Trans geometric isomers of EPA decrease LXRα-induced cellular triacylglycerol via suppression of SREBP-1c and PGC-1β

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Abstract **Dietary mono- or di-*trans* fatty acids with chain lengths of 18–22 increase the risk of cardiovascular diseases because they increase LDL cholesterol and decrease HDL cholesterol in the plasma. However, the effects of *trans* isomers of PUFAs on lipid metabolism remain unknown. Dietary PUFAs, especially eicosapentaenoic acid (EPA) in marine oils, improve serum lipid profiles by suppressing liver X receptor α (LXRα) activity in the liver. In this study, we compared the effects of *trans* geometric isomers of eicosapentaenoic acid (TEPA) on triacylglycerol synthesis induced by a synthetic LXRα agonist (T0901317) with the effects of EPA in HepG2 cells. TEPA significantly decreased the amount of cellular triacylglycerol and the expression of mRNAs encoding fatty acid synthase, stearoyl-CoA desaturase-1, and glycerol-3-phosphate acyltransferase induced by T0901317 compared with EPA. However, there was no significant difference between the suppressive effect of TEPA or EPA on the expression of sterol-regulatory element binding protein-1c (SREBP-1c) induced by T0901317. We found that TEPA, but not EPA, decreased the mRNA expression of peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β), which is a coactivator of both LXRα and SREBP-1. These results suggest that the hypolipidemic effect of TEPA can be attributed to a decrease not only in SREBP-1 but also in PGC-1β expression.**—Zaima, N., T. Sugawara, D. Goto, and T. Hirata. ***Trans* geometric isomers of EPA decrease LXRα-induced cellular triacylglycerol via suppression of SREBP-1c and PGC-1β.** ***J. Lipid Res.*** **2006.** 47: **2712–2717.**

Supplementary key words eicosapentaenoic acid • liver X receptor α • sterol-regulatory element binding protein-1c • peroxisome proliferator-activated receptor γ coactivator 1β

Most naturally occurring unsaturated fatty acids have only *cis* double bonds. However, *trans* fatty acids are found in several foods, including infant formulas, shortenings, vegetable oils and fish oils (1–3), because partial hydrogenation of edible oils converts some *cis* double bonds to *trans* double bonds without changing their location. Dietary *trans* fatty acids increase the risk of cardiovascular diseases. The intake of mono- or di-*trans* fatty acids with chain lengths of 18–22, from partially hydrogenated edible oils and fats, significantly increases LDL cholesterol and reduces HDL cholesterol in the plasma (4, 5). The *trans* fatty acid of C18:1 can also stimulate the expression of lipogenic genes in the livers of mice (6). In contrast, there is little information about the effects of *trans* geometric isomers, without changing the double bond location, of PUFAs on lipid metabolism.

Dietary PUFAs, especially eicosapentaenoic acid (EPA) in marine oils, improve serum lipid profiles (7). The hypolipidemic effect of PUFAs is attributable both to a decrease in lipogenesis and an increase in fatty acid β-oxidation. It has been well established that liver X receptor α (LXRα) and sterol-regulatory element binding protein-1c (SREBP-1c) play crucial roles in the transcriptional regulation of lipogenic genes in the liver (8, 9). Oxysterols, such as 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol, and 24(*S*),25-epoxycholesterol, are endogenous LXRα ligands that serve as modulators of LXRα activities. PUFAs compete with LXRα ligands in the activation of the ligand binding domain (LBD) (10, 11). LXRα regulates the expression of many lipogenic genes, including SREBP-1c (8), which is synthesized as a 125 kDa precursor protein attached to the endoplasmic reticulum (12). In response to insulin stimulation (13), the membrane-bound precursor is cleaved to a 68 kDa N-terminal fragment that translocates to the nucleus and activates the expression of lipogenic genes such as FAS, stearoyl-coenzyme A desaturase-1 (SCD-1), and glycerol-3-phosphate acyltransferase (GPA). PUFAs suppress SREBP-1c expression by inhibiting LXRα binding to liver X receptor response elements (LXREs), which leads to the decrease in expression of lipogenic genes. A recent study reported that peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β) coactivates the LXRα and SREBP families and increases circulating triacylglycerol and cholesterol in VLDL particles (6). PGC-1β may be a

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key cofactor linking the dietary intake of *trans* fatty acids with hyperlipidemia.

Trans geometric isomers of eicosapentaenoic acid (TEPA) have been found as a minor component in vivo (14). *Trans* isomers of α-linolenic acid are elongated and are desaturated to TEPA in rats fed a diet enriched with those isomers (15). In addition, *trans* isomerization of EPA may occur in vivo, because *trans* isomers of arachidonic acid are generated by NO_2-mediated isomerization (16) and are found in human blood plasma (17). TEPA has different physiological effects on platelet aggregation (18), oxidative stability, and anti-inflammation (19) compared with EPA. However, the effect of TEPA on lipid metabolism remains unknown.

The aim of this study was to clarify the effects of TEPA on lipid metabolism. We compared the effect of TEPA on triacylglycerol synthesis induced by a synthetic LXRα agonist (T0901317) with that of EPA in HepG2 cells. We used a mixture of TEPA prepared from *p*-toluenesulfinic acid, because there are many possible structures of TEPA.

MATERIALS AND METHODS

Preparation of TEPA

The mixture of TEPA was prepared using a chemical catalyst (*p*-toluenesulfinic acid) and was analyzed by HPLC and GC-MS according to a previously described method (19). The TEPA used in this study consisted of 71% *trans* isomers, without changing the double bond location, and 29% *cis* isomers (EPA). The sample contained 0.25% conjugated dienes, with no detectable conjugated trienes, conjugated tetraenes, or conjugated pentaenes.

Lipid extraction and triacylglycerol quantification

HepG2 cells (JCRB 1054; Health Science Research Resources Bank, Osaka, Japan) were plated on six-well plates at 2.0×10^5 cells/ml for 24 h in DMEM supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin). The cells were then treated with EPA, TEPA, and/or T0901317 (50 nM) in serum-supplemented medium. Fatty acids and T0901317 were dissolved in ethanol. After incubation for 72 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 an N_2 stream, and triacylglycerol was quantified using a triglyceride E-test kit (Wako Pure Chemical Industries, Osaka, Japan).

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

HepG2 cells were plated on 12-well plates at 2.0×10^5 cells/ml in DMEM supplemented with 10% fetal calf serum and antibiotics as detailed above. After 24 h of incubation, each fatty acid was added to HepG2 cells with T0901317 (50 nM) in serum-free medium containing 0.1% BSA. After 24 h of incubation, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNAs were treated with RNase-free DNase (Invitrogen) to remove contaminating genomic DNA. After inactivating DNase by adding 20 mM EGTA and heating at 65°C for 10 min, each RNA was transcribed to cDNA using SuperScript RNase H^- reverse transcriptase (Invitrogen) 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, then 0.08 μl of the mixture was added to 4 μl of iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA) and 1.6 μl of gene-specific primers in a final volume of 8 μl. Primers used for the quantification of each gene are listed in **Table 1**. Primer pairs were selected to yield gene-specific single amplicons based on analyses by melting curves and by agarose gel electrophoresis. Real-time quantitative PCR was performed using a DNA Engine Opticon system (Bio-Rad Laboratories). The thermal cycler parameters were as follows: 3 min at 95°C for one cycle, followed by amplification of the cDNA for 40 cycles with melting for 15 s at 95°C and with annealing and extension for 30 s at 60°C. Values were normalized using 18s rRNA as an endogenous internal standard.

Plasmid constructs used for luciferase assay

The LXRE in the human SREBP-1c promoter was cloned into the pGL3-Promoter vector (Promega, Madison, WI) according to

TABLE 1. Real-time RT-PCR primers for the quantification of human mRNA

mRNA	Sequence	Reference or Accession Number
SREBP-1c	5′-GGAGGGGTAGGGCCAACGGCCT-3′ 5′-CATGTCTTCGAAAGTGCAATCC-3′	20
SREBP-la	5′-TCAGCGAGGCGGCTTTGGAGCAG-3′ 5′-CATGTCTTCGATGTCGGTCAG-3′	20
FAS	5′-ACAGGGACAACCTGGAGTTCT-3′ 5′-CTGTGGTCCCACTTGATGAGT-3′	20
Stearoyl-coenzyme A desaturase-l	5′-TGGTTTCACTTGGAGCTGTG-3′ 5′-GGCCTTGGAGACTTTC'TTCC-3′	NM_005063
Glycerol-3-phosphate acyltransferase	5′-TTGGGTTTGCGGAATGTTAT-3′ 5′-GGCAGAACCATCAGGGTTTA-3′	NM_020918
Apolipoprotein A-II	5′-GAGCTTTGGTTCGGAGACAG-3′ 5′-TGTGTTCCAAGTTCCACGAA-3′	NM_001643
Peroxisome proliferator-activated receptor γ coactivator 1β	5′-ATGACTCCGAGCTCTTCCAG-3′ 5′-CGAAGCTGAGGTGCATGATA-3′	NM_133263
18s	5′-TAAGTCCCTGCCCTTTGTACACA-3′ 5′-GATCCGAGGGCCTCACTAAAC-3′	20
Liver X receptor α	5′-GGAGGTACAACCCTGGGAGT-3′ 5′-AGCAATGAGCAAGGCAAACT-3′	NM_005693

SREBP, sterol-regulatory element binding protein.

the method of Oberkofler et al. (21). The LXRE construct contained two LXREs in the SREBP-1c promoter (pLXREs-Luc). The cDNA for LXRα (hLXRα) was generated from HepG2 cells by RT-PCR amplification of the entire LXRα coding region (GenBank accession number U22662). A 1,350 bp cDNA fragment was amplified using 5′-TG**GGTACC**AAAGAGATGTCCTTGTGGCTGG-3′ (+29 to +50) as the forward primer and 5′-TG**CTCGAG**TCATTCGTGGACATCCCAG-3′ (+1,361 to +1,379) as the reverse primer and was ligated into the pcDNA(+) 3.1 vector (Invitrogen). *Asp*718 and *Xho*I restriction enzyme sites were introduced into the primer sequences, as shown in boldface. Each construct was verified by sequencing.

Transient transfection and luciferase assay

Before transfection, HepG2 cells and HEK293 cells (JCRB 9068; Health Science Research Resources Bank) were grown to confluence on 96-well culture plates. Vectors, including pLXREs-Luc (25 ng), hLXRα (10 ng), and an internal *Renilla* luciferase control vector (40 ng), were cotransfected using Lipofectamine 2000 reagent (2 μl/μg DNA; Invitrogen) in serum-free medium. After overnight transfection, cells were treated with EPA, TEPA, and/or T0901317 for 24 h in serum-free medium containing 0.1% BSA. After incubation, luciferase activities were measured with a luminometer using the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega).

Cell fractionation and immunoblotting

HepG2 cells were plated on six-well plates at 2.0×10^5 cells/ml for 24 h in DMEM supplemented with 10% fetal calf serum and antibiotics as detailed above. The cells were then treated with EPA, TEPA, and/or T0901317 (50 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. (22). Protein concentrations were measured according to the Bradford method (23). For immunoblot analysis, given amounts of membrane fractions (25 μg) and nuclear extracts (30 μg) were separated by 7% and 10% SDS-PAGE, respectively, and were then transferred to Clear Blot Membrane-P polyvinylidene difluoride filters (ATTO, Tokyo, Japan). The filters were incubated with rabbit polyclonal anti-SREBP-1 antibody (H-160, 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were visualized with alkaline phosphatase-conjugated anti-rabbit IgG (1:500 dilution; Cell Signaling Technology, Danvers, MA). The intensity of each band was quantified using the Scion Image program (Scion Corp.; available as a free download at http://www.scioncorp.com).

Statistical analysis

Data are reported as means ± SD. The results were analyzed by one-way ANOVA with Fisher's protected least significant difference using Stat View software (SAS Institute, Cary, NC).

RESULTS

Suppression of triacylglycerol synthesis by TEPA in HepG2 cells

We determined increased triaclyglycerol levels in HepG2 cells to estimate the effect of TEPA on lipogenesis (**Fig. 1**). The synthetic LXRα agonist, T0901317, significantly increased cellular triaclyglycerol levels (164.6 μg/mg protein) after 72 h of incubation. TEPA at 10 μM significantly suppressed the increase of T0901317-induced triacylglycerol levels more than EPA.

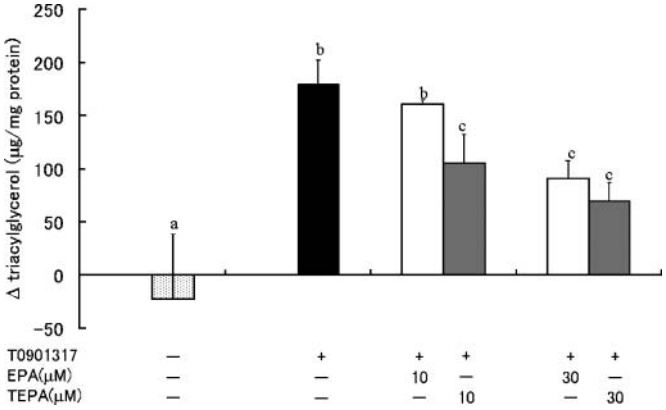

Fig. 1. Effect of *trans* geometric isomers of eicosapentaenoic acid (TEPA) on triacylglycerol synthesis in HepG2 cells. Each fatty acid was added to HepG2 cells with T0901317 (50 nM) in serum-supplemented medium for 72 h. The final ethanol concentration was 0.2%. The increased triacylglycerol level per milligram of protein (triacylglycerol level after 72 h of incubation minus that of reference cells) is shown. Data are reported as means ± SD (n = 3). Values with different letters are significantly different ($P < 0.05$). EPA, eicosapentaenoic acid.

Regulation of lipogenic gene mRNA levels by TEPA

To investigate the mechanism by which TEPA decreased cellular triacylglycerol levels, FAS, SCD-1, and GPA mRNA levels were measured (**Fig. 2A–C**). Both EPA and TEPA decreased the expression of FAS and SCD-1 mRNAs in a dose-dependent manner. TEPA significantly decreased the T0901317-induced expression of FAS, SCD-1, and GPA mRNAs more than EPA. No difference in cell viability was observed with TEPA or EPA treatment. Neither EPA nor TEPA had any effect on apolipoprotein A-II mRNA expression as a control gene (Fig. 2D).

Regulation of LXRE in the SREBP-1c promoter by TEPA

We generated a luciferase construct containing the two LXREs that exist in the human SREBP-1c promoter and performed luciferase reporter gene assays in both HepG2 cells and HEK293 cells (**Fig. 3**), because LXRα and SREBP-1c are key regulators of FAS, SCD-1, and GPA mRNA expression. In the presence of T0901317, pLXREs-Luc activity in HepG2 cells and in HEK293 cells was increased by 160% and 223%, respectively, compared with the vehicle control (Fig. 3A, B). EPA and TEPA suppressed the T0901317 induction of pLXREs-Luc activity (Fig. 3C, D). TEPA suppressed pLXREs-Luc in HepG2 cells and in HEK293 cells maximally at 30 and 20 μM, respectively. There were no significant differences in the suppressive effects of TEPA and EPA.

Regulation of SREBP-1c expression by TEPA

The expression of SREBP-1c mRNA in cells treated with T0901317 was 7.2-fold higher than that in vehicle-treated control cells (**Fig. 4A**). TEPA significantly downregulated the T0901317 induction of SREBP-1c mRNA in a dose-dependent manner, and the suppressive effect of TEPA

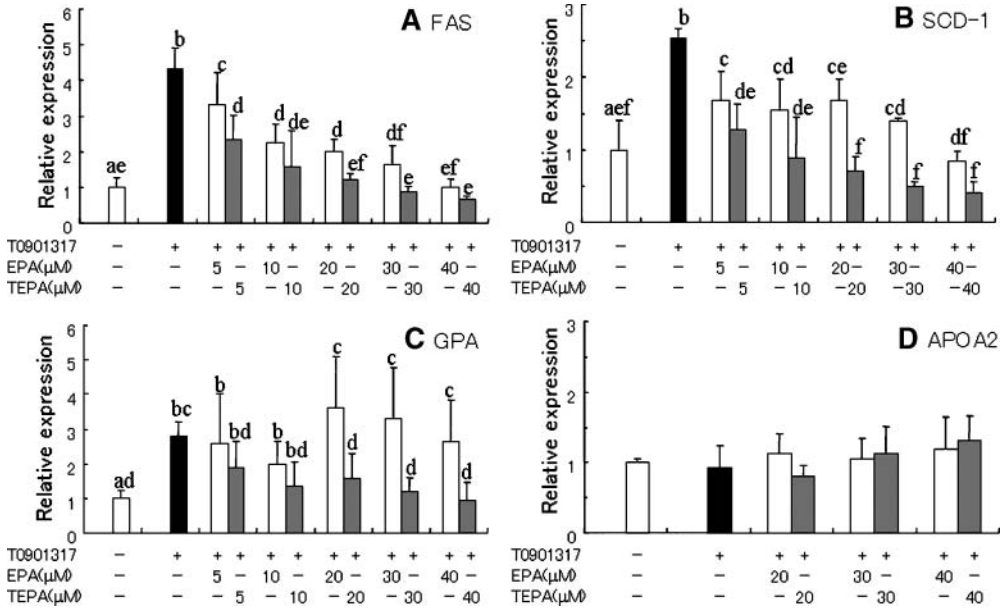

Fig. 2. Effects of EPA or TEPA on the expression of FAS (A), stearoyl-coenzyme A desaturase-1 (SCD-1; B), glycerol-3-phosphate acyltransferase (GPA; C), and apolipoprotein A-II (APOA2; D) mRNAs. Each fatty acid was added to HepG2 cells with T0901317 (50 nM) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.2%. The expression levels are presented as fold induction relative to the vehicle control (ethanol). Data are reported as means ± SD (n = 3). Values with different letters are significantly different ($P < 0.05$).

was equal to that of EPA. To investigate the effect of TEPA on SREBP-1 protein levels, the full-length precursor form in the cell membrane (125 kDa) and the cleaved mature form (68 kDa) in the nuclear extract were estimated by immunoblotting. Because the antibody used cannot distinguish between the SREBP-1c and -1a isoforms, we use the general term SREBP-1 to refer to the results. Representative blots are shown in Fig. 4B. T0901317 increased the levels of both the precursor and the mature forms of SREBP-1. TEPA as well as EPA decreased the T0901317 induction of both the precursor and mature forms of SREBP-1. The decrease of SREBP-1 can be attributed to the suppression of SREBP-1c protein, because neither TEPA nor EPA affected the expression of SREBP-1a mRNA (data not shown).

Downregulation of PGC-1β mRNA level by TEPA

To investigate the mechanism by which TEPA significantly decreased the expression of lipogenic gene mRNAs more than EPA, we examined the effect of TEPA on the

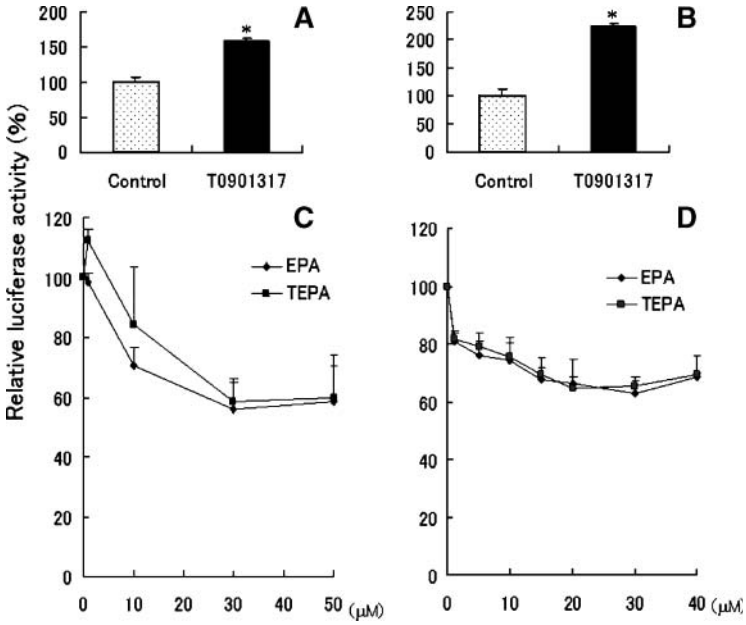

Fig. 3. Suppression of pLXREs-Luc expression by EPA or TEPA. A, B: Ethanol (vehicle control) or T0901317 (50 nM) was added to transfected HepG2 cells (A) and HEK293 cells (B) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.2%. The relative luciferase activity compared with the control is shown. Data are reported as means ± SD (n = 3). * Significant difference from control ($P < 0.05$). C, D: The indicated concentration of each fatty acid was added to transfected HepG2 cells (C) and HEK293 cells (D) with T0901317 (50 nM) in serum-free medium containing 0.1% BSA. The relative luciferase activity compared with the T0901317-induced control is shown. Data are reported as means ± SD (n = 3).

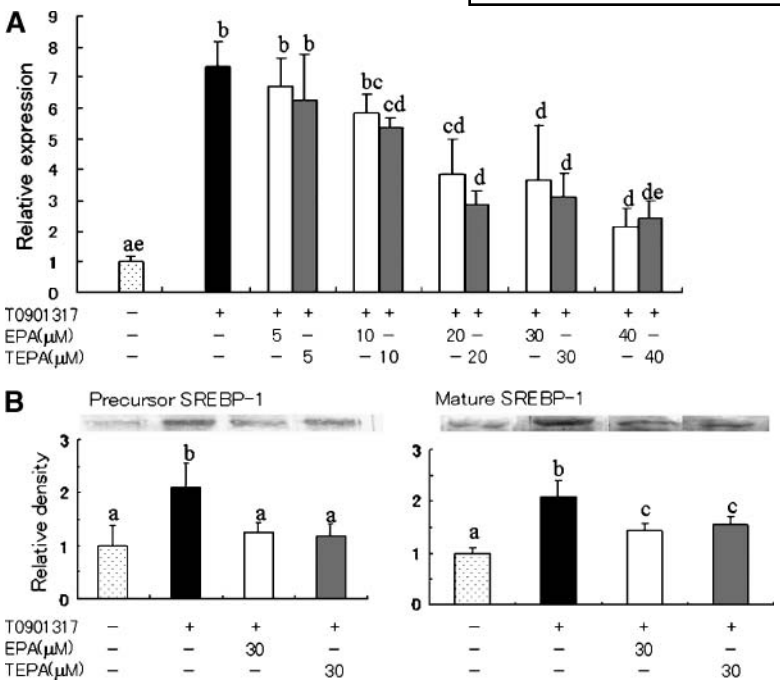

Fig. 4. Effects of EPA or TEPA on sterol-regulatory element binding protein-1 (SREBP-1) expression. A: Effect of EPA or TEPA on SREBP-1c mRNA expression. Each fatty acid was added to HepG2 cells with T0901317 (50 nM) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.2%. The expression levels are presented as fold induction relative to the vehicle control (ethanol). B: Effect of EPA or TEPA on SREBP-1c protein expression. Each fatty acid was added to HepG2 cells with T0901317 (50 nM) in serum-free medium containing 0.1% BSA. Representative Western blots are shown. 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). Values with different letters are significantly different ($P < 0.05$).

mRNA expression of PGC-1β, the coactivator of LXRα and SREBPs (**Fig. 5**). We found a significant difference between the effects of TEPA and EPA. TEPA downregulated the level of PGC-1β mRNA at 40 μM by 52% compared with the vehicle control. In contrast, EPA significantly increased the level of PGC-1β mRNA. T0901317 had no effect on PGC-1β mRNA expression.

DISCUSSION

This study shows that TEPA suppresses T0901317-induced triacylglycerol synthesis in HepG2 cells and downregulates the expression of lipogenic genes (such as FAS, SCD-1, and GPA) more than EPA (Figs. 1, 2A–C). TEPA also decreases pLXREs-Luc activity stimulated by T0901317 (Fig. 3) and SREBP-1c expression (Fig. 4), which suggests that TEPA, like EPA, suppresses SREBP-1c expression by competing with T0901317 in the activation of LXRα. Moreover, TEPA decreases PGC-1β mRNA expression, whereas EPA does not.

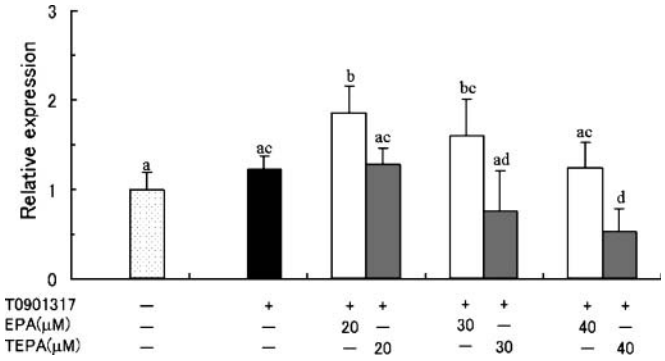

Fig. 5. Effects of EPA or TEPA on the expression of peroxisome proliferator-activated receptor γ coactivator 1β mRNA. Each fatty acid or ethanol (vehicle control) was added to HepG2 cells with T0901317 (50 nM) in serum-free medium containing 0.1% BSA. The final ethanol concentration was 0.2%. The expression levels are presented as fold induction relative to the vehicle control (ethanol). Data are reported as means ± SD (n = 3). Values with different letters are significantly different ($P < 0.05$).

It has been proposed that the arginine in the LBD of LXRα (R305) interacts with the hydroxyl group of LXRα ligands (24, 25). Svensson et al. (25) reported that R305 is the coordinator of the carboxylate group in fatty acids. The interaction between R305 and the carboxylate group of fatty acids may be important for antagonist function. Yoshikawa et al. (11) proposed that the degree of unsaturation of fatty acids is a factor for the inhibitory effect of SREBP-1c expression, but whether they are n-3 or n-6 is irrelevant. The magnitude of inhibition of each PUFA on LXRα LBD activation, using an expression plasmid of the LBD of LXRα fused to the Gal4 DNA binding domain, is as follows: arachidonic acid (C20:4) ≈ docosahexaenoic acid (C22:6) ≈ EPA > linoleic acid (C18:2) > oleic acid (C18:1) >> stearic acid (C18:0) (11). In this study, the suppressive effect of TEPA on T0901317-activated pLXREs-Luc activity was equal to that of EPA. An important factor required for PUFA to antagonize LXRα activation appears to be the number of double bonds and the carbon chain length, but not the double bond configuration. PUFAs having a 20–22 carbon length and more than three double bonds might be suitable antagonists of LXRα.

Unexpectedly, TEPA significantly decreased FAS, SCD-1, and GPA mRNA more than EPA, although there was no significant difference between the suppressive effects of TEPA or EPA on SREBP-1c expression or on pLXREs-Luc activity (Figs. 1A, 2). These results could be attributable in part to the suppression of PGC-1β expression by TEPA

(Fig. 5), because PGC-1β stimulates hepatic lipid synthesis by coactivating SREBP-1 and LXRα (6). Our data suggest that PGC-1β is important for the full induction of lipogenic genes regulated by SREBP-1 and LXRα; thus, PGC-1β provides a therapeutic target for the metabolic syndrome. Despite the EPA increase of PGC-1β mRNA, EPA decreased the expression of lipogenic genes in this study. This result is caused by the downregulation of SREBP-1c, which is an essential nuclear factor for PGC-1β-mediated transcription of lipogenic genes. The mechanism by which EPA increases PGC-1β mRNA is unclear, but the induction of PGC-1β by EPA is consistent with a previous report (6).

Consequently, *trans* isomerization of EPA increases the favorable effect of EPA on the expression of lipogenic genes, which leads us to speculate that TEPA decreases serum lipid concentrations more than EPA in vivo. The biological activity of TEPA on lipid metabolism may be distinguished from that of other unfavorable *trans* fatty acids having a few double bonds with chain lengths of 18–22. The adverse effect of *trans* fatty acids on serum lipid profiles may depend on their number of carbon atoms and double bonds. Because the TEPA used in this study was a mixture of *trans* isomers, it will be important to clarify the structure-activity relationships among a variety of *trans* isomers in future studies. In addition, insulin is an important inducer of SREBP-1c in vivo. It will be essential to examine the effect of TEPA on insulin-mediated lipogenesis both in vivo and in vitro.

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