Lipids
DOI 10.1007/s11745-014-3972-x

ORIGINAL ARTICLE

13-Oxo-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic Acid Activates Peroxisome Proliferator-Activated Receptor γ in Adipocytes

Haruya Takahashi · Hideyuki Hara · Tsuyoshi Goto · Kosuke Kamakari · Nomura Wataru · Shinsuke Mohri · Nobuyuki Takahashi · Hideyuki Suzuki · Daisuke Shibata · Teruo Kawada

Received: 5 September 2014 / Accepted: 10 November 2014

Abstract Peroxisome proliferator-activated receptor (PPAR)γ is expressed in adipose tissue and plays a key role in the regulation of adipogenesis. PPARγ activators are known to have potent antihyperglycemic activity and are used to treat insulin resistance associated with diabetes. Therefore, many natural and synthetic agonists of PPARγ are used in the treatment of glucose disorders. In the present study, we found that 13-oxo-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-oxo-OTA), a linolenic acid derivative, is present in the extract of tomato (*Solanum lycopersicum*), Mandarin orange (*Citrus reticulata*), and bitter gourd (*Momordica charantia*). We also found that 13-oxo-OTA activated PPARγ and induced the mRNA expression of PPARγ target genes in adipocytes, thereby promoting differentiation. Furthermore, 13-oxo-OTA induced secretion of adiponectin and stimulated glucose uptake in adipocytes. To our knowledge, this is the first study to report that 13-oxo-OTA induces adipogenesis through PPARγ activation and to present 13-oxo-OTA as a valuable food-derived compound that may be applied in the management of glucose metabolism disorders.

H. Takahashi · H. Hara · T. Goto · K. Kamakari · N. Wataru · S. Mohri · N. Takahashi · T. Kawada
Laboratory of Molecular Functions of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

T. Goto · N. Takahashi · T. Kawada (✉)
Research Unit for Physiological Chemistry, Kyoto University, Kyoto 606-8501, Japan
e-mail: fat@kais.kyoto-u.ac.jp

H. Suzuki · D. Shibata
Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan

Keywords PPARγ · Oxylipin · Adipocyte · LC–MS · Adiponectin · Glucose uptake

Abbreviations

13-oxo-ODA	13-Oxo-9,11-octadecadienoic acid
13-Oxo-OTA	13-Oxo-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid
9-oxo-ODA	9-Oxo-10,12-octadecadienoic acid
aP2	Fatty acid-binding protein
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CEBP	CCAAT/enhancer binding protein
DMEM	Dulbecco's modified Eagle's medium
EtOH	Ethanol
HAD	*cis*-10-Heptadecenoic acid
HKR	HEPES-Krebs–Ringer buffer
HMW	High molecular weight
HPLC	High-performance liquid chromatography
IBMX	1-Methyl-3-isobutylxanthine
LC–MS	Liquid chromatography–mass spectrometry
LIA	Linoleic acid
LMW	Low molecular weight
LNA	Linolenic acid
LPL	Lipoprotein lipase
PBS	Phosphate-buffered saline
PPAR	Peroxisome proliferator-activated receptors
PPRE	PPAR response elements
PVDF	Polyvinylidene difluoride
QTOF-MS	Quadrupole-time-of-flight MS
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG	Triglyceride
TZD	Thiazolidinedione
UPLC	Ultra performance LC

Published online: 27 November 2014

Introduction

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily [1–5]. PPAR are expressed in many tissues including adipocytes, hepatocytes, muscles, and endothelial cells, and play an important role in lipid and glucose homeostasis [6]. The PPAR family comprises three isoforms: PPARα, PPARβ/δ, and PPARγ. PPARγ is the master regulator of adipocyte differentiation, and functions to enhance the numbers of insulin-sensitive small adipocytes [7]. It is well known that the activation of PPARγ in mature adipocytes regulates several genes involved in glucose metabolism including *fatty acid-binding protein* (*aP2*), *lipoprotein lipase* (*LPL*), and *CCAAT/enhancer binding protein* (*CEBP*) *α* [8, 9].

PPARγ and retinoid X receptor (RXR) heterodimers bind lipophilic activators, which induce a conformational change in PPARγ and subsequently regulate gene transcription. The specific DNA regions of PPARγ target genes that bind PPAR are termed PPAR response elements (PPRE) [6]. As such, PPRE are found in the promoters of PPARγ-responsive genes. PPARγ activators, such as thiazolidinediones (TZD), are known to have potent antihyperglycemic activity and are used to treat insulin resistance associated with diabetes [10]. Moreover, recent studies revealed that many compounds derived from food or other natural sources enhance adipocyte differentiation and glucose uptake by activating PPARγ [11–16]. These compounds have PPARγ ligand activity, suggesting that intake of these compounds is valuable for maintaining health.

Many plant oxylipins are formed from unsaturated fatty acids in the lipoxygenase cascade. Oxylipins are known to be potent bioregulators and are involved in the regulation of growth, differentiation, and morphogenesis [17–19]. Recently, it has been reported that various oxylipins isolated from microalgae can inhibit inflammation [20]. Furthermore, our previous studies have revealed that tomatoes contain the oxidized linoleic acid (LIA) derivatives, 9-oxo-10,12-octadecadienoic acid (9-oxo-ODA) and 13-oxo-9,11-octadecadienoic acid (13-oxo-ODA), which serve as PPARα agonists in mouse liver [21, 22]. Interestingly, previous studies have revealed that 13-oxo-ODA can activate not only PPARα, [22] but also PPARγ [23].

Plants contain linolenic acid (LNA) as well as LIA, and various oxylipins have also been identified [24]. In this study, we found that 13-oxo-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-oxo-OTA) which is an LNA derivative is present in tomato (*Solanum lycopersicum*), Mandarin orange (*Citrus reticulata*), and bitter gourd (*Momordica charantia*). Although the structure of 13-oxo-OTA is similar to that of 13-oxo-ODA, little is known about the biofunction involved in the regulation of PPARγ activity. We hypothesized that 13-oxo-OTA is a PPARγ activator. Therefore, the aim of the present study was to evaluate the effect of 13-oxo-OTA on PPARγ activity and to elucidate its role in adipocyte differentiation and glucose uptake.

In the present study, we show that 13-oxo-OTA activated PPARγ and induced mRNA expression of PPARγ target genes, which promoted adipocyte differentiation. Furthermore, 13-oxo-OTA induced the secretion of adiponectin and glucose uptake in adipocytes. To the best of our knowledge, this is the first study to report that 13-oxo-OTA induced adipogenesis through PPARγ activation, and the findings suggest that 13-oxo-OTA might be a valuable food-derived compound to improve glucose metabolism disorders.

Materials and Method

Materials

Authentic 13-oxo-ODA and 13-oxo-OTA were purchased from Indofine Chemical Company (Hillsborough, USA) and Laroden Fine Chemicals (Malmö, Sweden), respectively. Authentic *cis*-10-heptadecenoic acid (HDA) was purchased from Sigma (St. Louis, MO). All other chemicals used were from Sigma or Wako (Osaka, Japan), and were guaranteed to be high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC–MS) grade. Tomatoes, Mandarin oranges, and bitter gourds were obtained from the local market.

13-Oxo-OTA Analysis by Ultra Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC–QTofMS)

Each freeze-dried sample (10 mg) was homogenized with a mixer in 1 mL of extraction solvent (99.5 % EtOH containing 1 μg/mL of HDA). After centrifugation (15,000 rpm, 10 min, 4 °C), the supernatant was collected as an extract. Using an additional 1 mL of extraction solvent, the same procedure was then repeated. The pooled extracts were filtered through a 0.2-μm-pore polyvinylidene difluoride (PVDF) membrane (Whatman, Brentford, UK), and the filtrates were used for LC–MS.

LC–MS was performed using a Waters Acquity UPLC system coupled to a Xevo QTOF-MS (Waters), equipped with an electrospray source, operating in negative ion mode, with a lock-spray interface for accurate mass measurements. The capillary, sampling cone, and extraction cone voltages were set at 2,700, 20, and 1 V respectively. The source and desolvation temperatures were 120 and 450 °C respectively. The mass scan range was set at 50–1,000 m/z. The cone and desolvation gas flow rates were

set at 50 and 800 L/h respectively, and leucine enkephalin was employed as the lock-mass compound. It was infused straight into the MS at a flow rate of 30 μL/min at a concentration of 200 μg/mL (in 50 % acetonitrile, 50 % water, 0.1 % formic acid). The data were acquired with MassLynx software (Waters). External mass calibration was performed following the manufacture's protocol. An aliquot of the extracted sample (3 μL) was injected into an Acquity UPLC BEH-C18 reversed-phase column (column size 2.1 × 100 mm, particle size 1.7 μm). Mobile phases A (water and 0.1 % formic acid) and B (acetonitrile and 0.1 % formic acid) were used. The column temperature was set to 40 °C. The buffer gradient consisted of 30–50 % B for 0–4 min, 50–85 % B for 4–14 min, and was held at 99 % B for 14–17 min and returned to 30 % B for 3 min before the next injection, at a flow rate of 300 μL/min.

Luciferase Assay

Luciferase assays were performed as previously described, using a GAL4/PPAR chimera system [25, 26]. We transfected p4xUASg-tk-luc (a reporter plasmid), pM-hPPARa (an expression plasmid for a chimera protein of the GAL4 DNA-binding domain and each human PPAR-ligand-binding domain), and pRL-CMV (an internal control for normalizing transfection efficiency) into monkey CV1 kidney cells by using Lipofectamine (Life Technologies Japan Ltd.) according to manufacturer's protocol. Luciferase activity was assayed using the dual luciferase system (Promega, MO, USA) according to the manufacturer's protocol.

3T3-L1 Adipocyte Cell Culture

The 3T3-L1 cells were cultured, maintained, and differentiated according to a previously described method [27]. Briefly, 3T3-L1 murine pre-adipocytes were cultured in growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10 % (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin) at 37 °C in 5 % CO_2. At 2 days after reaching confluence, the cells were incubated in a differentiation medium (containing 0.25 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine [IBMX]) for 48 h and then in growth medium containing 5 μg/ml insulin for an additional 2 days. On the 4th day, this was replaced with fresh growth medium. 13-Oxo-OTA, 13-oxo-ODA, LNA, LIA, or troglitazone were treated from day 0 to day 6. Total adiponectin was determined using the Mouse Adiponectin ELISA kit (R&D Systems, Minneapolis, MN). Oil-Red O staining was performed as described previously [28]. Quantification of triglyceride (TG) using the TG E-test Kit (Wako) was performed as described previously [29].

Quantification of mRNA Expression Levels

Total RNA was prepared from 3T3-L1 cells by using Sepasol (Nacalai Tesque), according to the manufacturer's protocols. Using M-MLV reverse transcriptase (Life Technologies Japan Ltd.), total RNA was reverse-transcribed using a thermal cycler (Takara PCR Thermal Cycler SP: Takara Bio Inc., Shiga, Japan). To determine mRNA expression levels, real-time quantitative RT-PCR analysis was performed with a Light Cycler System (Roche Diagnostics) using SYBR green fluorescence signals as described previously [25, 30]. The oligonucleotide primer sets of mouse *36B4* and *PPARγ* target genes were designed using a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database as follows: mouse *PPARγ* (Fwd: 5′-ggagatctccagtgatatcgacca-3′; Rev: 5′-acggcttctacggatcgaaact-3′), mouse *aP2* (Fwd: 5′-aagacagctcctcctcgaaggtt-3′; Rev: 5′-tgaccaaatccccatttacgc-3′), mouse *LPL* (Fwd: 5′-atccatggatggacggtaacg-3′; Rev: 5′-ctggattccaatacttcgacca-3′), mouse *CEBPα* (Fwd: 5′-tggacaagaacagcaacgag-3′; Rev: 5′-tcactggtcaactccagcac-3′), and mouse *36B4* as an internal control (Fwd: 5′-tccttcttccaggctttggg-3′; Rev: 5′-gacaccctccagaaagcgag-3′). All data indicating mRNA expression levels are presented as a ratio relative to a control in each experiment.

Glucose Uptake into 3T3-L1 Adipocytes

2-Deoxy-D-[3*H*] glucose (Amersham Biosciences, Piscataway, NJ, USA) uptake was assayed as described previously with modifications [31]. Briefly, prior to the assay, the cells were deprived of serum for 18 h. The cells were then incubated in HEPES-Krebs–Ringer (HKR) buffer (with 0.1 % BSA) in the presence or absence of 100 nM insulin for 20 min at 37 °C. At the end of the incubation period, 2-deoxy-D-[3*H*] glucose was added to a final concentration of 0.5 μCi/mL. After incubation for 10 min, the reaction was terminated by three washes of ice-cold phosphate-buffered saline (PBS). The cells were solubilized in 0.1 N NaOH, and aliquots of the cell lysate were transferred to scintillation vials for radioactivity counting. Non-specific deoxyglucose uptake was measured in the presence of 10 μM cytochalasin B.

The remainder of the lysate was used for measuring protein content with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The radioactivity measurements were normalized by protein concentrations.

Immunoblotting

Total cellular proteins were solubilized in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 % Triton X-100, 0.5 % deoxycholate, 0.1 % sodium dodecyl sulfate [SDS]; pH

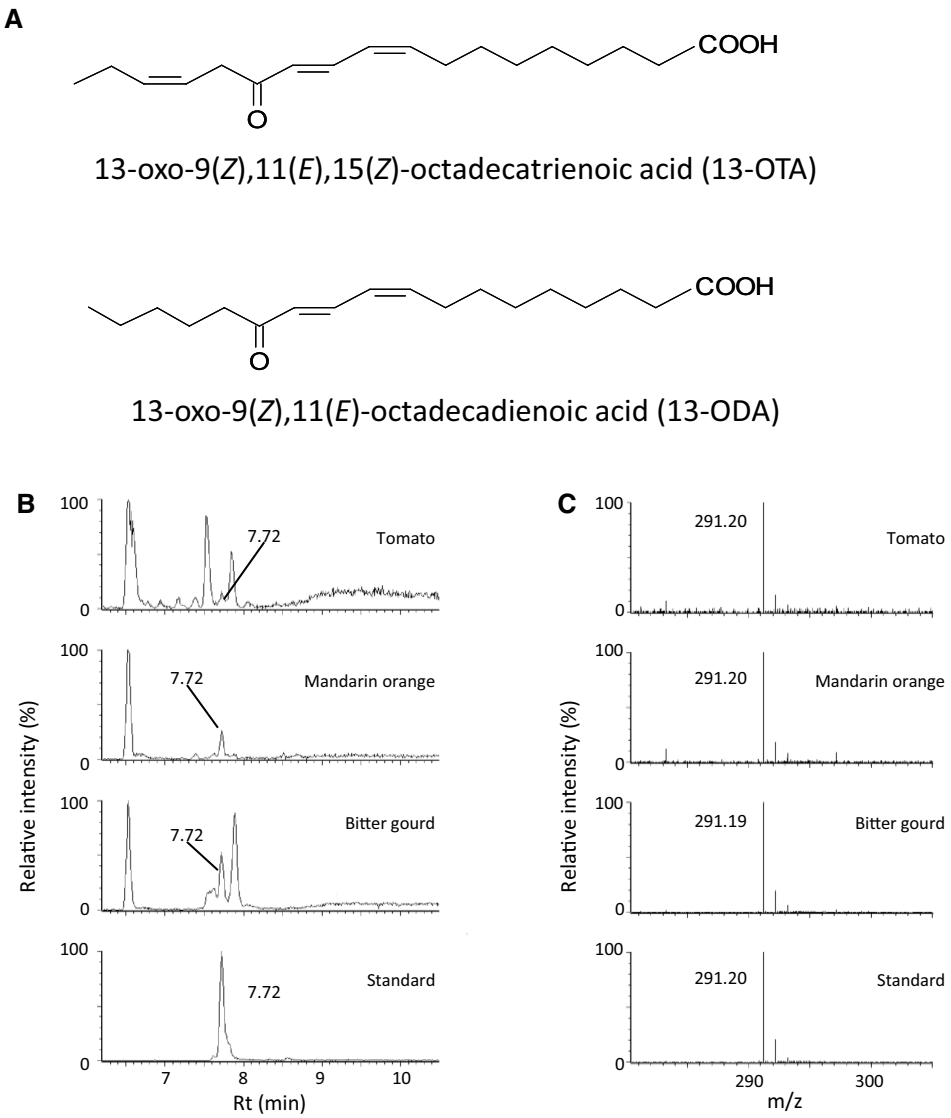

Fig. 1 Identification of 13-oxo-octadecatrienoic acid (13-oxo-OTA) by LC–MS. **a** The structure of 13-oxo-OTA and 13-oxo-ODA. **b** Extracted ion chromatogram ($m/z = 291.20$) data and **c** full mass data of 13-oxo-OTA in authentic sample and plant extraction sample. The elution peak (Rt = 7.7 min, $m/z = 291.20$) is 13-oxo-OTA

7.4) containing a protease inhibitor cocktail. The protein concentration of samples was determined using a protein assay kit (Bio-Rad Laboratories). Protein samples (20 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 5 % gel. Separated proteins were transferred electrophoretically to PVDF membranes (Millipore, MA), which were blocked with 5 % nonfat dried milk in PBS. The membranes were incubated with antibodies to PPARγ (Cell Signaling Technology, MA) and β-actin (Cell Signaling Technology, MA), and then with peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (Santa Cruz, CA), respectively. To study adiponectin isoforms, we performed western blotting with Mini-PROTEAN® TGX™ Gels 4–15 % (BIO RAD, Tokyo, Japan) and used an antibody against adiponectin (Affinity BioReagents™, Golden, CO). Proteins were detected using an ECL western blotting detection system (GE Healthcare, NJ).

Statistical Analysis

Data are presented as means ± SEM. Differences between groups were determined with one-way analysis of variance (ANOVA), followed by Duncan's test. Values of $p < 0.05$ were considered statistically significant.

Results

Identification of 13-Oxo-OTA in Tomato, Mandarin Orange, and Bitter Gourd Extraction

We first explored the oxylipins induced LNA from plants extract using an LC–MS system. LC–MS was performed to reveal that 13-oxo-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-oxo-OTA, Fig. 1a), which is analogous to 13-oxo-9(*Z*),11(*E*)-octadecadienoic acid (13-oxo-ODA, Fig. 1a), was

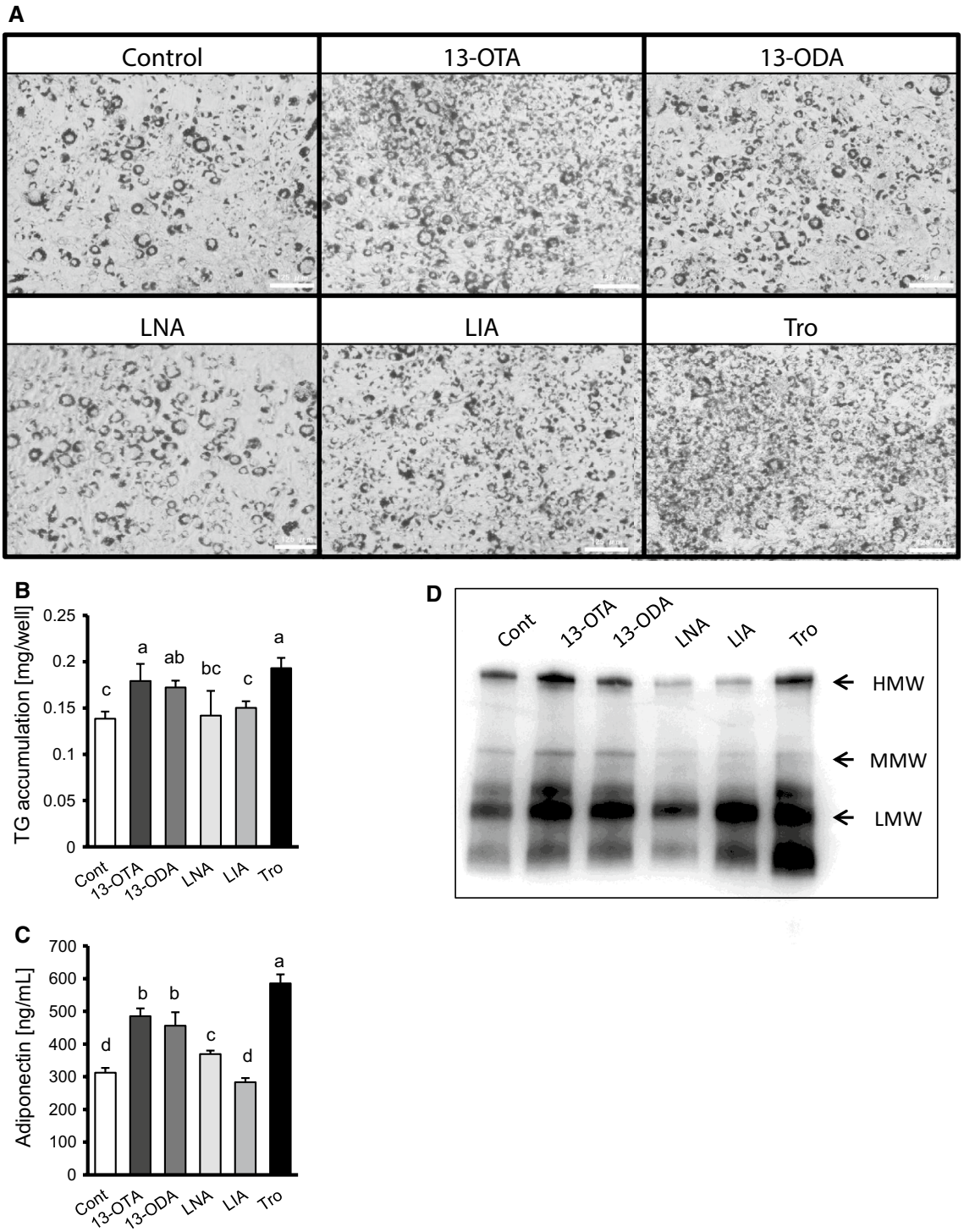

Fig. 2 13-Oxo-OTA promoted adipogenesis and induced the secretion of adiponectine in 3T3-L1 adipocytes. **a, b** lipid accumulation and the secretion of **c** total-adiponectin or **d** HMW-adiponectin in 3T3-L1 adipocytes treated with 13-oxo-OTA, 13-oxo-ODA, LNA, LIA (10 μM), or Tro (5 μM). Data are presented as the mean ± SEM ($n = 3$–5). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Cont. Magnification is × 200 (*white bar* is 125 μm). *Cont* Control, *Tro* troglitazone, *LNA* linolenic acid, *LIA* linoleic acid

contained in the extract of tomato (*Solanum lycopersicum*), Mandarin orange (*Citrus reticulata*), and bitter gourd (*Momordica charantia*) (Rt = 7.72 min, *m/z* = 291.20, Fig. 1b, c). The peak of 13-oxo-OTA was detected as unfragmented deprotonation ion ($C_{18}H_{27}O_3^-$, $[M\text{-}H]^-$, Fig. 1c) under conditions of electrospray negative ionization mass spectrometry.

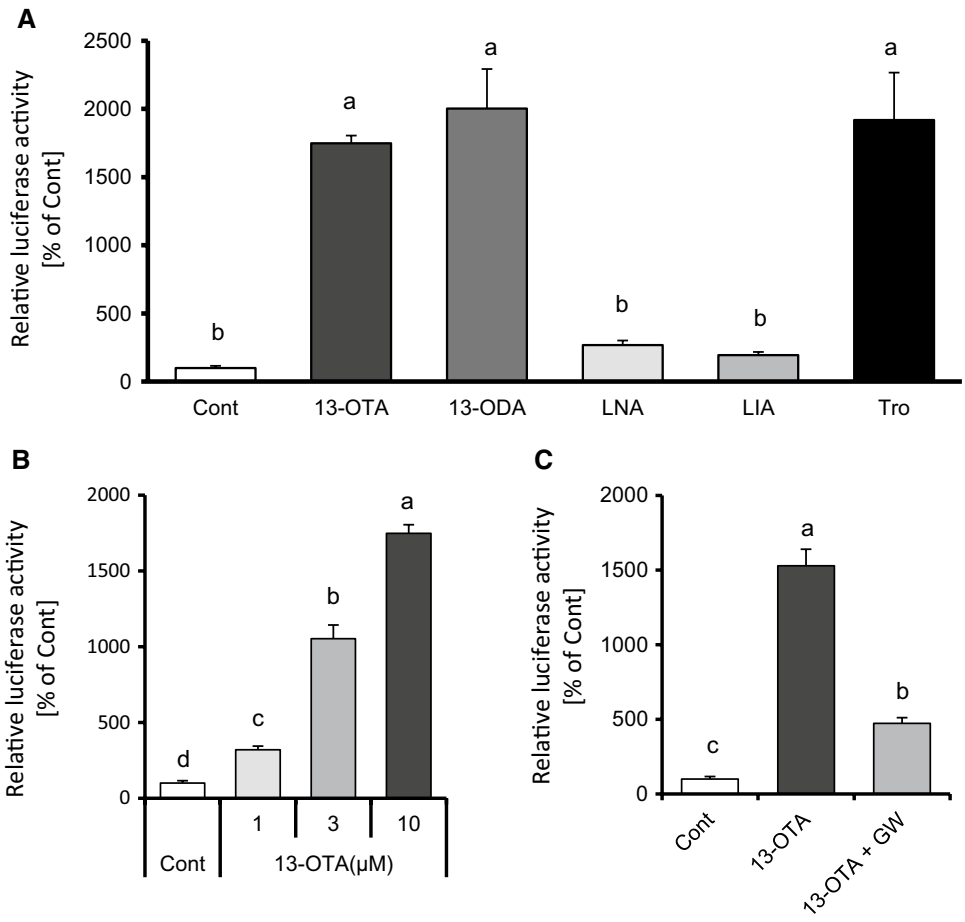

Fig. 3 13-Oxo-OTA activated PPARγ. **a** Evaluation of PPARγ activity in luciferase reporter assay treated with 13-oxo-OTA, 13-oxo-ODA, LNA, LIA (10 μM respectively), or Tro (5 μM). **b** The dose-dependent effect of 13-oxo-OTA on PPARγ activity. **c** evaluation of PPARγ activity in luciferase reporter assay treated with 13-oxo-OTA and/or PPARγ antagonist (10 μM respectively). Data are mean ± SEM ($n = 5$). *Cont* Control, *Tro* troglitazone, *LNA* linolenic acid, *LIA* linoleic acid, *GW* GW9662

13-Oxo-OTA Promoted TG Accumulation and Induced Secretion of Adiponectin in Adipocytes

To investigate the effects of 13-oxo-OTA, 13-oxo-ODA, LNA, and LIA on triglyceride (TG) accumulation, 3T3-L1 adipocytes were cultured in a medium containing these compounds. Oil Red-O staining revealed that lipid accumulation in 3T3-L1 adipocytes increased following 13-oxo-OTA treatment compared to that observed with LNA and LIA (approximately 1.3-fold, Fig. 2a, b). We also investigated the effect of 13-oxo-OTA on secretion of adiponectin in 3T3-L1 adipocytes and found that secretion of total adiponectin increased because of 13-oxo-OTA treatment (approximately 1.6-fold, Fig. 2c). In particular, 13-oxo-OTA increased the secretion of high-molecular-weight (HMW) adiponectin (Fig. 2d). These data suggest that 13-oxo-OTA promotes TG accumulation and induces adiponectin secretion in 3T3-L1 adipocytes.

13-Oxo-OTA Activated PPARγ and Induced mRNA Expression of PPARγ Target Genes

PPARγ is the master regulator of adipocytes differentiation [7]. To elucidate the contribution of 13-oxo-OTA to PPARγ activation, we investigated whether 13-oxo-OTA activated PPARγ in a luciferase ligand assay. 13-Oxo-OTA and 13-oxo-ODA increased luciferase activity remarkably compared to their precursors, LNA and LIA, respectively (Fig. 3a). This activity was increased in a concentration-dependent manner by 13-oxo-OTA treatment (Fig. 3b) and inhibited by PPARγ antagonist (GW9662) treatment (Fig. 3c). In 3T3-L1 adipocytes, the expression level of PPARγ was increased by 13-oxo-OTA treatment (approximately 1.6-fold, Fig. 4a). In addition to increased mRNA levels, up-regulation of PPARγ protein levels was observed (Fig. 4b). Furthermore, mRNA expression levels of PPARγ target genes, such as *aP2*, *LPL*, and *CEBPα,* were increased in 3T3-L1 adipocytes because of treatment with 13-OTA (approximately 1.9-, 2.1-, and 1.9-fold respectively, Fig. 4c–e). These findings suggest that 13-OTA promotes adipocyte differentiation via a PPARγ-dependent pathway.

13-Oxo-OTA Promotes Glucose Uptake in 3T3-L1 Adipocytes

To elucidate the contribution of 13-oxo-OTA to glucose uptake, we investigated whether 13-oxo-OTA promotes glucose uptake in a radio isotopic assay. We demonstrate here that 13-oxo-OTA

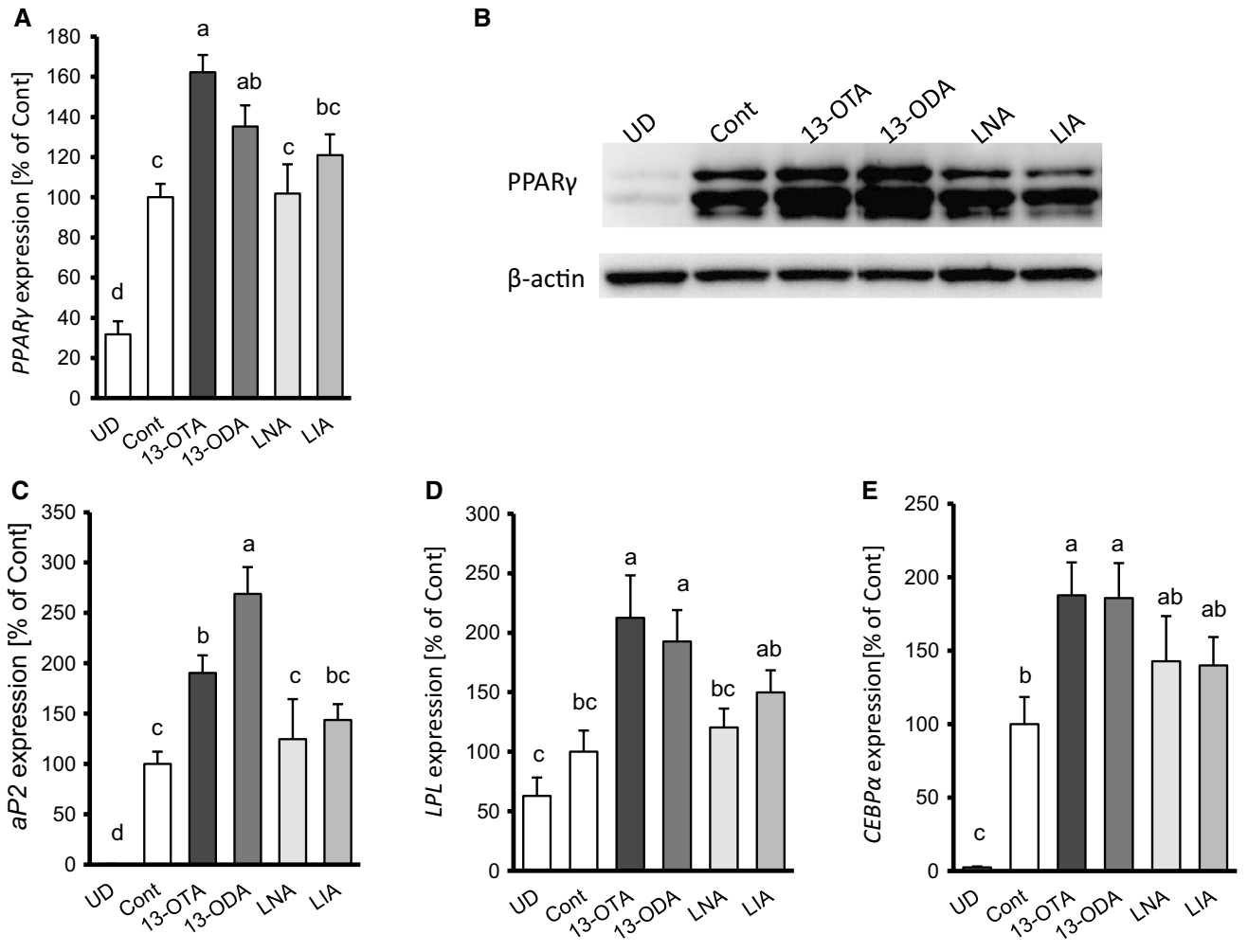

Fig. 4 13-Oxo-OTA activated PPARγ target genes expression in 3T3-L1 adipocytes. Effect of 13-oxo-OTA (10 μM) on **a** *PPARγ* gene expression and **b** protein level. Effect of 13-oxo-OTA on **c** *Ap2*, **d** *CEBPα,* and **e** *LPL*, PPARγ target genes expression. Data are presented as means ± SEM ($n = 3$–5). *$p < 0.05$, **$p < 0.01$ vs. Cont. *UD* undifferentiated 3T3-L1 cells

promoted glucose uptake in 3T3-L1 adipocytes treated with (approximately + 0.9 pmol/min/μg protein compared with control, Fig. 5b) or without (approximately + 0.3 pmol/min/μg protein compared with control, Fig. 5a) insulin. As compared with 13-oxo-ODA, 13-oxo-OTA has an intense effect on glucose uptake in adipocytes treated with insulin (13-oxo-OTA: approximately 1.6 pmol/min/μg protein, 13-oxo-ODA: approximately 1.0 pmol/min/μg protein, Fig. 5b). Although 13-oxo-OTA stimulated glucose uptake, LIN and LNA inhibited this phenomenon in 3T3-L1 adipocytes treated with or without insulin (Fig. 5a, b). These findings suggest that 13-oxo-OTA stimulates glucose uptake in 3T3-L1 adipocytes.

Discussion

In the present study, the analysis of metabolites in plant extracts by using LC–MS revealed that 13-oxo-OTA in extracts of tomato, Mandarin orange, and bitter gourd. 13-Oxo-OTA is an LNA derivative. We confirmed that LNA was present in these plants as well (data not shown). It is proposed that 13-oxo-OTA can be derived from LNA via a non-enzymatic and/or enzymatic reaction. It is possible that LNA is susceptible to oxidation at C13 by an auto-oxidation reaction and/or that the activity of 13-lipoxygenase is involved in the production of 13-oxo-OTA in these fruits.

PPARγ is expressed principally in adipose tissue and plays a key role in the regulation of adipogenesis, energy balance, and lipid biosynthesis [9, 32–34]. Many natural and synthetic agonists of PPARγ are used in the treatment of glucose disorders [6]. Although previous studies have reported that 13-oxo-ODA derived from LIA contributes to activate PPARγ [23], little is known about the effect of 13-oxo-OTA on PPARγ. The structure of 13-oxo-OTA is similar to that of 13-oxo-ODA, which is derived from LIA. We therefore hypothesized that 13-oxo-OTA also has an ability to activate PPARγ.

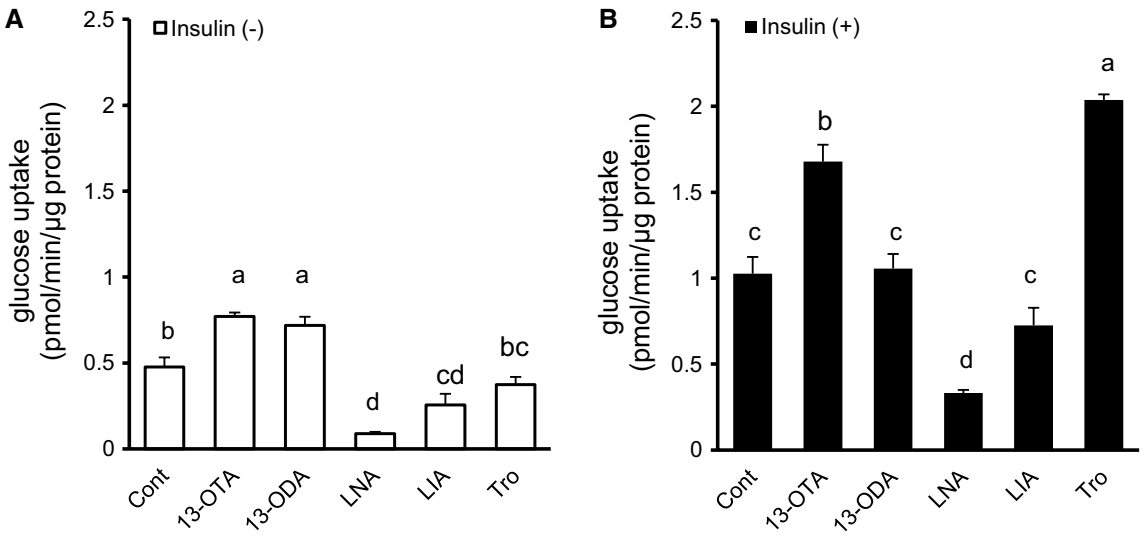

Fig. 5 13-Oxo-OTA enhanced glucose uptake in 3T3-L1 adipocytes. Effect of 13-oxo-OTA, 13-oxo-ODA, LNA, LIA (10 μM) or Tro (5 μM) on glucose uptake in 3T3-L1 adipocytes treated with **b** or without **a** insulin. Data are presented as the mean ± SEM ($n = 3$–5). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Cont. INS: insulin (100 nM)

We demonstrated for the first time that 13-oxo-OTA activates PPARγ and contributes to increased mRNA expression of PPARγ target genes including *aP2*, *LPL*, and *CEBPα,* which are differentiation markers in adipocytes [35]. These findings suggested that 13-oxo-OTA promotes differentiation in 3T3-L1 adipocytes via a PPARγ-dependent pathway. Oil Red-O staining definitively demonstrated that lipid accumulation in 3T3-L1 adipocytes increased owing to 13-oxo-OTA treatment, corroborating our observations following analysis of gene expression. Although 13-oxo-OTA and 13-oxo-ODA both activated PPARγ, LIA and LNA had little effect on PPARγ, suggesting that the keto group (C13 position) plays an important role in binding to PPARγ. In a previous study, it was demonstrated that LIA and LNA have no effect on the expression of PPARγ [36]. Our data confirm the finding from this previous study.

Our present study also showed that 13-OTA induced the secretion of adiponectin and promoted glucose uptake in 3T3-L1 adipocytes. PPRE exists in the adiponectin promoter region [37]. PPARγ agonists therefore stimulate the release of adiponectin from adipocytes. Adiponectin stimulates fatty acid oxidation and contributes to improving glucose metabolism disorders, including insulin resistance [38]. In the present study, 13-oxo-OTA increased the secretion of high-molecular-weight (HMW) adiponectin. Recently, several studies have designated adiponectin isoforms as low (LMW)- or HMW-adiponectin [39]. It has also been suggested that the treatment of diabetic subjects with TZD (PPARγ ligands) specifically increases blood levels of the HMW-adiponectin, and HMW-adiponectin correlates strongly with increased insulin sensitivity [39, 40]. This evidence suggests that HMW-adiponectin induced by 13-OTA treatment contributes to an improvement in glucose metabolism disorders. Indeed, our findings demonstrate that 13-oxo-OTA stimulates glucose uptake in 3T3-L1 adipocytes.

Interestingly, 13-oxo-OTA promotes glucose uptake in 3T3-L1 adipocytes treated with or without insulin, suggesting that 13-oxo-OTA is capable of enhancing glucose uptake via both insulin-dependent and -independent pathways. In a previous study, it was reported that coyote melon (*Ibervillea sonorae*) extract induces glucose uptake in adipocytes without insulin and that this effect is exerted by activation of a PI3 K-independent pathway [41]. This finding raises the possibility that the effect of 13-oxo-OTA is also exerted by a similar mechanism. The preceding study also revealed that the oxidative stress in adipocytes might be the origin of obesity [42]. Oxidative stress in adipocytes impaired insulin signals and decreased insulin-stimulating glucose uptake [43]. Our data showed the possibility that 13-oxo-OTA is effective in improving oxidative stress in adipocytes. Although 13-oxo-OTA and 13-oxo-ODA contributed to the stimulation of glucose uptake, LIA and LNA inhibited glucose uptake in adipocytes, with or without insulin. Further examination is necessary to determine the mechanism of glucose uptake in adipocytes treated with 13-oxo-OTA or LNA.

Although our present study reveals that 13-oxo-OTA is involved in PPARγ activation *in vitro*, little is known about 13-oxo-OTA bioactivation *in vivo*. It is expected that 13-oxo-OTA contained in food (exogenous 13-oxo-OTA) effects adipocytes throughout the bloodstream. 13-Oxo-OTA is one of the oxylipins that have various patterns

including fatty acid hydroperoxides, hydroxyl-, oxo-, epoxy, or keto fatty acids, divinyl ethers, aldehydes, or jasmonic acid [17]. Oxylipins are widespread in nature, occurring in plants, animals, mosses, algae, bacteria, and fungi [20]. Indeed, several oxygenated derivative of fatty acids in animal have been found to be biological activity [44, 45]. In a previous study, it was demonstrated that nitroalkene derivatives of LIA serves as an endogenous PPARγ ligand [46]. These findings raise the possibility that not only exogenous, but also endogenous 13-oxo-OTA, is involved in bioactivity in animals. Therefore, it is necessary to evaluate oxylipins *in vivo* to investigate the effect of both exogenous and endogenous oxylipins.

In conclusion, 13-oxo-OTA contained in tomato, Mandarin orange, and bitter gourd activates PPARγ and induces mRNA expression of PPARγ target genes. These, in turn, promote differentiation and induce secretion of adiponectin and glucose uptake in 3T3-L1 adipocytes. These findings provide the first evidence that 13-oxo-OTA induces adipogenesis through a PPARγ-dependent pathway and contributes to improving treatments for glucose metabolism disorder. These data suggest that 13-oxo-OTA might be a valuable, food-derived compound for maintaining health.

Acknowledgments The authors thank S. Shinoto and M. Sakai for secretarial and technical support, respectively. This work was supported in part by Research and Development Projects for Application in Promoting New Policies Agriculture, Forestry, and Fisheries of Japan, by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (22228001 and 24688015), and by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (24521).

References

1. Escher P, Wahli W (2000) Peroxisome proliferator-activated receptors: insight into multiple cellular functions. Mutat Res 448:121–138
2. Desvergne B, Wahli W (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev 20:649–688
3. Chinetti G, Fruchart JC, Staeles B (2000) Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. Inflamm Res 49:497–505
4. Duval C, Chinetti G, Trottein F, Fruchart JC, Staeles B (2002) The role of PPARs in atherosclerosis. Trends Mol Med 8:422–430
5. Goldenberg I, Benderly M, Goldbourt U (2008) Update on the use of fibrates: focus on bezafibrate. Vasc Health Risk Manag 4:131–141
6. Grygiel-Górniak B (2014) Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications—a review. Nutr J 13:17
7. Brun RP, Spiegelman BM (1997) PPAR gamma and molecular control of adipogenesis. J Endocrinol 155:217–218
8. Kintscher U, Law RE (2005) PPARgamma-mediated insulin sensitization: the importance of fat versus muscle. Am J Physiol Endocrinol Metab 288:E287–E291
9. Lehrke M, Lazar MA (2005) The many faces of PPARgamma. Cell 123:993–999
10. Nolan JJ, Ludvik B, Beerdsen P, Joyce M, Olefsky J (1994) Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. N Engl J Med 331:1188–1193
11. Han KL, Jung MH, Sohn JH, Hwang JK (2006) Ginsenoside 20(S)-protopanaxatriol (PPT) activates peroxisome proliferator-activated receptor γ (PPARγ) in 3T3-L1 adipocytes. Bio Pharm Bull 29:110–113
12. Yang Y, Shang W, Zhou L, Jiang B, Jin H, Chen M (2007) Emodin with PPARγ ligand-binding activity promotes adipocyte differentiation and increases glucose uptake in 3T3-L1 cells. Biochem Biophys Res Commun 353:225–230
13. Shang W, Yang Y, Jiang B, Jin H, Zhou L, Liu S, Chen M (2007) Ginsenoside Rb1 promotes adipogenesis in 3T3-L1 cells by enhancing PPARγ2 and C/EBPα gene expression. Life Sci 80:618–625
14. Choi SS, Cha BY, Lee YS, Yonezawa T, Teruya T, Nagai K, Woo JT (2009) Magnolol enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells. Life Sci 84:908–914
15. Shin DW, Kim SN, Lee SM, Lee W, Song MJ, Park SM, Lee TR, Baik JH, Kim HK, Hong JH, Noh M (2009) (−)-Catechin promotes adipocyte differentiation in human bone marrow mesenchymal stem cells through PPAR gamma transactivation. Biochem Pharmacol 77:125–133
16. Takahashi N, Yao R, Kang MS, Senda M, Ando C, Nishimura K, Goto T, Hirai S, Ezaki Y, Kawada T (2011) Dehydroabietic acid activates peroxisome proliferator-activated receptor-γ and stimulates insulin-dependent glucose uptake into 3T3-L1 adipocytes. BioFactors 37:309–314
17. Grechkin A (1998) Recent developments in biochemistry of the plant lipoxygenase pathway. Prog Lipid Res 37:317–352
18. Farmer EE, Almeras E, Krishnamurthy V (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol 6:372–378
19. Gardner AN, Tarchevsky IA (1999) The lipoxygenase signaling system. Russ J Plant Physiol 46:114–123
20. Reyes C, Ávila-Román J, Ortega MJ, Jara A, García-Mauriño S, Motilva V, Zubía E (2014) Oxylipins from the microalgae *Chlamydomonas debaryana* and *Nannochloropsis gaditana* and their activity as TNF-α inhibitors. Phytochemistry 102:152–161
21. Kim YI, Hirai S, Takahashi H, Goto T, Ohyane C, Tsugane T, Konishi C, Fujii T, Inai S, Iijima Y, Aoki K, Shibata D, Takahashi N, Kawada T (2011) 9-oxo-10(E),12(E)-Octadecadienoic acid derived from tomato is a potent PPAR α agonist to decrease triglyceride accumulation in mouse primary hepatocytes. Mol Nutr Food Res 55:585–593
22. Kim YI, Hirai S, Goto T, Ohyane C, Takahashi H, Tsugane T, Konishi C, Fujii T, Inai S, Iijima Y, Aoki K, Shibata D, Takahashi N, Kawada T (2012) Potent PPARα activator derived from tomato juice, 13-oxo-9,11-octadecadienoic acid, decreases plasma and hepatic triglyceride in obese diabetic mice. PLoS One 7:e31317
23. Reinhold A, Martin H, Tanja S, Michael G, Arthur WB, Katrin M, Daniela V, Hans H, Jürgen S, Werner F, Gerhard R (2007) 13-Oxo-ODE is an endogenous ligand for PPARγ in human colonic epithelial cells. Biochem Pharmacol 74:612–622
24. Smilowitz JT, Zivkovic AM, Wan YJ, Watkins SM, Nording ML, Hammock BD, German JB (2013) Nutritional lipidomics: molecular metabolism, analytics, and diagnostics. Mol Nutr Food Res 57:1319–1335
25. Takahashi N, Kawada T, Goto T, Yamamoto T, Taimatsu A, Matsui N, Kimura K, Saito M, Hosokawa M, Miyashita K, Fushiki T (2002) Dual action of isoprenols from herbal medicines on both PPARγ and PPARα in 3T3- L1 adipocytes and HepG2 hepatocytes. FEBS Lett 514:315–322

26. Takahashi N, Kawada T, Goto T, Kim CS, Taimatsu A, Egawa K, Yamamoto T, Jisaka M, Nishimura K, Yokota K, Yu R, Fushiki T (2003) Abietic acid activates peroxisome proliferator-activated receptor-γ (PPARγ) in RAW264.7 macrophages and 3T3-L1 adipocytes to regulate gene expression involved in inflammation and lipid metabolism. FEBS Lett 550:190–194
27. Goto T, Nagai H, Egawa K, Kim YI, Kato S, Taimatsu A, Sakamoto T, Ebisu S, Hohsaka T, Miyagawa H, Murakami S, Takahashi N, Kawada T (2011) Farnesyl pyrophosphate regulates adipocyte functions as an endogenous PPARγ agonist. Biochem J 438:111–119
28. Iwata M, Haruta T, Usui I, Takata Y, Takano A, Uno T, Kawahara J, Ueno E, Sasaoka T, Ishibashi O, Kobayashi M (2001) Pioglitazone ameliorates tumor necrosis factor-alpha-induced insulin resistance by a mechanism independent of adipogenic activity of peroxisome proliferator–activated receptor-gamma. Diabetes 50:1083–1092
29. Kobayashi T, Fujimori K (2012) 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
30. Goto T, Takahashi N, Kato S, Egawa K, Ebisu S, Moriyama T, Fushiki T, Kawada T (2005) Phytol directly activates peroxisome proliferator-activated receptor α (PPARα) and regulates gene expression involved in lipid metabolism in PPARα-expressing HepG2 hepatocytes. Biochem Biophys Res Commun 337:440–445
31. Stephens JM, Pekala PH (1991) Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. J Biol Chem 266:21839–21845
32. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Whali W, Willson TM, Lenhard JM, Lehmann JM (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator- activated receptors alpha and gamma. Proc Natl Acad Sci USA 94:4318–4323
33. Sheu SH, Kaya T, Waxman DJ, Vajda S (2005) Exploring the binding site structure of the PPAR gamma ligand-binding domain by computational solvent mapping. Biochemistry (Mosc) 44:1193–1209
34. Medina-Gomez G, Gray SL, Yetukuri L, Shimomura K, Virtue S, Campbell M, Curtis RK, Jimenez-Linan M, Blount M, Yeo GSH, Lopez M, Seppänen-Laakso T, Ashcroft FM, Orešič M, Vidal-Puig A (2007) PPAR gamma 2 prevents lipotoxicity by controlling adipose tissue expandability and peripheral lipid metabolism. PLoS Genet 3:e64
35. Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79:1147–1156
36. Chambrier C, Bastard JP, Rieusset J, Chevillotte E, Bonnefont-Rousselot D, Therond P, Hainque B, Riou JP, Laville M, Vidal H (2002) Eicosapentaenoic acid induces mRNA expression of peroxisome proliferator-activated receptor γ. Obes Res 10:518–525
37. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M, Shimomura I (2003) Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 521:1655–1663
38. Kadowaki T, Yamauchi T (2005) Adiponectin and adiponectin receptors. Endocr Rev 26:439–451
39. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE (2004) Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. J Biol Chem 279:12152–12162
40. Tonelli J, Li W, Kishore P, Pajvani UB, Kwon E, Weaver C, Scherer PE, Hawkins M (2004) Mechanisms of early insulin-sensitizing effects of thiazolidinediones in type 2 diabetes. Diabetes 53:1621–1629
41. Zapata-Bustos R, Alonso-Castro ÁJ, Gómez-Sánchez M, Salazar-Olivo LA (2014) *Ibervillea sonorae* (Cucurbitaceae) induces the glucose uptake in human adipocytes by activating a PI3 K-independent pathway. J Ethnopharmacol 152:546–552
42. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 114:1752–1761
43. Houstis N, Rosen ED, Lander ES (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440:944–948
44. Delerive P, Furman C, Teissier E, Fruchart J, Duriez P, Staels B (2000) Oxidized phospholipids activate PPARalpha in a phospholipase A2-dependent manner. FEBS Lett 471:34–38
45. Kozak KR, Gupta RA, Moody JS, Ji C, Boeglin WE, DuBois RN, Brash AR, Marnett LJ (2002) 15-Lipoxygenase metabolism of 2-arachidonylglycerol. Generation of a peroxisome proliferator-activated receptor alpha agonist. J Biol Chem 277:23278–23286
46. Schopfer FJ, Lin Y, Baker PR, Cui T, Garcia-Barrio M, Zhang J, Chen K, Chen YE, Freeman BA (2005) Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor gamma ligand. Proc Natl Acad Sci USA 102:2340–2345