Very long-chain-fatty acids enhance adipogenesis through coregulation of Elovl3 and PPARγ in 3T3-L1 cells

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Submitted 6 December 2011; accepted in final form 15 March 2012

Kobayashi T, Fujimori K. Very long-chain-fatty acids enhance adipogenesis through coregulation of Elovl3 and PPARγ in 3T3-L1 cells. Am J Physiol Endocrinol Metab 302: E1461–E1471, 2012. First published March 20, 2012; doi:10.1152/ajpendo.00623.2011.—Here, we show that Elovl3 (elongation of very long-chain fatty acids 3) was involved in the regulation of the progression of adipogenesis through activation of peroxisome proliferator-activated receptor (PPAR)γ in mouse adipocytic 3T3-L1 cells. The expression of the Elovl3 gene increased during adipogenesis, the expression pattern of which was similar to that of the PPARγ gene. Troglitazone, a PPARγ agonist, enhanced Elovl3 expression in adipocytes, as did that of other PPARγ target genes. Promoter-reporter analysis demonstrated that three PPAR-responsive elements in the Elovl3 gene promoter had the potential to activate its expression in 3T3-L1 cells. Moreover, a chromatin immunoprecipitation assay revealed that PPARγ bound these PPAR-responsive elements of the Elovl3 promoter. When the Elovl3 mRNA level was suppressed by its siRNAs, the level of other PPARγ target genes was also repressed. In a mammalian two-hybrid assay, C18:1 and C20:1 very long-chain fatty acids (VLCFAs), which are the products of Elovl3 and activated PPARγ function. In addition, these same VLCFAs could prevent the Elovl3 siRNA-mediated suppression of adipogenesis by enhancing the expression of adipogenic, lipolytic, and lipogenic genes. Moreover, this VLCFAs-mediated activation was repressed by a PPARγ antagonist. These results indicate that the expression of the Elovl3 gene was activated by PPARγ during adipogenesis. Elovl3-produced C18:1 and C20:1 VLCFAs acted as agonists of PPARγ in 3T3-L1 cells. Thus, the Elovl3-PPARγ cascade is a novel regulatory circuit for the regulation of adipogenesis through improvement of PPARγ function in adipocytes.

ADIPOCYTES HAVE BEEN IDENTIFIED as a type of endocrine cell that secretes various biologically active molecules, termed adipokines, that regulate metabolism (28). Obesity is a critical health problem throughout the world and serves as a significant risk factor for a number of diseases such as diabetes, heart disease, and hypertension (27).

Adipogenesis is regulated through complex processes including coordinated changes in hormone sensitivity and gene expression. The molecular mechanisms in the regulation of adipogenesis have been extensively studied, and many transcription factors involved in this regulation (6). Among a number of transcription factors involved in the regulation of adipogenesis, peroxisome proliferator-activated receptor (PPAR)γ and CCAAT/enhancer-binding proteins (C/EBPs) are master regulators that control many adipogenic and lipogenic genes (18, 29, 44). PPARγ belongs to the nuclear receptor superfamily of transcription factors, which are activated in a ligand-mediated fashion (3, 31, 33, 35). The activity of PPARγ is exploited by synthetic PPARγ ligands, notably the thiazolidinediones such as pioglitazone and rosiglitazone, to improve insulin sensitivity (10).

Fatty acids are synthesized from acetyl-CoA through two enzymatic steps, including acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). ACC is the rate-limiting enzyme of fatty acid biosynthesis, and fatty acids are further elongated to palmitic acid by the action of FAS. Both of these enzymes are localized in the cytosol. Further elongation of fatty acids is catalyzed by long-chain fatty acid elongases (elongation of very long-chain fatty acids [Elov1s]) that are predominantly localized in the membranes of the endoplasmic reticulum (ER) (4, 12, 14, 41) and exert substrate-specific elongation with respect to fatty acid length and level of unsaturation (5, 12, 19). The members of the Elovl family are mammalian homologs of yeast ELOs and catalyze the elongation of very long-chain fatty acids (VLCFAs) (19). Seven Elovl family enzymes (Elov1–7) have been identified in mammals, and these elongases are the rate-limiting enzymes for the elongation of fatty acids (40).

Elov3 is expressed in liver and adipose tissues and is involved in response to high-fat diet and exposure to cold stress (42, 43). Elov3 catalyzes the elongation of C16, C18, or C20 saturated and monounsaturated VLCFAs (9, 16, 26, 42, 46). The expression of the Elovl3 gene is activated by PPAR agonists (11, 13) and norepinephrine (13) in brown adipose tissue (BAT). In contrast, a liver RXRα agonist, T0901317, represses the expression of the Elovl3 gene in BAT (11). Elovl3-ablated mice show impaired formation of triglycerides and lipid droplets in both skin and BAT (43, 46). However, the roles and regulation of Elovl3 in white adipose tissue have been poorly studied.

In the present study, we demonstrate that Elovl3 was transcriptionally regulated by PPARγ in mouse adipocytic 3T3-L1 cells, and, in turn, that Elovl3-produced C18:1 and C20:1 VLCFAs activated the expression of PPARγ. Knockdown of Elovl3 caused decreases in fatty acid biosynthesis, adipogenesis, and lipolysis along with a reduction in the level of intracellular triglycerides. This is the first evidence that the Elovl3-PPARγ cascade is involved in the regulation of adipogenesis through improvement of PPARγ function in adipocytes.

MATERIALS AND METHODS

Cell culture. Mouse adipocytic 3T3-L1 cells and human HeLa cells (Human Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St.

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Lus, MO) containing 10% (vol/vol) fetal calf serum and antibiotics and maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Adipocyte differentiation of 3T3-L1 cells was initiated by incubation for 2 days in DMEM containing insulin (10 μg/ml; Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). On day 2, the medium was replaced with DMEM containing insulin (10 μg/ml) alone and changed every 2 days. Oil red O staining was carried out as described previously (8).

**RNA preparation and quantification of RNA level.** Total RNA was extracted by use of Sepasol-RNAI (Nacalai Tesque, Kyoto, Japan). First-strand cDNAs were synthesized from total RNA with random hexamer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) at 42°C for 60 min after initial denaturation at 72°C for 3 min, followed by heat denaturation of enzyme at 99°C for 5 min. The cDNAs were diluted and further utilized as the templates for quantitative PCR analysis.

Expression levels were quantified using a LightCycler system (Roche Diagnostics, Mannheim, Germany) with THUNDERBIRD SYBR PCR Mater Mix (Toyobo) and primer sets (Table 1). The expression level of the target genes was normalized to that of TATA-binding protein (TBP).

**Construction of reporter-promoter vectors and luciferase assay.** The luciferase reporter vectors carrying the mouse Elovl3 promoter were generated as follows: an ~1150-bp [corresponding to ~970 to +172; transcription initiation site is defined as +1 (1)] region of the Elovl3 promoter and sequentially deleted regions of it were cloned into the pGL3-Enhancer (E) vector (Promega, Madison, WI). Site-directed mutations were introduced using a QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) of the constructs were determined to verify the correct sequences.

3T3-L1 cells were caused to differentiate into adipocytes for 6 days, and then cotransfected with each construct (0.9 μg) and pRL-SV40 (0.1 μg, Promega) in 12-well plates, the latter plasmid carrying the Renilla luciferase gene under the control of the SV40 promoter as the transfection control, along with FuGENE6 Transfection Reagent (Roche Diagnostics) according to the manufacturer’s instructions. At 1 day after transfection, the cells were trypsinized and seeded into a 96-well assay plate and further cultured for one more day. The luciferase activities were measured using a Dual-Glo Luciferase Assay System (Promega). The reporter activity was calculated relative to that of pGL3(E) vector, which was defined as “1”. Data were performed from three independent experiments, and each experiment was performed in triplicate. The relative promoter activities were presented as the mean ± SD.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assays were performed as described previously (7). Briefly, 3T3-L1 cells were caused to differentiate into adipocytes in the presence of the potent PPARγ agonist troglitazone (10 μM) as the positive control for 6 days. The cells were cross-linked with 1% (vol/vol) formaldehyde for 10 min and then collected and lysed in SDS lysis buffer containing 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% (wt/vol) SDS, and protease inhibitor cocktail (Nacalai Tesque). Chromatin was sonicated and then incubated with a polyclonal antibody against PPARγ (H-100; Santa Cruz Biotechnology, Santa Cruz, CA) or normal IgG (Cell Signaling Technology, Danvers, MA) at 4°C overnight, followed by further incubation with protein G-Sepharose (GE Healthcare, Buckinghamshire, UK) at 4°C for 1 h. The Sepharose was washed, and the immunoprecipitated DNA-protein complexes were eluted and then reverse-cross-linked. The purified DNA fragments were used for PCR analysis with the following specific primer sets: 5'-TTTGCATCTTCTCATAGTC-3' and 5'-ACTCTTCTAGGGCTTACAGTTCTC-3' for PPRE1 and 5'-AAAGCTGCGACCCATATCTGG-3' and 5'-CCTTAGTGTGACTCATAAGGCAC-3' for PPAR response element (PPRE)/3. PCR was conducted with KOD FX DNA Polymerase (Toyobo) under the following conditions: initial denaturation at 94°C for 2 min, followed by 40 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 30 s. The resultant PCR products (expected amplified size was 220 bp for PPRE1 and 219 bp for PPRE2/3) were analyzed by performing 2% (wt/vol) agarose gel electrophoresis. Band intensity was measured with an LAS-3000 Lumimaging Analyzer (Fujiﬁlm, Tokyo, Japan) and analyzed with Multi Gauge software (Fujiﬁlm).

**Suppression by RNAi.** The following stealth siRNA for Elovl3–1 and -2 and stealth negative control (NC) siRNA were obtained from Invitrogen (Carlsbad, CA): Elovl3 siRNA 1: 5'-CAAGCUGUUGA-UCAAGAAAUU-3' and Elovl3 siRNA 2: 5'-CAACUGAAG-CUCAUGUUU-3'. 3T3-L1 cells were caused to differentiate into adipocytes for 2 days. The cells were transfected with either siRNA or NC siRNA (20 nM) using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics) according to the methods prescribed by the supplier and caused to differentiate into adipocytes as described above. A change of medium containing insulin alone and transfection with siRNA were carried out every 2 days.

**Measurement of intracellular triglyceride level.** 3T3-L1 cells were caused to differentiate into adipocytes for 8 days. Medium was changed every 2 days as described above. The cells were lysed with PBS containing 0.5% (vol/vol) Triton X-100, and incubated for 90°C for 30 min. After centrifugation at 12,000 g for 10 min to remove cell debris, the supernatant was further used for measurement of the intracellular triglyceride using a WAKO LabAssay Triglyceride Kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer’s instructions. Protein concentrations were measured with a Pierce BCA protein assay reagent (Thermo Scientific, Rockford, IL) according to the protocols prescribed by manufacturer.

**Western blot analysis.** The cells were lysed in RIPA buffer consisting 50 mM Tris-Cl, pH 8.0, containing 150 mM NaCl, 0.1% (wt/vol) SDS, 0.5% (wt/vol) sodium deoxycholate, 1% (vol/vol) NP-40, and 1% (vol/vol) Triton X-100 with protease inhibitor cocktail (Nacalai Tesque). Cell lysates were sonicated, and then centrifuged for 20 min at 12,000 g at 4°C to remove cell debris. Protein concentrations were measured as described above. The proteins (20 μg/lane) were separated on SDS-PAGE gels and transferred onto PVDF membranes. The following antibodies were used: 1α 25-hydroxyvitamin D₃ (Santa Cruz), 17β estradiol (Santa Cruz), and human PPARγ antibody (Sigma). The blots were detected using a chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK) at 4°C for 1 min. The membranes were washed, and the immunoprecipitated DNA-protein complexes were eluted and then reverse-cross-linked.

### Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Acc.No.</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elovl3</td>
<td>NM_007703</td>
<td>5'-ACTTTGGAAGAGTTTCCAGAATT-3'</td>
<td>5'-CCACTATGAGAAATAGGCTTACCC-3'</td>
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<tr>
<td>PPARγ</td>
<td>NM_011146</td>
<td>5'-CAGAGAAATACCAAAATGCGAGAAA-3'</td>
<td>5'-GGCCTGTCGGTCTCGAAGA-3'</td>
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<tr>
<td>aP2</td>
<td>NM_024406</td>
<td>5'-CACAGCCTTCTCAGCTGGAAAG-3'</td>
<td>5'-TTGGCGCAGAAGCCCACTC-3'</td>
</tr>
<tr>
<td>SCD</td>
<td>NM_009127</td>
<td>5'-TGCTGCTCTGTTGCTCT-3'</td>
<td>5'-GGTCCTGAGGCGAAGA-3'</td>
</tr>
<tr>
<td>ACC</td>
<td>NM_133360</td>
<td>5'-GCTGCGGTAGATCCGATTT-3'</td>
<td>5'-CTGACTGGGCCAGATCCTG-3'</td>
</tr>
<tr>
<td>FAS</td>
<td>NM_009788</td>
<td>5'-GCTGAGTGTGTGGAGATCGACG-3'</td>
<td>5'-AGTGGTGCTTCTCGGAGA-3'</td>
</tr>
<tr>
<td>ATGL</td>
<td>AY894805</td>
<td>5'-GCGCTGATCCTGGTCTCCAGA-3'</td>
<td>5'-TGAGTGTCGGGAGGAAACA-3'</td>
</tr>
<tr>
<td>HSL</td>
<td>NM_010719</td>
<td>5'-GCACTTGAGCTGGTGTGCTGTTG-3'</td>
<td>5'-CTGACGACGATCCTGCA-3'</td>
</tr>
<tr>
<td>MGL</td>
<td>NM_011844</td>
<td>5'-TCGAGAAGAGGTAGTGAGGAAGG-3'</td>
<td>5'-TCGACGAGCTGATGCGAAA-3'</td>
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<tr>
<td>TBP</td>
<td>NM_013684</td>
<td>5'-GTTGATGATGAGTTCCCCTACCAAGG-3'</td>
<td>5'-CTACGCAATGCTGGTGCTCA-3'</td>
</tr>
</tbody>
</table>

See text for definitions.
membranes (Immobilon P; Millipore, Bedford, MA) for Western blot analysis by use of a SNAP I.D. protein detection system (Millipore). The blots were incubated with anti-PPARγ (H-100, 1:1,000), anti-SCD (S-15; 1:1,000), or anti-FAS (H-300; 1:1,000) polyclonal antibody (SantaCruz Biotechnology) or with anti-ap2 (1:1,000), anti-ACC (1:1,000; Epitomics, Burlingame, CA), or anti-actin (AC-15; 1:5,000; Sigma) monoclonal antibody, followed by incubation with the appropriate secondary antibody, i.e., anti-rabbit or anti-mouse IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive signals were detected by use of a Luminata Forte Western HRP substrate (Millipore) and an LAS-3000 Luminoimage Analyzer (Fujifilm) and analyzed with Multi Gauge software (Fujifilm). Each expression level was normalized by that of actin.

Glycerol release assay. 3T3-L1 cells were transfected with Elovl3 siRNA and further cultured for 6 days. The culture medium was then collected and utilized for the measurement of glycerol released from the cells by the use of Free Glycerol Assay Reagent (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. Absorbance was measured at 492 nm using a Microplatereader EnBio RCAS (receptor cofactor assay system) for PPARγ agonists and GW-9662 (1 μM) as PPARγ antagonist were utilized. After 24 h of transfection, the cells were trypsinized and seeded into a 96-well assay plate. After 6 h, the medium was discarded, and 1% (wt/vol) BSA-DMEM containing one of the various BSA:fatty acid complexes was added; and the cells were further cultured for 1 day. Luciferase reporter activities were measured using the Bright-Glo Luciferase Assay System (Promega) according to the protocol prescribed by the manufacturer. The relative promoter activities were expressed as means ± SD from three independent experiments.

Nuclear receptor cofactor assay system. In vitro binding between PPARγ and fatty acids was investigated by use of ELISA-based EnBio RCAS (receptor cofactor assay system) for PPARγ (Fujikura Kasei, Ibaraki, Japan) according to the manufacturer’s instructions. The relative activity was calculated using the formula: B/Bmax = (A - B)/(A - B)%, where A is the OD450 value of the positive control in a CBP(+) well minus the value of the positive control in a CBP(-) well; B is the value of the NC in a CBP(+) well minus the OD450 value of the positive control in a CBP(-) well; and C is the value of the sample in a CBP(+) well minus the value of the sample in a CBP(-) well. All experiments were performed in triplicate.

Statistical analysis. Comparison of two groups was analyzed by Student’s t-test. For comparison of more than two groups with...
comparable variances, one-way ANOVA and a Tukey’s post hoc test were carried out. \( P < 0.05 \) was considered significant.

**RESULTS**

Expression of Elovl3 during adipocyte differentiation. Elovl3 mRNA level was under the detection limit in preadipocytes under our experimental conditions and increased during adipogenesis (Fig. 1A). Interestingly, the expression pattern of the Elovl3 gene during adipogenesis was similar to that of the PPAR\( \gamma \) gene (Fig. 1A).

To examine whether PPAR\( \gamma \) is involved in the transcriptional regulation of the Elovl3 gene, we investigated the responsiveness of the expression of the Elovl3 gene to a PPAR\( \gamma \) agonist. 3T3-L1 cells were caused to differentiate into adipocytes for 8 days in medium containing various concentrations (0–10 \( \mu \)M) of the PPAR\( \gamma \) agonist troglitazone. The expression levels of fatty acid-binding protein-4 (aP2) and stearoyl-CoA desaturase (SCD), which are PPAR\( \gamma \) target genes, were enhanced by this agonist during adipogenesis, being upregulated \( \sim 2.7- \) and 7.6-fold (for aP2; 1 and 10 \( \mu \)M agonist, respectively), and 2.6- and 5.4-fold (for SCD; 1 and 10 \( \mu \)M agonist, respectively) compared with those of the vehicle-treated differentiated cells (Fig. 1B). Likewise, the expression of the Elovl3 gene was increased during adipogenesis, \( \sim 6.5- \) and 13.2-fold in 1 and 10 \( \mu \)M Troglitazone-treated cells, respectively, compared with that of the vehicle-treated differentiated cells (Fig. 1B). These results reveal that the expression of the Elovl3 gene was enhanced during adipogenesis and that its expression profile was similar to that of the PPAR\( \gamma \) gene during adipocyte differentiation. Moreover, Elovl3 is upregulated upon troglitazone-induced adipogenesis, suggesting possible regulation by PPAR\( \gamma \), like that of the aP2 and SCD genes.

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**Fig. 2.** Identification of the functional PPAR-responsive elements (PPREs) in the Elovl3 promoter. A: deletion and mutation analyses of the mouse Elovl3 promoter in 3T3-L1 cells. Cells were caused to differentiate into adipocytes. At 3 days, cells were transfected with each construct and further incubated for 1 day. Then, cells were trypsinized and seeded into a 96-well assay plate and cultured for 1 more day in the absence (gray bars) or presence of troglitazone (10 \( \mu \)M, filled bars). Data are presented as mean \( \pm \) SD of 3 independent assays. Putative cis-regulatory elements are indicated at the top of the diagram. *\( P < 0.01 \), as indicated by brackets. B: schematic representation of cis-regulatory elements of the mouse Elovl3 promoter. Transcription initiation site was defined as +1. Three putative PPREs (PPRE1–3), TATA box, and translational initiation codon (ATG) are indicated on the top. Amplicon of ChIP assay for PPRE1 and PPRE2/3 are also shown. C: ChIP assay of PPRE of mouse Elovl3 promoter in 3T3-L1 cells. Cells were caused to differentiate into adipocytes for 6 days in the absence (gray bars) or presence of Troglitazone (10 \( \mu \)M; filled bars), and the ChIP assay was then carried out. The profile of the amplicon is shown at the left; input control (input) means that a small aliquot before immunoprecipitation was used for PCR amplification. Data are representative of those of 3 independent experiments. Band intensity was measured by Multi Gauge software. *\( P < 0.01 \) vs. vehicle-treated cells.
Binding of PPARγ in the promoter of Elovl3 gene in adipocytes. Next, we investigated the transcriptional regulation of the Elovl3 gene by PPARγ in adipocytes. At first, we searched for PPREs within a 1-kb region of the promoter of the mouse Elovl3 gene using MatInspector software (2) and found three putative PPREs starting at positions −965, −458, and −430 (transcription initiation site defined as position +1) of the mouse Elovl3 gene promoter (Fig. 2A). These PPREs were designated PPRE1 for −965 to −953, PPRE2 for −458 to −446, and PPRE3 for −430 to −418.

To examine the involvement of these PPREs in the transcriptional regulation of the Elovl3 gene, we carried out the luciferase reporter assay (Fig. 2A). When the construct carrying the promoter region from −970 to +172, named −970/+172, was used for the transfection, efficient reporter activity was detected, and its activity was increased by 2.6-fold by the treatment with the troglitazone compared with that for the vehicle-treated cells (Fig. 2A). When the region from −970 to −800 was deleted, troglitazone-dependent elevation was decreased by 14% of that of the −970/+172 construct, although the basal promoter activity was not altered (Fig. 2A). Further deletion from −800 to −500 resulted in no significant change in luciferase activity even in the presence or absence of troglitazone (Fig. 2A). On the contrary, further deletion to −400 caused a significant decrease in the promoter activity, which was 33% of that of the −970/+172 construct, and troglitazone-dependent enhancement was completely absent, indicating that the regions from −970 to −800 and −500 to −400 contained critical cis-elements for the troglitazone-dependent transcriptional activation. These results are well consistent with our prediction that the three PPREs at −961, −454, and −426 would be critical for PPARγ-mediated activation of the Elovl3 gene expression in 3T3-L1 cells. Then, a mutation was introduced into each of the three PPREs of the promoter region of the Elovl3 gene (−970/+172) to obtain the −970/+172 PPRE1mu, −970/+172 PPRE2mu, and −970/+172 PPRE3mu (Fig. 2A). The luciferase reporter activity of each of these three mutant constructs was decreased compared with that of the wild-type −970/+172 construct with respect to the responsiveness to troglitazone (Fig. 2A). Moreover, we introduced the mutations into all three PPREs to make −970/+172 PPRE1/2/3mu. In the presence of troglitazone, the luciferase reporter activity of −970/+172 PPRE1/2/3mu was decreased to 36% of that of the wild-type −970/+172 construct, and the responsiveness to troglitazone was lost (Fig. 2A); however, the basal promoter activity was not changed (Fig. 2A). These results indicate that troglitazone activated Elovl3 gene expression synergistically through these three PPREs at position starting at −965, −458, and −430 of the Elovl3 promoter in 3T3-L1 cells.

Next, to determine whether PPARγ bound each of these PPREs of the Elovl3 promoter, we carried out the ChIP assay. The ChIP assay was performed by using each of two regions containing PPRE at −965 (PPRE1) and two PPREs at −458 and −430 (PPRE2–3) of the Elovl3 promoter, because the latter two PPREs at −458 and −430 were located too near each other to distinguish individual binding by the ChIP-PCR analysis (Fig. 2B). 3T3-L1 cells were caused to differentiate into adipocytes for 8 days in the presence or absence of troglitazone. In ChIP-PCR analysis, the PCR primers specific for Elovl3 promoter produced an amplicon of predicted size (220 bp for PPRE1 and 219 bp for PPRE2–3; Fig. 2B) from both total input DNA and immunoprecipitates obtained with the anti-PPARγ antibody, although when normal IgG was utilized instead of anti-PPARγ antibody, no amplified signals were detected (Fig. 2C). On the other hand, the primers for the region without any PPRE did not produce an amplicon (data not shown). Moreover, the signal intensity of the amplicons was increased ~1.4-fold for PPRE1 and 1.3-fold for PPRE2/3 by the treatment with troglitazone compared with these intensities for the vehicle-treated cells (Fig. 2C). These results, taken together, indicate that PPARγ bound at least two PPREs of the Elovl3 promoter region and that binding efficiencies were enhanced by the treatment with Troglitazone.

Involvement of Elovl3 in the regulation of triglyceride storage in adipocytes. To examine the roles of Elovl3 in the regulation of adipogenesis, we conducted a siRNA-mediated knockdown study on the Elovl3 mRNA. We transfected 3T3-L1 cells with one of two siRNAs, i.e., no. 1 or no. 2 for Elovl3 or with NC siRNA. The mRNA level of the Elovl3 gene was significantly decreased more than 60% by the transfection with either no. 1 or no. 2 siRNA compared with that of the NC siRNA-transfected cells (Fig. 3A). Elovl3 siRNA did not affect the expression of other Elovl family genes, i.e., Elovl1, -2, and -4. Elovl3 siRNA treatment decreased lipid accumulation in adipocytes (Fig. 3B). Elovl3 siRNA knocked down Elovl3 mRNA in 3T3-L1 cells (Fig. 3C).
-4 through -7 (data not shown). These results suggest that Elovl3 siRNA suppressed the Elovl3 expression in the adipocytes. The more suppressive Elovl3 siRNA, no. 2, was chosen for use in further study.

Next, we investigated the function of Elovl3 in the accumulation of intracellular lipids in adipocytes. 3T3-L1 cells were transfected with Elovl3 siRNA or NC siRNA and caused them to differentiate into adipocytes for 8 days. Accumulation of the intracellular lipids was observed in the NC siRNA-transfected cells (Fig. 3B), and this accumulation was decreased by transfection with Elovl3 siRNA (Fig. 3B). In addition, we measured the intracellular triglyceride level in these cells. When 3T3-L1 cells were transfected with Elovl3 siRNA, the intracellular triglyceride level was decreased by 61% compared with that in NC siRNA-transfected cells (Fig. 3C). These results indicate that Elovl3 activated the accumulation of the intracellular lipids in adipocytes.

Suppression of expression of adipogenic, lipogenic, and lipolytic genes in Elovl3 knockdown cells. We examined the expression of the PPARγ gene and its target genes aP2 and SCD genes in Elovl3 knockdown adipocytes. The cells were caused to differentiate into adipocytes for 8 days, and transfection with Elovl3 siRNA was performed every 2 days. The expression levels of the PPARγ, aP2, and SCD genes decreased to be ∼44, 27, and 37%, respectively, of those in the NC siRNA-transfected cells (Fig. 4A). Moreover, when the cells were transfected with Elovl3 siRNA,

Fig. 4. Repressed expression of adipogenic, lipogenic, and lipolytic genes by suppression of Elovl3.

A: suppression of adipogenic gene expression in Elovl3 siRNA knockdown cells. Cells were transfected with Elovl3 (filled bars) or NC (gray bars) siRNA, and caused to differentiate into adipocytes for 8 days. Transfection was carried out every 2 days during this period. RNA was prepared and used for quantitative PCR analysis. Data are means ± SD from 3 independent experiments. *P < 0.01 vs. NC siRNA-transfected cells. B: protein levels of PPARγ, aP2, and SCD in Elovl3 siRNA-transfected cells. 3T3-L1 cells were cultured as described in A. Crude cell extracts (20 μg/lane) were utilized for Western blot analysis. Band intensities were measured by Multi Gauge software. Data are presented as means ± SD. *P < 0.01 vs. NC siRNA-transfected cells. C: siRNA-mediated suppression of lipogenic gene expression in 3T3-L1 cells. 3T3-L1 cells were cultured as described in A. Data are means ± SD from 3 independent experiments. *P < 0.01 vs. NC siRNA-transfected cells. D: protein levels of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in Elovl3 siRNA-transfected 3T3-L1 cells. Cells were cultured as described in B. Crude cell extracts (20 μg/lane) were utilized for Western blot analysis. Band intensities were measured by Multi Gauge software. Data are presented as means ± SD. *P < 0.01, as indicated by brackets. E: suppression of lipolytic gene expression in Elovl3 knockdown cells. 3T3-L1 cells were cultured as described in A. Transcription levels of lipolytic genes were measured by quantitative PCR analysis. Data are presented as means ± SD from 3 independent experiments. *P < 0.01 vs. NC siRNA-transfected cells. F: measurement of glycerol released into medium. 3T3-L1 cells were transfected with Elovl3 (filled bar) or NC (gray bar) siRNA, and caused to differentiate into adipocytes for 6 days. Data are means ± SD from 3 independent experiments. *P < 0.01 vs. NC siRNA-transfected cells.
the protein levels of PPARγ, aP2, and SCD were reduced ~61, 74, and 56%, respectively, compared with those of NC siRNA-transfected cells, although the actin level was not altered in any sample (Fig. 4B).

Next, we investigated the expression of the genes involved in fatty acid synthesis and lipolysis in Elovl3 knockdown cells. When 3T3-L1 cells were caused to differentiate into adipocytes for 8 days and transfected with Elovl3 siRNA every 2 days during this period, the mRNA levels of the ACC and FAS genes, which are involved in fatty acid synthesis, were decreased to ~57 and 47%, respectively, compared with those of the NC siRNA-transfected cells (Fig. 4C). In addition, the protein levels of ACC and FAS were reduced ~47 and 62%, respectively, in the Elovl3 knockdown cells compared with those in the NC siRNA-transfected cells, although the actin level was not altered in any sample (Fig. 4D). Furthermore, the mRNA levels of lipolytic genes such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL), were also decreased ~64, 59, and 77%, respectively, by the transfection with Elovl3 siRNA compared with their levels in the NC siRNA-transfected cells (Fig. 4E). Then, we measured the amount of glycerol released from these cells. 3T3-L1 cells were transfected with Elovl3 or NC siRNA and caused to differentiate into adipocytes for 6 days. When the cells were transfected with Elovl3 siRNA, the glycerol amount decreased ~38% compared with that in the NC siRNA-transfected cells (Fig. 4F). In addition, almost the same results were obtained when the other Elovl3 siRNA (no. 1) was used instead of Elovl3 siRNA (no. 2) (data not shown). These results, taken together, indicate that Elovl3 enhanced the expression of adipogenic, lipogenic, and lipolytic genes in the adipocytes.

Elovl3-produced fatty acids activate PPARγ function. To study the properties of Elovl3-synthesized products as the ligand of PPARγ, we carried out the mammalian two-hybrid assay using three plasmids, pGAL4-PPARγ(LBD), pVP16-TIF2, and pG5-Luc, in HeLa cells. At 1 day after the transfection, the medium was replaced with fresh medium containing various fatty acids (0–500 μM), which is consistent with the natural concentration of VLCFAs (oleic acid; ~100 μM) in serum (38) or synthetic PPARγ agonist troglitazone or GW-1929, and the cells were further cultured for 1 day. Troglitazone or GW-1929 enhanced the luciferase-derived luminescence ~4.1- and 10.3-fold, respectively, compared with that of the vehicle-treated cells (Fig. 5A), indicating the usefulness of this assay system or evaluating the ligand activity of PPARγ.

Next, we utilized various fatty acids as putative ligands for PPARγ, which include Elovl3-synthesized products, to inves-

Fig. 5. Activation of PPARγ function by Elovl3-produced very long-chain fatty acids (VLCFAs). A: PPARγ agonists activate PPARγ function in mammalian two-hybrid assay. HeLa cells were transfected with pGAL4-PPARγ(LBD), pVP16-TIF2, and pM-Luc vectors and cultured for 6 h. Medium was changed to 1% (wt/vol) BSA-DMEM containing troglitazone (Tro; 10 μM; filled bar) or GW-1929 (1 μM; filled bar) as PPARγ agonists and further incubated for 1 day. Luciferase reporter activity was measured as described in MATERIALS AND METHODS. Data are presented as means ± SD from 3 independent experiments. *P < 0.01 vs. vehicle (V; gray bar)-treated cells. B: VLCFAs enhance PPARγ function. HeLa cells were transfected as described in A. Medium was discarded, and 1% (wt/vol) BSA-DMEM containing a given VLCFA (500 μM; filled bars) was added, and cells were cultured for 1 more day. Luciferase reporter activity was measured as described in MATERIALS AND METHODS. Data are means ± SD from 3 independent experiments. *P < 0.01, **P < 0.05 vs. vehicle (V; gray bar)-treated cells. C: suppression of C18:1- and C20:1-mediated activation of PPARγ by PPARγ antagonist. HeLa cells were transfected as described in A and cultured in medium containing C18:1 or C20:1 (0, 100, or 500 μM) with or without GW-9662, a PPARγ antagonist (1 μM) for 2 days. Luciferase reporter activity was measured as described in MATERIALS AND METHODS. Data are mean ± SD from 3 independent experiments. *P < 0.01, as indicated by brackets.

Fig. 6. Direct binding of VLCFAs with PPARγ. Binding ability of VLCFAs with PPARγ. Various concentrations of VLCFAs (C18:1, ▲; C20:1, □; C22:1, △; C18:0, ○) or troglitazone (●), and GW-1929 (■) as positive control, were incubated with PPARγ. Binding activity was measured by receptor cofactor assay system (RCAS) as described in MATERIALS AND METHODS. The study was carried out in triplicate. Data are means ± SD. *P < 0.01 vs. basal level (DMSO).
ttigate the activation of PPARγ function in the mammalian two-hybrid assay. As indicated by the increased luminescence compared with that of the vehicle-treated cells, C18:1 and C20:1 VLCFAs could activate the PPARγ function in this assay, and C18:0 was slightly effective (Fig. 5B). On the contrary, the other fatty acids (C16:0, 20:0, 22:0, 22:1, and 24:0) examined in this study showed no significant effects on PPARγ function (Fig. 5B). Furthermore, either C18:1 or C20:1 VLCFA enhanced PPARγ function in a concentration-dependent manner, and the activation was completely suppressed by GW-9662 (Fig. 5C).

Next, we investigated the binding abilities of VLCFAs with PPARγ by RCAS. Lower concentrations of GW-1929 and troglitazone, PPARγ agonists, bound PPARγ compared with those of 18:1 and C20:1 (Fig. 6). Whereas C18:0 and C22:1 VLCFAs did not show significant binding activities (Fig. 6). These results, taken together, indicate that C18:1 and C20:1 VLCFAs, which are products of Elovl3, activated the PPARγ function.

**Progression of adipogenesis by treatment of Elovl3 knockdown cells with C18:1 or C20:1 VLCFA.** Next, we examined whether VLCFAs were able to compensate for the loss of Elovl3 by siRNA, which was shown above (Fig. 3) to suppress the accumulation of intracellular lipids. When 3T3-L1 cells were caused to differentiate into adipocytes for 6 days, the content of intracellular lipids increased compared with that of the undifferentiated cells (Fig. 7A), and this increase was decreased by the transfection with Elovl3 siRNA (Fig. 7A). Moreover, addition of C18:1 or C20:1 but not C22:1 VLCFA clearly increased the content of intracellular lipid droplets in the Elovl3 siRNA knockdown cells (Fig. 7A). Furthermore, this C18:1 or C20:1 VLCFA elevated accumulation of the intracellular lipid was repressed by cotreatment with GW-9662 (Fig. 7A). Then, we measured the intracellular triglyceride level. This level drastically increased during adipocyte differentiation, and this enhancement was decreased ~61% by transfection with Elovl3 siRNA compared with the triglyceride level of the differentiated cells (Fig. 7B). In contrast, when the cells were transfected with Elovl3 siRNA and cultured in medium containing C18:1 or C20:1 VLCFA, the intracellular triglyceride level was significantly elevated ~1.9- and 1.8-fold, respectively, compared with that of the Elovl3 siRNA-transfected cells (Fig. 7B). Moreover, this elevation was suppressed by cotreatment with GW-9662 (Fig. 7B), whereas C22:1 VLCFA did not show any increase of the intracellular triglyceride level in Elovl3 siRNA-transfected cells (Fig. 7B).

Then, we measured the expression of adipogenic genes in the Elovl3 siRNA knockdown cells cultured in medium for 6 days in the presence or absence of VLCFA with or without GW-9662. The transcription level of adipogenic genes such as PPARγ, aP2, SCD, and CD36 was suppressed by the transfection with Elovl3 siRNA (Fig. 8A). Although the expression of CD36, one of the fatty acid transporters, was repressed, the fatty acid uptake was not altered in these cells (data not shown). This suppressed expression of adipogenic genes was enhanced by adding C18:1 or C20:1 VLCFA (Fig. 8A). In turn, this VLCFA-mediated enhancement of the expression was repressed by cotreatment with GW-9662 (Fig. 8A).

**Fig. 7.** C18:1 and C20:1 VLCFAs compensate for loss of Elovl3 to allow progression of adipogenesis in Elovl3 knockdown cells. A: elevation of content of intracellular lipid droplets by addition of C18:1 or C20:1 VLCFA with or without GW-9662 to Elovl3 knockdown cell cultures. 3T3-L1 cells (U, undifferentiated) were transfected with Elovl3 siRNA and caused to differentiate (D) into adipocytes for 6 days. Transfection was performed every 2 days during this period. Intracellular lipid droplets were observed microscopically. B: increase in content of intracellular triglycerides by addition of C18:1 or C20:1 VLCFA with or without GW-9662 to cultures of Elovl3 siRNA-transfected cells. 3T3-L1 cells (open bar) were transfected with Elovl3 siRNA and caused to differentiate (D) into adipocytes for 6 days in the absence or presence of C18:1 or C20:1 VLCFA (filled bars). Data are means ± SD from 3 independent experiments. *P < 0.01, as indicated by brackets.
the transcription level of the Elovl3 gene was also enhanced and overwhelmed the effect of siRNA on Elovl3 mRNA levels by the addition of C18:1 or C20:1 VLCFA in Elovl3 knockdown cells (Fig. 8B), because these VLCFAs can enhance the expression of the Elovl3 gene through activation of PPARγ (Fig. 6B). In contrast, such changes were not observed when C22:1 VLCFA, instead of C18:1 or C20:1 VLCFA, was added (Fig. 8, A and B). This enhancement was also suppressed by GW-9662 (Fig. 8B). These results indicate that Elovl3-produced VLCFAs such as C18:1 and C20:1 were able to compensate for the lost expression of adipogenic genes in Elovl3 siRNA-transfected cells, thus permitting progression of adipogenesis by improving PPARγ function.

**DISCUSSION**

The metabolic pathways of VLCFAs play important roles in the maintenance of membrane lipid composition as well as in the generation of precursors for cell signaling molecules such as eicosanoids and sphingosine-1 phosphate (12, 41). Elovl3, a member of the Elovl family, was identified as a gene induced by cold stress in BAT and was named the cold-inducible glycoprotein of 30 kDa, Cig30 (36, 37). Ablation of Elovl3 prevents the formation and storage of triglycerides in the liver and adipose tissue, resulting in reduced adipose mass, impaired insulation, and elevated oxygen consumption mainly by increased fatty acid oxidation in BAT (42). In addition,
disruption of the Elovl3 gene reduces expansion of adipose tissue, leads to resistance against diet-induced obesity, and decreases intracellular triglycerides and de novo fatty acid synthesis and uptake (46). Thus, Elovl3 is indispensable for appropriate synthesis of triglycerides and fatty acid uptake and storage in adipose tissue. However, the functions of Elovl3 in white adipose tissues are not well understood. In this study, we found that the expression of the Elovl3 gene was transcriptionally regulated by PPARγ and that Elovl3-produced VLCFAs act as the ligands for PPARγ in 3T3-L1 cells.

The expression of the Elovl3 gene was activated by PPARγ through at least two PPREs in 3T3-L1 cells (Fig. 2, A–C). Moreover, troglitazone enhanced the expression of Elovl3 in 3T3-L1 cells (Fig. 1B). These data are supported by previous findings that the expression level of the Elovl3 gene is gradually enhanced with a peak at 4–6 days after the initiation of adipogenesis, whose expression profile is similar to that of PPARγ (39). In primary cultures of brown adipocytes, the expression of the Elovl3 gene is synergistically regulated by norepinephrine and the PPARγ ligand rosiglitazone (13). Moreover, expression of the Elovl3 gene is also regulated by sterol- regulatory element-binding proteins that are essential in both cholesterol and bile acid synthesis and fatty acid metabolism and are differentially regulated at multiple levels by sterol depletion (32).

The intracellular fatty acid pool is an important determinant in the control of lipogenesis and lipolysis that are regulated by PPARγ activity in adipocytes (15). Fatty acids enter the cells through transporters and bind to cytoplasmic fatty acid-binding proteins, which may play roles in directing fatty acids to various intracellular compartments for metabolism and gene expression (17, 24, 34). Although it is unclear whether ligands modulate PPARγ activity in the differentiated adipocytes, ligand-mediated activation of PPARγ appears to be required for transcriptional activity during adipogenesis (33). Fatty acids and their derivatives have been identified as endogenous ligands for PPARγ (22). Moreover, inhibition of ACC (20) or FAS (30), both involved in fatty acid biosynthesis, represses the progression of adipogenesis by suppressing de novo fatty acid biosynthesis. Thus, fatty acids are involved in the regulation of PPARγ-activated adipogenesis. Here, we demonstrated that Elovl3-produced VLCFAs such as C18:1 and C20:1 VLCFAs (100 or 500 μM) activated the function of PPARγ in a mammalian two-hybrid assay. Moreover, C18:1 and C20:1 VLCFAs could bind PPARγ in vitro (Fig. 6), whose data supported that C18:1 VLCFA acts as PPARγ ligand (21, 23, 25, 45) with increasing triglyceride and enhancement of the expression of adipogenic genes such as PPARγ and lipoprotein lipase (25). Our data presented here support the notion that VLCFAs are powerful molecules and suggest that, by manipulating the synthesis of saturated and monounsaturated C18–24 VLCFAs, it may be possible to influence adipose tissue expansion. However, substrate specificity of Elovl3 catalysis is controversial. An in vitro study demonstrated that Elovl3 uses saturated and monounsaturated C18–22 VLCFAs as the substrate (9). In contrast, Ohno et al. (26) demonstrated that saturated and unsaturated C18 and unsaturated C20 VLCFAs predominantly, and C16:0 VLCFA moderately, are used as the substrates of Elovl3 catalysis. Moreover, Zadravec et al. (46) and Westerberg et al. (42) showed that C20–24 saturated and monounsaturated VLCFAs are synthetized by Elovl3 in liver. Kitazawa et al. (16) reported that saturated and monounsaturated C16 and C18 VLCFAs are utilized as the substrates of Elovl3 catalysis. Moreover, saturated and monounsaturated C20 and C22 VLCFAs accumulate in Elovl3−/− mice (46). Thus, further precise investigation of the substrate specificity of Elovl3 catalysis in adipocytes should be elucidated. Moreover, we will elucidate in vivo function of Elovl3-produced VLCFAs in adipose tissues using aP2p-Elovl3 transgenic mouse.

In addition, lipolysis is also an important determinant of the intracellular lipid level. In the present study, although the expression of both lipogenic (ACC and FAS) and lipolytic (ATGL, HSL, and MGL) genes and the released glycerol level are also decreased by knockdown of Elovl3 (Fig. 4, E and F), the accumulation of intracellular lipid was clearly decreased (Fig. 3B), suggesting that reduced lipogenesis may overrun reduce lipolysis in Elovl3 knockdown cells.

In summary, Elovl3 expression was regulated by PPARγ in 3T3-L1 cells. siRNA-mediated suppression of Elovl3 decreased the expression of PPARγ and adipogenic, lipolytic, and lipogenic genes with a concomitant reduction in the intracellular lipid level, indicating that Elovl3-produced VLCFAs enhanced adipogenesis through activation of PPARγ function by serving as endogenous ligands in 3T3-L1 cells. Therefore, Elovl3 has possible therapeutic implications for the treatment of metabolic disorders such as diabetes and obesity.

ACKNOWLEDGMENTS

We thank Dr. Fumio Amano (Osaka University of Pharmaceutical Sciences) for valuable discussion.

GRANTS

This work was supported in part by a Grant-in-Aid for Scientific Research (21570151) and Scientific Research on Innovative Areas (23116516) from The Ministry of Education, Culture, Sports, Science and Technology of Japan, and by grants from the Japan Foundation for Applied Enzymology, the Takeda Science Foundation, the Research Foundation for Pharmaceutical Sciences, and the Naito Foundation.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.K. and K.F. performed experiments; T.K. and K.F. prepared figures; T.K. and K.F. edited and revised manuscript; T.K. and K.F. approved final version of manuscript; K.F. conception and design of research; K.F. drafted manuscript.

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