTTTCTTCC
FAS	[1]	ACAGGGACAACCTGGAGTTCT	CTGTGGTCCCACTTGATGAGT
ACC1	NM_198834	ATCCCGTACCTTCTTCTACTG	CCCAAACATAAGCCTTCACTG
ACC2	NM_001093	CTCTGACCATGTTCGTTCTC	ATCTTCATCACCTCCATCTC
18s	[1]	TAAGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC

[1] F.J. Field, E. Born, S. Murthy, S.N. Mathur, Poly-unsaturated fatty acids decrease the expression of sterol regulatory element-binding protein-1 in CaCo-2 cells: effect on fatty acid synthesis and triacylglycerol transport, Biochem. J. 368 (2002) 357-363.

Table 2. Composition of high sucrose diet

Ingredient	(weight %)
β-Cornstarch	10
Casein	20
Dextrinized cornstarch	13.2
Sucrose	39.7486
Soybean oil	7
Cellulose powder	5
AIN-93 mineral mix	3.5
AIN-93 vitamin mix	1
L-Cystine	0.3
Choline bitartrate	0.25
Butyl hydroxy toluene	0.0014

Table 3. 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	[2]	AAACTGCAGGTATCCCAACTCTTC	CTGTGGAACATTTAAGATACGTTTCGAAAA
ACC2	[2]	GACGCCCGAGGATCTGAAG	GGGACAGGGACGTACTGATC
GADPH	NM_008084.2	CGTCCCGTAGACAAAATGGT	TGCCGTGAGTGGAGTCATAC

[2] J.C. Castle, Y. Hara, C.K. Raymond, P. Garret-Engee, K. Ohwaki, Z. Kan, J. Kusunoki, J.M. Johnson, ACC2 is expressed at high levels human white adipose and has an isoform with a novel N-Terminus, PloS One 4 (2009) e4369.

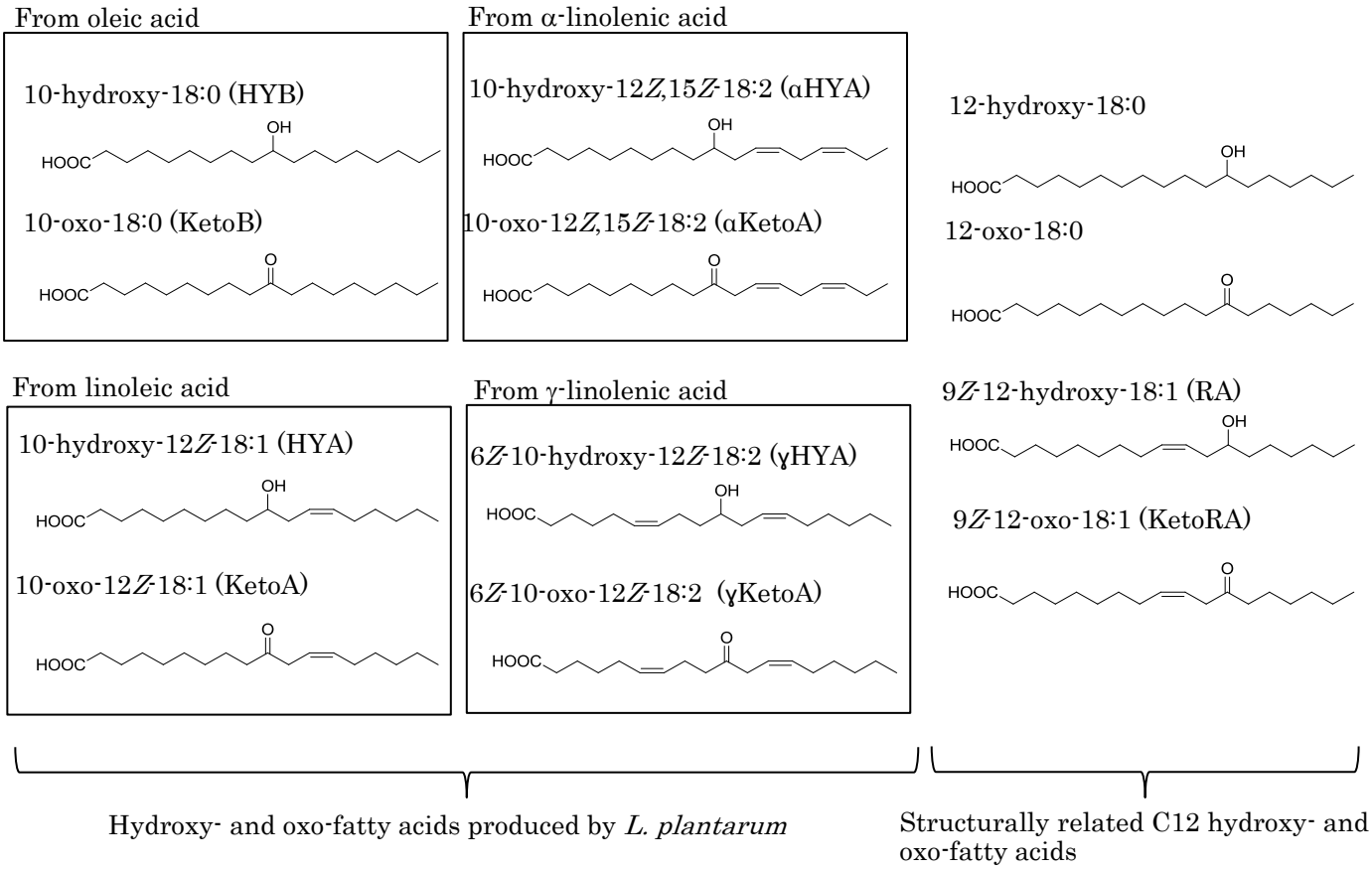


Fig. 1.

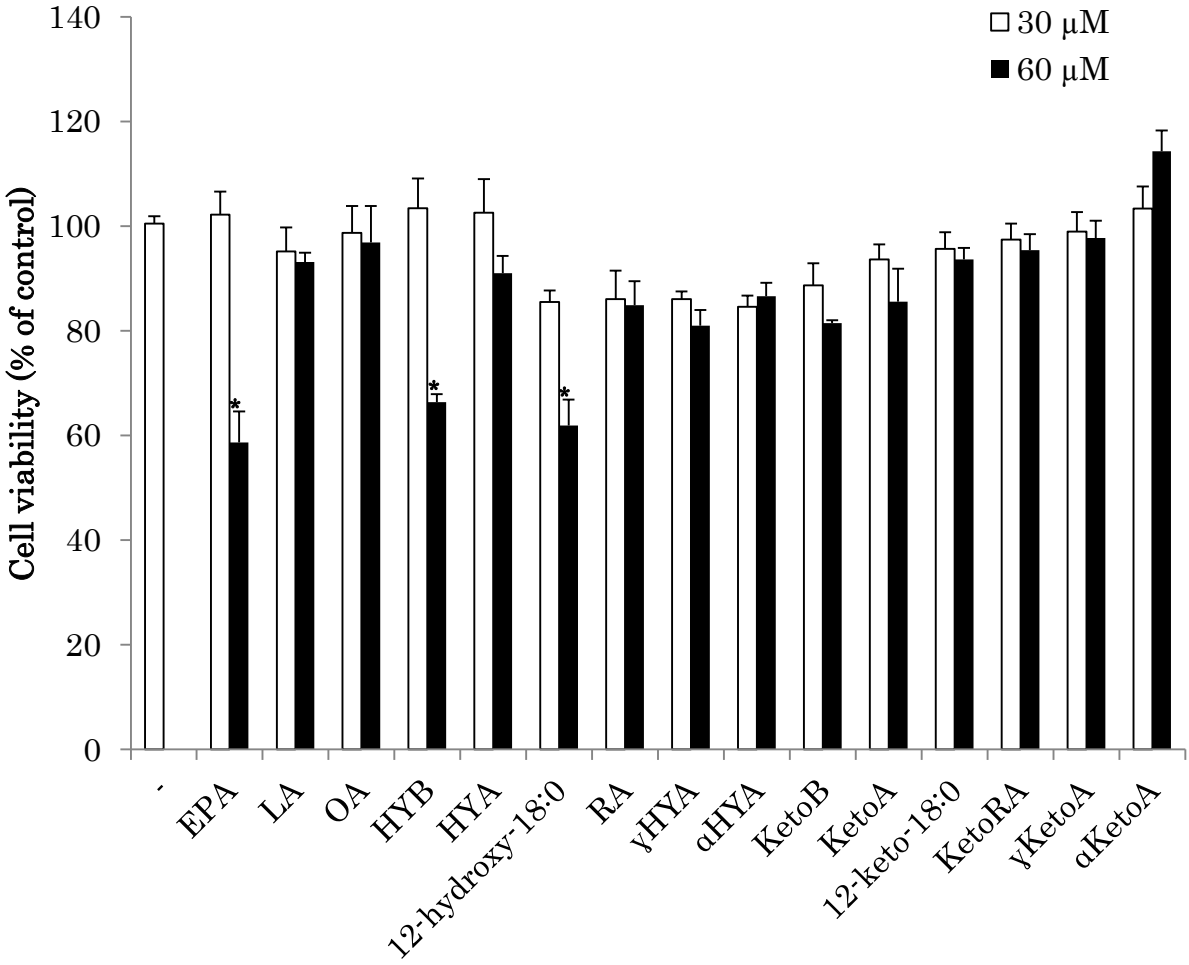


Fig. 2.

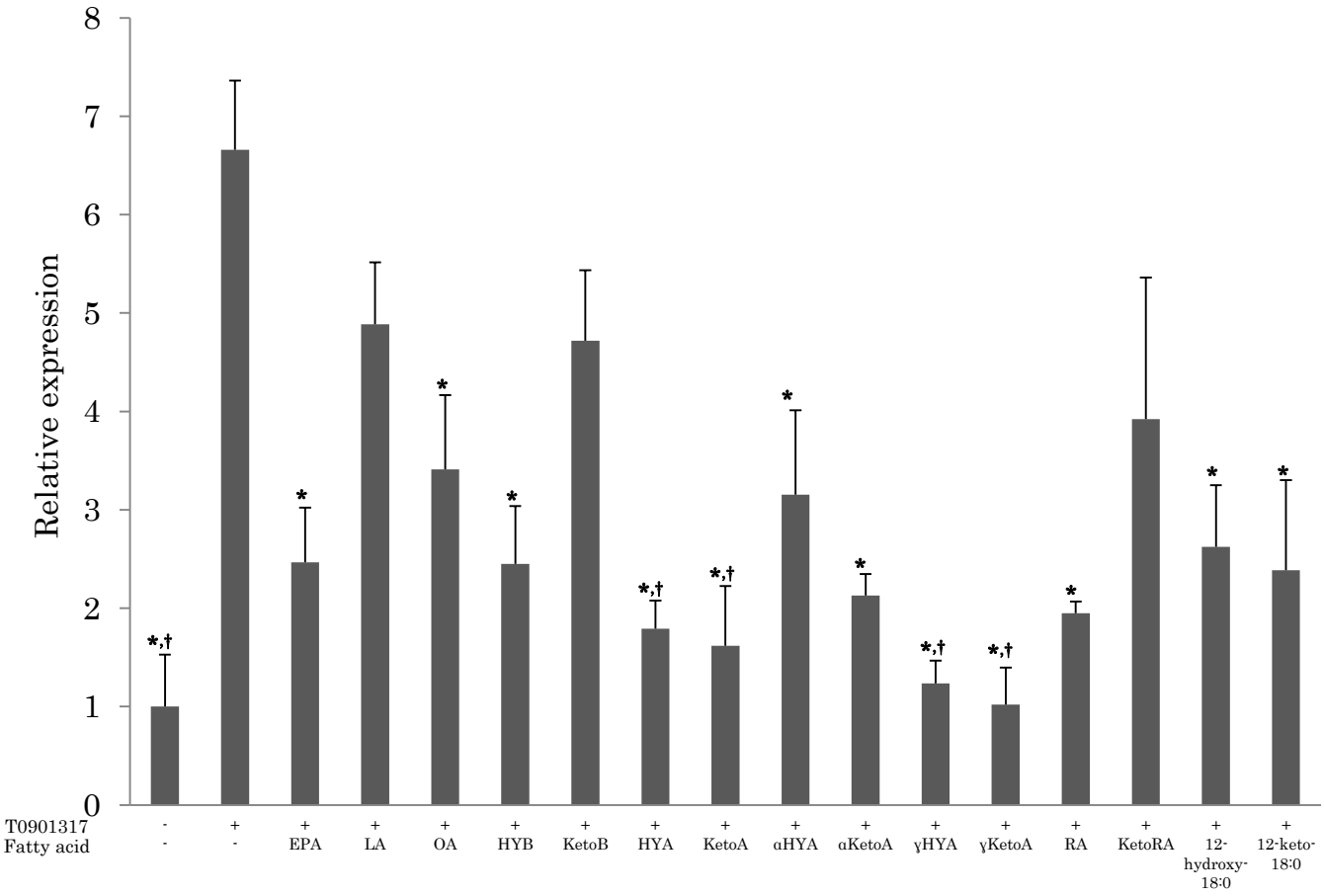


Fig. 3.

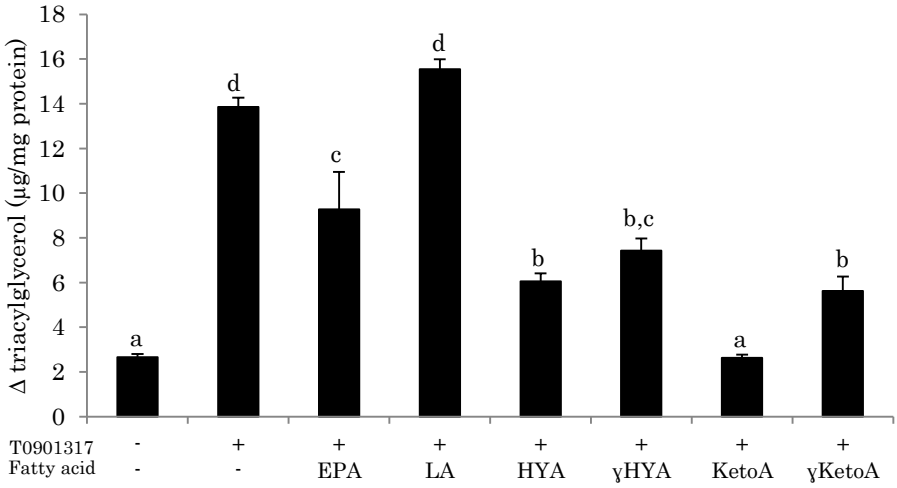


Fig. 4.

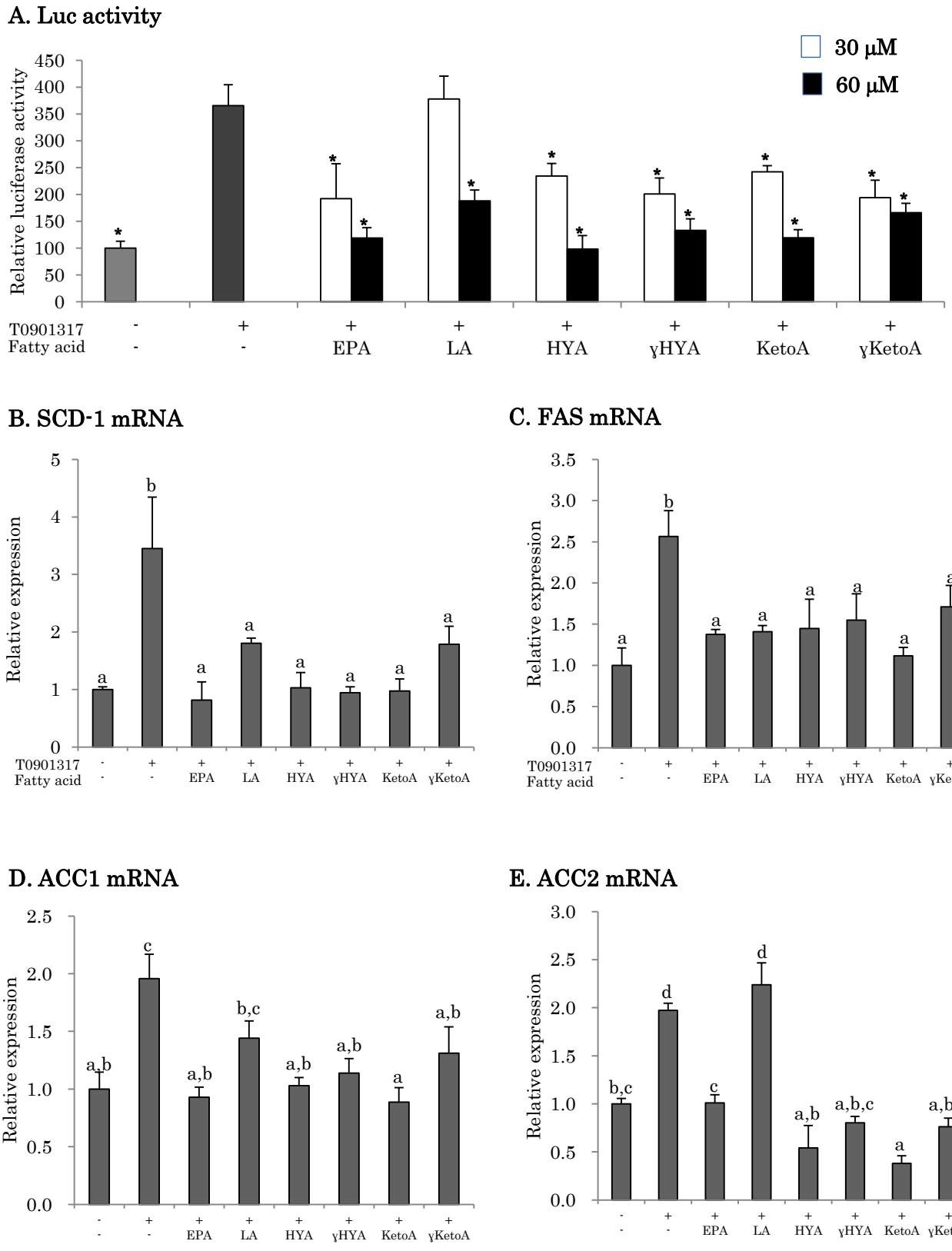
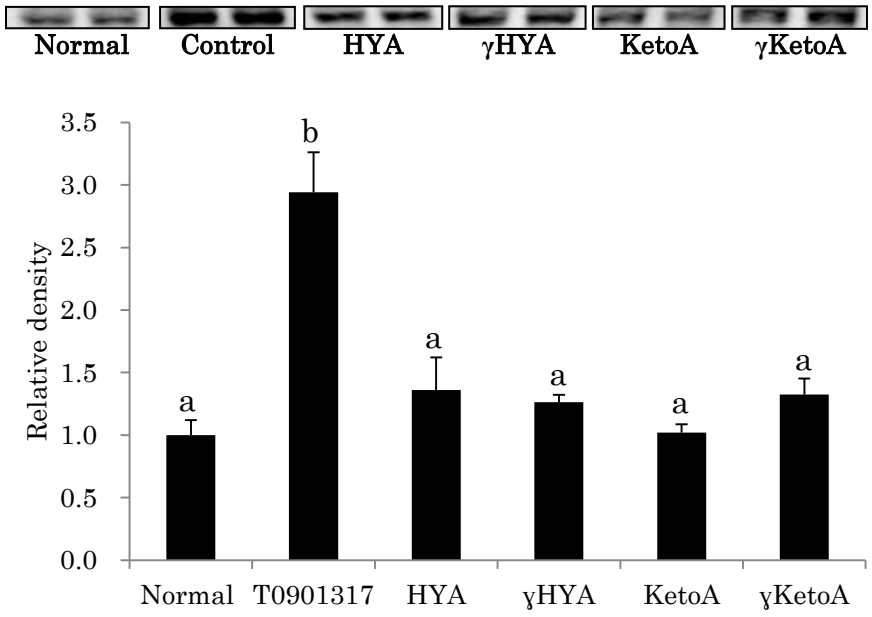


Fig. 5.

(A) Precursor SREBP-1



(B) Mature SREBP-1

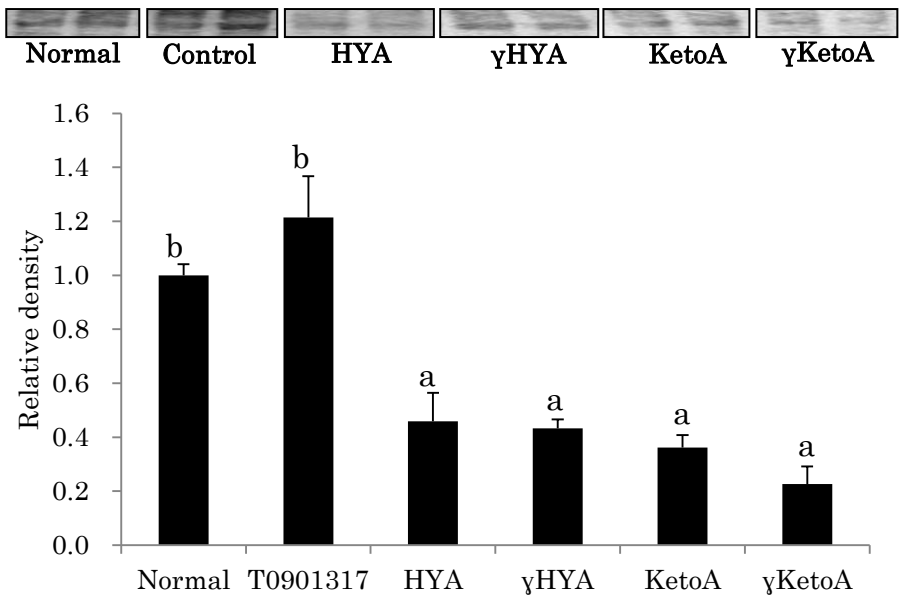


Fig. 6.

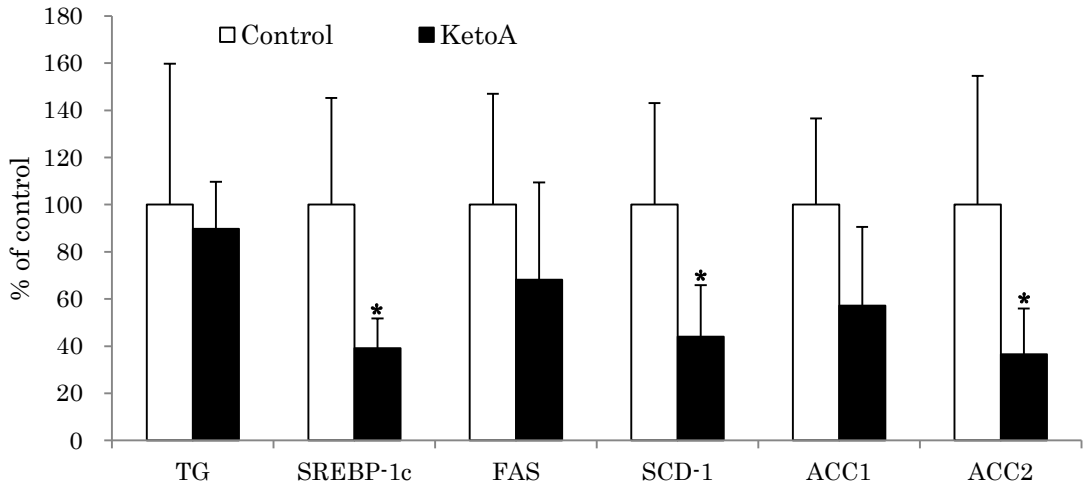


Fig. 7.