RESEARCH ARTICLE

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

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Scope: Tomato is one of the most common crops worldwide and contains many beneficial compounds that improve abnormalities of lipid metabolism. However, the molecular mechanism underlying the effect of tomato on lipid metabolism is unclear. It has been commonly accepted that peroxisome proliferator-activated receptor α (PPARα) is one of the most important targets for ameliorating abnormalities of lipid metabolism. Therefore, we focused on the activation of PPARα and attempted to detect active compounds activating PPARα in tomato.

Methods and results: To identify such active compounds, we screened fractions of tomato extracts using PPARα luciferase reporter assay. One fraction, rechromatographed-fraction eluted in 57 min (RF57), significantly increased PPARα reporter activity, in which a single compound is detected by LC/MS analysis. On the basis of LC/MS and NMR analyses, we determined the chemical structure of the active compound in RF57 as 9-oxo-10(E),12(E)-octadecadienoic acid (9-oxo-ODA). The RF57 fraction significantly increased the mRNA expression levels of PPARα target genes involved in fatty acid oxidation and O_2 consumption in mouse primary hepatocytes. Furthermore, RF57 inhibited cellular triglyceride accumulation in the hepatocytes.

Conclusion: These findings suggest that tomatoes containing 9-oxo-ODA that acts on PPARα are valuable for ameliorating abnormalities of lipid metabolism.

Keywords:
Dyslipidemia / Fatty acid oxidation / Oxylipin / PPARα / Tomato

1 Introduction

Obesity is a major risk factor for chronic diseases such as diabetes, cardiovascular diseases, and hypertension [1, 2]. This is because an enhanced adiposity contributes to insulin resistance, hyperglycemia, and dyslipidemia [3, 4]. In particular, dyslipidemia is one of the direct risk factors for arteriosclerosis and cirrhosis. It has been considered that

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Abbreviations: 9-oxo-ODA, 9-oxo-10(E),12(E)-octadecadienoic acid; **ACS,** acyl-CoA synthase; **AOX,** acyl-CoA oxidase; **CLA,** conjugated linoleic acid; **CPT-1,** carnitine-palmitoyl transferase-1; **F42,** fraction eluted in 42 min; **FBS,** fetal bovine serum; **OCR,** O_2 consumption rate; **ODS,** octa decyl silyl; **P/S,** penicillin-streptomycin mixed solution; **PPAR,** peroxisome proliferator-activated receptor; **RF57,** rechromatographed-fraction eluted in 57 min; **TG,** triglyceride

dyslipidemia is partially due to the dysfunction of lipid metabolism in the liver. Therefore, it is important to ameliorate the dysfunction of hepatic lipid metabolism to suppress arteriosclerosis and cirrhosis.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily, which regulate energy homeostasis (glucose and lipid metabolisms), inflammation, proliferation, and differentiation [5–9]. In particular, PPARα is a master regulator of fatty acid oxidation by controlling transcription of its target genes such as *carnitine-palmitoyl transferase-1* (*CPT-1*), *acyl-CoA oxidase* (*AOX*), and *acyl-CoA synthase* (*ACS*) [10, 11]. Consistent with this function, PPARα is mainly expressed in tissues with high lipid catabolic capacities such as the liver, skeletal muscle, and brown adipose tissue [7, 12]. It has been reported that activation of PPARα enhances fatty acid β-oxidation in the liver and decreases the level of circulating or cellular lipids in obese diabetes patients [13, 14]. Therefore, the regulation of PPARα activity is one of the most important means of managing chronic diseases with respect to the dysfunction of hepatic lipid metabolism.

Tomato is one of the most popular and extensively consumed crops in the world. Furthermore, tomatoes contain many beneficial nutrients and phytochemicals that are considered to suppress chronic diseases. Many previous studies have shown that the dietary intake of tomato is associated with a reduced risk of chronic diseases such as cardiovascular diseases, cancer, and type-2 diabetes [15–18]. Now, the prevention and amelioration of such chronic diseases by foodstuffs are socially required, although many therapeutic medicines for chronic diseases are widely used. Thus, tomato is a candidate for preventing and ameliorating the dysfunction of lipid metabolism. However, the molecular mechanism underlying the effect of tomato on lipid metabolism is unclear.

We have identified various food-derived compounds activating PPARα *in vitro* and *in vivo* [19–22]. In this study, we focused on the activation of PPARα, and attempted to identify a new anti-dyslipidemia compound that activates PPARα in tomato. Screening of HPLC fractions derived from tomato extracts revealed that one fraction, rechromatographed-fraction eluted in 57 min (RF57), activated PPARα in a luciferase assay. Then, we analyzed the RF57 fraction by NMR and LC/MS analyses, and finally identified 9-oxo-10(E),12(E)-octadecadienoic acid (9-oxo-ODA) as the active compound in RF57. RF57 increased the mRNA expression levels of PPARα target genes involved in fatty acid β-oxidation in mouse hepatocytes concomitantly with an increase in O_2 consumption rate (OCR). Furthermore, RF57 decreased the amounts of cellular triglyceride (TG) in the hepatocytes. These results indicate that RF57 suppresses lipid accumulation through PPARα activation in mouse primary hepatocytes, suggesting that the effect of RF57 on PPARα transactivation ameliorates dysfunction of hepatic lipid metabolism.

2 Materials and methods

2.1 Plant materials and chemicals

In this study, we used the *Furikoma* tomato cultivar, which is widely used for processing. *Furikoma* tomatoes from the 2007 harvest were provided by the Agriculture and Forestry Research Center (Chiba, Japan). Authentic 9- and 13-oxo-ODA were from Laroden Fine Chemicals (Malmö, Sweden). GW7647 and GW6471, a PPARα-specific agonist and antagonist, respectively [23], were from Sigma (MO, USA). All other chemicals used were from Invitrogen (CA, USA), Nacalai Tesque (Kyoto, Japan), Wako (Osaka, Japan), or Dainippon Sumitomo Pharma (Osaka, Japan), and were guaranteed to be of reagent grade, HPLC grade, or tissue-culture grade.

2.2 Extract preparation and fractionation of crude extract

Furikoma tomatoes were blended in an electric mixer and freeze-dried. First, lipophilic components were extracted from the freeze-dried *Furikoma* powder using ethanol at room temperature for 24 h. Ethanol in the extract was evaporated under vacuum at 40°C using a rotary evaporator. Furthermore, the ethanol extract was extracted using hexane at room temperature for 6 h and hexane was evaporated under vacuum at 40°C using a rotary evaporator. The crude *Furikoma* extract was stored at −20°C until analysis. The crude *Furikoma* extract was fractionated by reverse-phase HPLC on a $5C_{18}$-AR-2 octa decyl silyl (ODS) column (6.0 × 150 mm; Nacalai Tesque) using a mobile phase of water (solvent A) and ACN (solvent B) with 0.1% v/v formic acid added to both solvents. A gradient elution program at a 1.0 mL/min flow rate was used to achieve the separation. The program began with an isocratic step at 30% solvent B in solvent A followed by a linear elution gradient from 30 to 90% solvent B in solvent A for 60 min. To monitor HPLC elution, DAD was used in the range of 200–700 nm. The flow rate was set at 1.0 mL/min. Eluted fractions were collected at 1 mL/min with a CHF 100AA fraction collector (Advantec, Tokyo, Japan). For determining an active compound, an active fraction (F42) was further separated by reverse-phase HPLC on another column, a TSK-gel column ODS-100V (4.6 × 250 mm; TOSOH, Tokyo, Japan). The mobile phase, gradient program, and monitor were the same as those in the method using the $5C_{18}$-AR-2 ODS column. The flow rate was set at 0.5 mL/min. Eluted fractions were collected at 0.5 mL/min using the same collector.

2.3 LC/MS

An Agilent 1100 system (Agilent Technologies, CA, USA) coupled to a Finnigan LTQ-FT (Thermo Fisher Scientific,

MA, USA) was used for LC-FTICR-MS analysis. The data were acquired and browsed using Xcalibur software version 2.0 (Thermo Fisher Scientific). RF57 was applied to the TSK-gel column ODS-100V. Water (solvent A) and ACN (solvent B) were used as the mobile phase with 0.1% v/v formic acid added to both solvents. The program begins with an isocratic step at 30% solvent B in solvent A followed by a linear elution gradient from 30 to 90% solvent B in solvent A for 60 min. The flow rate was set at 0.5 mL/min, and the column oven temperature was set at 40°C; 20 μL of each sample was injected. To monitor HPLC elution, a photodiode array detector was used in the wavelength range of 200–650 nm. The ESI setting was as follows: spray voltage, 4.0 kV and capillary temperature, 300°C for negative-ionization modes. Nitrogen sheath gas and auxiliary gas were set at 40 and 15 arbitrary units, respectively. A full MS scan with internal standards was performed in the *m/z* range 100–1500 at a resolution of 100 000 (at *m/z* 400). MS/MS fragmentation was carried out at a normalized collision energy of 35.0% and an isolation width of 4.0 (*m/z*) in the ion trap mode. The relative accumulation level of RF57 was estimated by dividing the peak area of the metabolite by that of an internal standard (*cis*-10-heptadecenoic acid).

2.4 Luciferase assay

Monkey CV1 kidney cells (American Type Culture Collection, USA) used in luciferase assay were cultured in DMEM with 10% fetal bovine serum (FBS) (JRH Bioscience, Kansas, USA) and 1% penicillin–streptomycin mixed solution (P/S; Invitrogen) at 37°C under a humidified 5% CO_2 atmosphere. Luciferase assay was performed using a GAL4/PPAR chimera system, as previously described [19, 22]. Briefly, we transfected p4xUASg-tk-luc (a reporter plasmid), pM-hPPARα (an expression plasmid for a chimera protein for the GAL4 DNA-binding domain and each human PPAR-ligand-binding domain), and pRL-CMV (an internal control for normalizing transfection efficiencies) into CV1 cells cultured on 60 mm tissue culture dishes. The transfection was performed using LipofectAMINE (Invitrogen) following the manufacturer's protocol. Five hours after the transfection, the transfected cells were transferred to 96-well plates and cultured in a medium containing each compound for 24 h. Luciferase assay was performed using the dual luciferase system (Promega, MO, USA) following the manufacturer's protocol.

2.5 Preparation of mouse primary hepatocytes

Isolation and culture of mouse hepatocytes were performed in accordance with Watanabe's protocol [24, 25]. Briefly, C57/BL/6J male mice, 5–6 weeks old, (Nihon SLC, Shizuoka, Japan) were anesthetized with Nembutal (Dainippon Sumitomo Pharma) intraperitonealy and the livers were perfused with 40 mL of Liver Perfusion Medium (Invitrogen) followed by 30 mL of Liver Digestion Medium (Invitrogen), both at a flow rate of 5 mL/min. Hepatocytes were dispersed in Hepatocyte Wash Medium (Invitrogen) supplemented with 1% P/S by dissection and gentle shaking. After filtration through a 150 μm nylon mesh filter, hepatocytes were isolated by repeated centrifugation (3–5 times) at $50 \times g$ for 3 min. The isolated hepatocytes were resuspended in William's E Medium (Invitrogen) supplemented with 10% FBS, 0.1 μM insulin, 1 μM dexamethasone, and 1% P/S, and cultured in type-1 collagen-coated 12-well or 24-well plates (Iwaki, Chiba, Japan) at a cell density of 3×10^5 cells/well. After a 2-h incubation at 37°C in a 5% CO_2 atmosphere, the hepatocytes were used for viability assay (cell viability was usually more than 90%), mRNA quantification assay, immunoblotting assay, OCR assay, and TG accumulation assay.

2.6 Quantification of mRNA expression levels

To investigate the effects of *Furikoma* extract on the mRNA expression of PPARα target genes, mouse primary hepatocytes in 12-well plates (3×10^5 cells/mL) were treated with a *Furikoma* fraction sample at various concentrations in serum-free DMEM. After 24-h incubation, cells were washed with PBS and harvested for RNA isolation using Sepasol (Nacalai Tesque) in accordance with the manufacturer's instructions. Using M-MLV reverse transcriptase (Invitrogen), total RNA was reverse-transcribed in accordance with the manufacturer's instructions using a thermal cycler (Takara PCR Thermal Cycler SP from Takara Bio, Shiga, Japan). To determine mRNA expression levels, real-time quantitative RT-PCR analysis was performed with a LightCycler System (Roche Diagnostics) using SYBR Green fluorescence signals, as described previously [19, 22]. The oligonucleotide primer sets of PPARα-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 *CPT-1* (accession number, AF320000; Fwd, ctgttaggcctcaacaccgaac; Rev, ctgtcatggctaggcggtacat), mouse *AOX* (NM_015729, Fwd, gcaccattgccattcgataca, Rev, acggctattctcacagcagtgg), mouse *ACS* (AJ223958; Fwd, acatccacgtgtatgagttctacgc; Rev, agtagacgaagttctcacggtcgat), and mouse *36B4* (BC011291; Fwd, tgtgtgtctgcagatcgggtac; Rev, ctttggcgggatttagtcgaag) as an internal control. All data indicating mRNA expression levels were presented as a ratio relative to the internal control in each experiment.

2.7 Immunoblotting assay

Immunoblotting was carried out as previously described [20, 26]. The anti-mouse AOX antibody was from Abcam (MA, USA). Anti-mouse β-actin and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Cell

Signaling Technology (MA, USA) and Santa Cruz Biotechnology (CA, USA), respectively. Protein bands were detected by chemiluminescence using an enhanced chemiluminescence (ECL) system (NEN Lifescience Products) in accordance with the manufacturer's instructions.

2.8 OCR assay

To investigate the effects of *Furikoma* extract on OCR in mouse primary hepatocytes, seeded cells in customized 24-well plates (5×10^5 cells/ml) were treated with the *Furikoma* fraction sample at various concentrations in serum-free DMEM. After 24-h incubation, OCR in the treated cells was measured in real-time using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, N. Billerica, MA, USA) in accordance with the manufacturer's instructions.

2.9 Measurement of TG accumulation

To examine TG accumulation, mouse primary hepatocytes on 12-well plates (3×10^5 cells/mL) were treated with 500 μM oleic acid coupled to fatty acid-free BSA (molar ratio, 5:1) in 10% FBS DMEM for 24 h. Then, the cells were treated with the *Furikoma* fraction sample at various concentrations and/or 10 μM GW6471, a PPARα-specific antagonist, in serum-free DMEM. After an additional 24-h incubation, the cells were washed with PBS and placed immediately in lysis buffer containing 50 mM PIPES (pH 6.5) and 1% Triton X-100 (Nacalai Tesque). The lysate was centrifuged at 10 000 rpm for 5 min, and supernatants were collected. The TG level in the supernatants was measured using a TG E-test Wako kit (Wako).

2.10 Statistical analysis

The data are presented as mean ± SEM. Data were assessed by one-way ANOVA and Dunnet's multiple comparison tests. Differences were considered significant when p was < 0.05.

3 Results

3.1 Identification of *Furikoma* fraction activating PPARα

To identify active compounds activating PPARα, we screened fractions of *Furikoma* extracts using PPARα luciferase reporter assay. An ethanol extract of *Furikoma* tomatoes was fractionized by HPLC using an analytical ODS column, and then we examined the effects of the fractions on PPARα activation in the reporter assay. The assay revealed that one fraction, which was eluted in 42 min (F42), significantly increased PPARα activity (data not shown). To identify the active compound in F42, we further separated F42 to obtain a single peak by HPLC using another analytical ODS column. As a result, a single peak at 57 min (RF57) was obtained (inset of Fig. 1), which showed PPARα activation (Fig. 1) as well as GW7647, a PPARα-specific agonist [23].

3.2 Determination of the chemical structure of active compound in *Furikoma* RF57

To determine the chemical structure of the active compound in RF57, we analyzed it by LC/MS. In the negative ESI mode, a protonated ion at m/z 295.23 $[M+H]^+$ was detected (data not shown), suggesting that a possible molecular formula for RF57 is $C_{18}H_{30}O_3$ with two sites of unsaturation. To further analyze the chemical structure of RF57, we used ^{1}H-NMR and ^{13}C-NMR analyses. NMR analysis revealed that RF57 is 9-oxo-ODA or 13-oxo-9,11-octadecadienoic acid (13-oxo-ODA), but we could not identify the chemical structure of RF57 by NMR (data not shown). Therefore, we compared RF57 with authentic 9-oxo-ODA and 13-oxo-ODA by LC/MS. The MS/MS pattern of RF57 agreed with that of the 9-oxo-ODA isomer (Fig. 2A). Furthermore, the retention time (Fig. 2B) and absorption wavelength (Fig. 2C) of RF57 completely agreed with those of 9-oxo-10(E),12(E)-ODA (Fig. 2D). RF57 increased PPARα

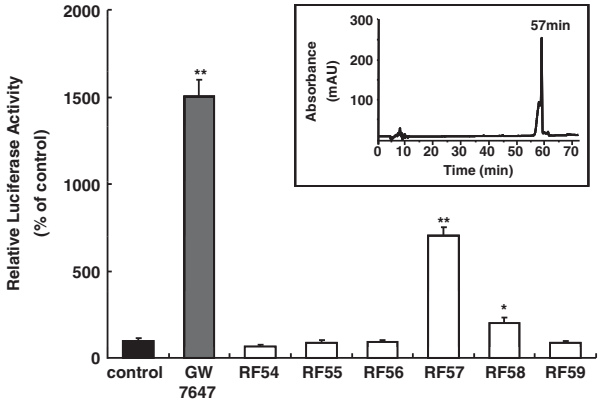

Figure 1. Screening of *Furikoma* fractions in PPARα luciferase reporter assay. A reporter plasmid (p4xUASg-tk-luc) and an expression vector for a GAL4 PPARα chimera protein (pM-hPPARα) were transfected into CV1 cells together with an internal control reporter plasmid (pRL-CMV). Twenty-four hours after the transfection, the cells were treated with *Furikoma* fractions for 24 h. GW7647 (10 nM), which is a PPARα-specific agonist, was used as a positive control. Luciferase activity was measured using a dual luciferase system. The activity of a vehicle control was set at 100%, and the relative luciferase activities are presented as fold induction with respect to that of the vehicle control. Data are presented as mean ± SEM ($n = 4$–5). $^*p < 0.05$, $^{**}p < 0.01$ *versus* control. The inset figure shows an HPLC chromatogram (276 nm) during the purification of F42.

reporter activity in a dose-dependent manner (Fig. 3). Furthermore, we compared the PPARα reporter activities of RF57 and conjugated linoleic acid (CLA), which is a precursor of 9-oxo-ODA [27] and is well known as a PPARα agonist [28]. The activity of RF57 as a PPARα ligand was similar to that of CLA, as shown in luciferase reporter assay (RF57 and CLA showed 26.4- and 12.8-fold increases at 20 μM in the PPARα reporter assay, respectively).

Moreover, to investigate whether this effect of RF57 on the luciferase activity is dependent on PPARα, we coadministered GW6471, a PPARα-specific antagonist [23]. The increase in luciferase activity by RF57 was abolished by GW6471, although the basal level of the cellular TG was unchanged by GW6471 (data not shown). These findings indicated that RF57 is 9-oxo-ODA and acts as a PPARα agonist.

3.3 Effects of RF57 on mRNA expression levels of PPARα-target genes in mouse primary hepatocytes

To elucidate whether *Furikoma* RF57 actually induces PPARα activation, we examined the mRNA expression levels of PPARα-target genes in mouse primary hepatocytes in the presence of various concentrations of RF57. Treatment with RF57 for 24 h significantly increased the mRNA expression levels of PPARα target genes such as *CPT-1*, *AOX*, and *ACS* in primary hepatocytes (Fig. 4A), as well as that of GW7647, a synthetic agonist (*CPT-1*, *AOX*, and *ACS* mRNA expressions were significantly increased 3.66-, 1.60-, and 1.19-fold increases in 100 nM GW7647, respectively). Furthermore, the expression level of the AOX protein was increased by the addition of 20 μM RF57, as well as 100 nM GW7647 (Fig. 4B). Moreover, we examined the effects of

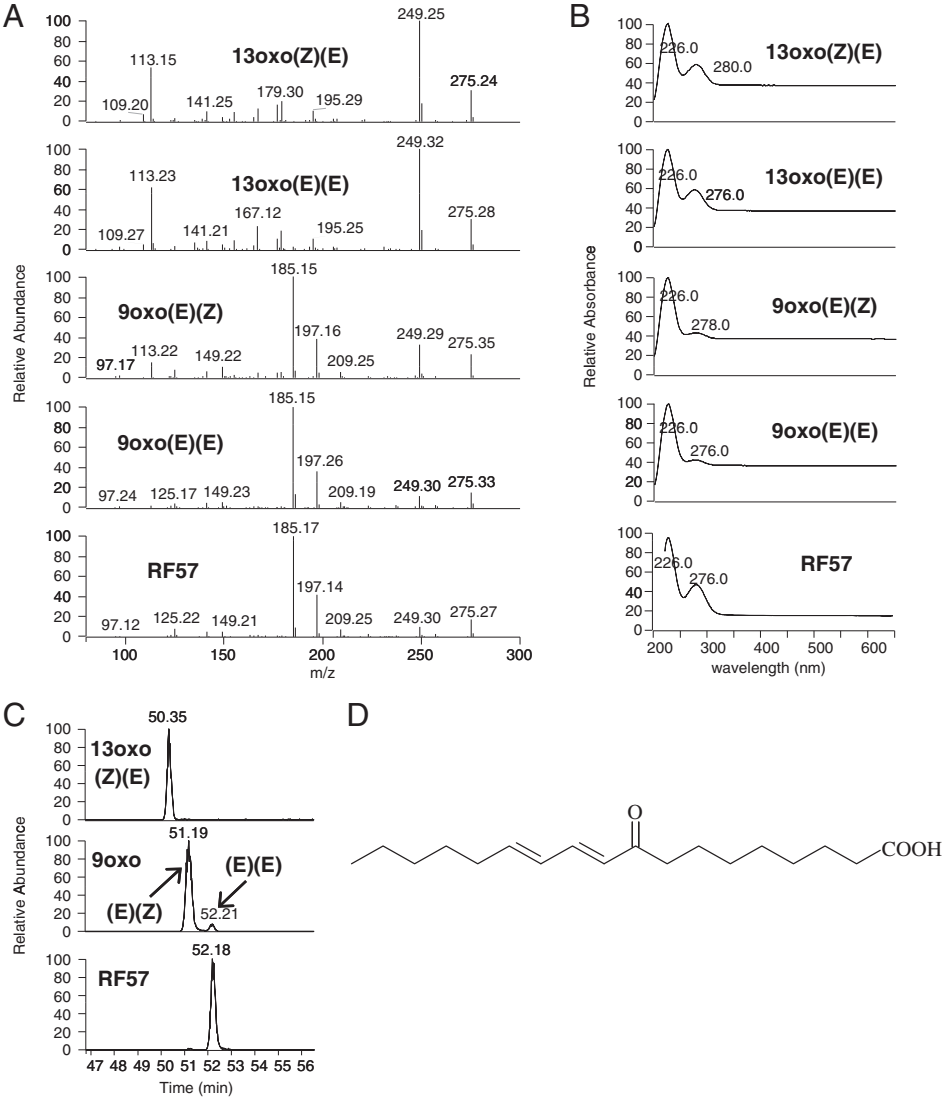

Figure 2. Determination of the chemical structure of active compound in RF57. To determine the chemical structure of the active compound in RF57, the MS/MS patterns (A), retention time (B), and absorption wavelength (C) of RF57 were compared with those of authentic 9-oxo-ODA, or 13-oxo-ODA by LC/MS analysis. (D) Chemical structure of 9-oxo-10(E),12(E)-ODA.

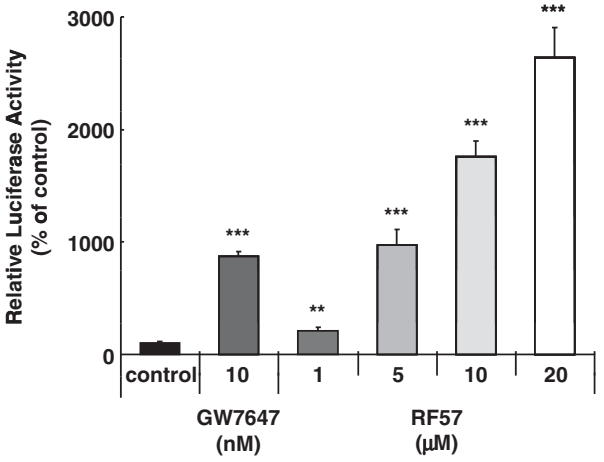

Figure 3. Effects of RF57 on PPARα activation in luciferase assay. Luciferase assay was performed using the same protocol described in Fig. 1 legend. The transfected CV1 cells were treated with RF57 at various concentrations or 10 nM GW7647 for 24 h. The activity of a vehicle control was set at 100%, and the relative luciferase activities are presented as fold induction with respect to that of the vehicle control. Data are presented as mean ± SEM ($n = 4$–5). $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control.

RF57 on OCR in parallel to the fatty acid β-oxidation rate in hepatocytes. The OCRs of hepatocytes treated with RF57 for 24 h were significantly increased in comparison with that of control cells (Fig. 4C). These results indicated that RF57 induced both the mRNA and protein expression of PPARα target genes and increased OCR in mouse primary hepatocytes, suggesting that fatty acid oxidation is enhanced by the addition of RF57 in hepatocytes.

3.4 Effect of RF57 on the amount of cellular TG

To confirm the observed induction of PPARα activation by *Furikoma* RF57, we examined the effect of RF57 on the amount of cellular TG. As shown in Fig. 5, treatment with RF57 for 24 h significantly decreased the amount of cellular TG in primary hepatocytes (the treatment with 10 and 20 μM RF57 reduced cellular TG level to 89.7 and 83.7%, respectively). Furthermore, to verify that this effect of RF57 on the amount of cellular TG is dependent on PPARα activation, we coadministered GW6471, a PPARα-specific antagonist. The decrease in cellular TG level by RF57 was abolished by GW6471, although the basal level of the cellular TG was unchanged by GW6471 (Fig. 5). The effect

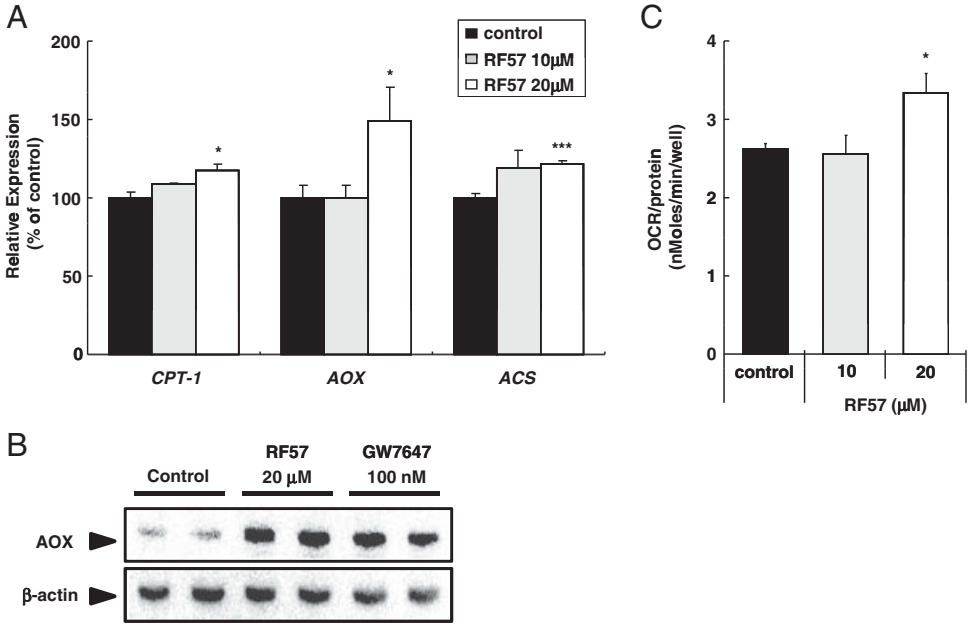

Figure 4. Effects of RF57 on gene expression, protein expression and O_2 consumption in mouse primary hepatocytes. (A) mRNA expression levels of *CPT-1*, *AOX*, and *ACS* in mouse primary hepatocytes treated with 10 or 20 μM RF57 for 24 h. The amounts of mRNAs were quantified by real-time PCR. The relative amount of each transcript was normalized to the amount of the *36B4* transcript. The activity of a vehicle control was set at 100%, and the relative luciferase activities are presented as fold induction with respect to that of the vehicle control. (B) Protein expression of AOX in the hepatocytes treated with 20 μM RF57 and GW7647 for 24 h. The same amounts of protein (20 μg/lane) were loaded and blotted onto PVDF membranes. The membranes were sequentially treated with primary antibodies as indicated and secondary antibodies conjugated with horseradish peroxidase. The enhanced chemiluminescence system was used for visualization of membranes. (C) Effect of RF57 treatment on OCR in hepatocytes. The hepatocytes were treated with 10 or 20 μM RF57 for 24 h. The OCR was measured using an XF24 Extracellular Flux Analyzer. Data are presented as mean ± SEM ($n = 4$–5). $^{*}p < 0.05$, $^{***}p < 0.001$ *versus* each control.

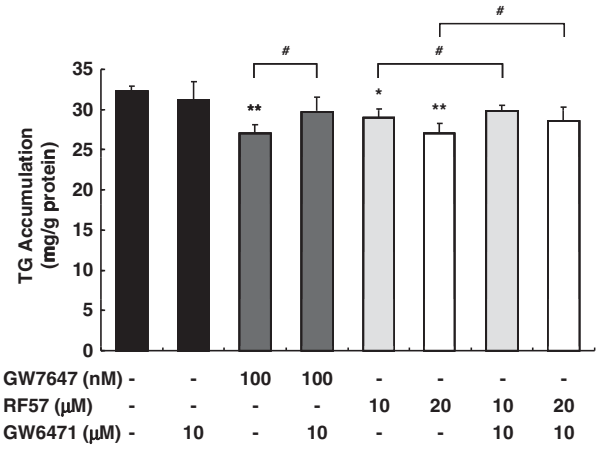

Figure 5. Effects of RF57 on cellular TG accumulation in mouse primary hepatocytes. Mouse primary hepatocytes were incubated with oleic acid (coupled to BSA) for 24 h, and then treated with 10 or 20 μM RF57 or 100 nM GW7647, and/or 10 μM GW6471 for an additional 24 h. GW6471 is a PPARα-specific antagonist. The amounts of TG in the hepatocytes were measured by an enzymatic method. Data are presented as mean ± SEM ($n = 4$–5). $^{*}p<0.05$, $^{**}p<0.01$ *versus* each control without GW6471. ♯; $p<0.05$ compared between indicated groups.

of RF57 on cellular TG amount was almost the same as that of GW7647 (Fig. 5). These findings indicate that *Furikoma* RF57 decreases the amount of cellular TG via PPARα activation in mouse primary hepatocytes.

4 Discussion

Many previous studies have shown that tomatoes contain many beneficial natural compounds that suppress various chronic diseases such as cardiovascular diseases, cancer, and type-2 diabetes [15–18]. These beneficial effects of tomato are generally attributed to different compounds such as carotenoids, vitamins, and flavonoids. Lycopene, one of the major carotenoids in tomato, has been well researched as a beneficial compound. Lycopene has anti-oxidation and anti-inflammatory properties, which result in the suppression of chronic diseases [29–33]. It has also been reported that administration of tomato products changes hepatic lipid metabolism [34, 35], suggesting a possibility that these effects of tomato on lipid metabolism are due to the properties of lycopene. However, the molecular mechanism is unknown. Hepatic lipid metabolism is regulated by various steps such as transcription and enzymatic activation. We focused on the PPARα-dependent transcriptional regulation of hepatic lipid metabolism. Activation of PPARα in the liver enhanced β-oxidation, resulting in a decrease in liver and plasma TG levels [14, 36] and contributing to beneficial effects such as anti-atherogenic and anti-diabetogenic effects [37–39]. Indeed, a crude extract of *Furikoma* activated PPARα in luciferase assay (data not shown). However, we previously reported that lycopene does not activate PPARα, as determined by luciferase reporter assay [19]. Therefore, we hypothesized that there are other compounds that activate PPARα in tomato, resulting in the regulation of hepatic lipid metabolism. In this study, we identified an active fraction, RF57, activating PPARα in the *Furikoma* tomato, and this study is the first to identify such a fraction from tomato. Therefore, this identification is very valuable for clarifying the molecular mechanism by which tomato consumption ameliorates the dysfunction of hepatic lipid metabolism.

In this study, we screened tomato extracts by PPARα luciferase reporter assay and finally identified 9-oxo-ODA as the activator of PPARα from tomato (Figs. 1 and 2). 9-oxo-ODA is a major oxylipin in plants and is synthesized from CLA by lipoxygenase [27, 40]. Many previous studies have shown that various fatty acids and their derivatives activate PPARα [41, 42]. In particular, CLA has been well reported as a potent naturally occurring ligand for PPARα [28]. Indeed, administration of CLA induces PPARα activation and results in an enhancement of fatty acid oxidation in the liver and a decrease in plasma TG levels [43–45]. Thus, CLA has been considered to be a valuable food-derived compound for the management of dysfunctions of lipid metabolism. In this study, 9-oxo-ODA activated PPARα as determined by luciferase assay and increased the mRNA expression levels of PPARα target genes such as *CPT-1*, *AOX*, and *ACS* in mouse primary hepatocytes. These findings suggest that 9-oxo-ODA enhances fatty acid oxidation in hepatocytes through PPARα activation. This possibility was confirmed by the observation that the addition of 9-oxo-ODA increased OCR in mouse primary hepatocytes. Interestingly, the activity of 9-oxo-ODA as a PPARα ligand was similar to that of CLA, as shown in luciferase reporter assay. These results indicate that 9-oxo-ODA is a potent ligand for PPARα in hepatocytes as well as CLA, suggesting that 9-oxo-ODA ameliorates abnormalities of lipid metabolism in the liver, although further investigation is required to elucidate the *in vivo* effects of 9-oxo-ODA.

In conclusion, we found that tomato contains the anti-dyslipidemic component 9-oxo-ODA. In addition, it was shown that 9-oxo-ODA increased the mRNA expression levels of PPARα target genes involved in fatty acid oxidation and oxygen consumption, so that the cellular accumulation of TG was suppressed in mouse primary hepatocytes. These findings suggest that tomatoes containing 9-oxo-ODA activating PPARα are valuable for ameliorating abnormalities of lipid metabolism associated with obesity and diabetes.

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The authors have declared no conflict of interest.

5 References

[1] Reilly, M. P., Rader, D. J., The metabolic syndrome: more than the sum of its parts? *Circulation* 2003, *108*, 1546–1551.

[2] Miranda, P. J., DeFronzo, R. A., Califf, R. M., Guyton, J. R., Metabolic syndrome: definition, pathophysiology, and mechanism. *Am. Heart J.* 2005, *149*, 33–45.

[3] Kahn, B. B., Flier, J. S., Obesity and insulin resistance. *J. Clin. Invest.* 2000, *106*, 473–481.

[4] Ginsberg, H. N., Insulin resistance and cardiovascular disease. *J. Clin. Invest.* 2000, *106*, 453–458.

[5] Escher, P., Wahli, W., Peroxisome proliferator-activated receptors: insights into multiple cellular function. *Mutat. Res.* 2000, *448*, 121–138.

[6] Desvergne, B., Wahli, W., Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr. Rev.* 1999, *20*, 649–688.

[7] Chinetti, G., Fruchart, J. C., Staels, B., Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. *Inflamm. Res.* 2000, *49*, 497–505.

[8] Duval, C., Chinetti, G., Trottein, F., Fruchart, J. C., Staeles, B., The role of PPARs in atherosclerosis. *Trends Mol. Med.* 2002, *8*, 422–430.

[9] Goldenberg, I., Benderly, M., Goldbourt, U., Update on the use of fibrates: focus on bezafibrate. *Vasc. Health Risk Manag.* 2008, *4*, 131–141.

[10] Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R. *et al.*, The mouse peroxisome proliferator activated receptor recognizes a response element in the 5′ flanking sequence of the rat acyl CoA oxidase gene. *EMBO J.* 1992, *11*, 433–439.

[11] Roepstorff, C., Halberg, N., Hillig, T., Saha, A. K. *et al.*, Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. *Am. J. Physiol. Endocrinol. Metab.* 2005, *288*, E133-E142.

[12] Mandard, S., Müllar, M., Kersten, S., Peroxisome proliferator-activated receptors alpha target genes. *Cell. Mol. Life Sci.* 2004, *61*, 393–416.

[13] Steals, B., Dallongeville, J., Auwerx, J., Schoonjans, K. *et al.*, Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation* 1998, *98*, 2088–2093.

[14] Peters, J. M., Hennuyer, N., Staels, B., Fruchart, J. C. *et al.*, Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor a-deficient mice. *J. Biol. Chem.* 1997, *272*, 27307–27312.

[15] Pandey, D. K., Shekelle, R., Selwyn, B. J., Tangney, C., Stamler, J., Dietary vitamin C and β-carotene and risk of death in middle-aged men. *Am. J. Epidemiol.* 1995, *142*, 1269–1278.

[16] Lazarus, S. A., Bowen, K., Garg, M. L., Tomato juice and platelet aggregation in type 2 diabetes. *JAMA* 2004, *292*, 805–806.

[17] Giovannucci, E., Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. *J. Natl. Cancer Inst.* 1999, *91*, 317–331.

[18] Blum, A., Monir, M., Wirsansky, I., Ben-Arzi, S., The benefical effects of tomatoes. *Eur. J. Intern. Med.* 2005, *16*, 402–404.

[19] Takahashi, N., Kawada, T., Goto, T., Yamamoto, T. *et al.*, Dual action of isoprenols from herbal medicines on both PPARγ and PPARα in 3T3-L1 adipocytes and HepG2 hepatocytes. *FEBS Lett.* 2002, *514*, 315–322.

[20] Goto, T., Takahashi, N., Kato, S., Egawa, K. *et al.*, 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.* 2005, *337*, 440–445.

[21] Takahashi, N., Kang, M. S., Kuroyanagi, K., Goto, T. *et al.*, Auraptene, a citrus fruit compound, regulates gene expression as a PPARalpha agonist in HepG2 hepatocytes. *Biofactors* 2008, *33*, 25–32.

[22] Takahashi, N., Kawada, T., Goto, T., Kim, C. S. *et al.*, 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.* 2003, *550*, 190–194.

[23] Xu, H. E., Stanley, T. B., Montana, V. G., Lambert, M. H. *et al.*, Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARα. *Nature* 2002, *415*, 813–817.

[24] Watanabe, M., Houten, M. S., Wang, L., Moschetta, A. *et al.*, Bile acids lower triglyceride levels *via* a pathway involving FXR, SHR, and SREBP-1c. *J. Biol. Chem.* 2004, *113*, 1408–1418.

[25] Hiyoshi, H., Yanagimachi, M., Ito, M., Yasuda, N. *et al.*, Squalene synthase inhibitors suppress triglyceride biosynthesis through the farnesol pathway in rat hepatocytes. *J. Lipid Res.* 2003, *44*, 128–135.

[26] Takahashi, N., Kawada, T., Yamamoto, T., Goto, T. *et al.*, Overexpression and ribozyme-mediated targeting of transcriptional coactivators CREB-binding protein and p300 revealed their indispensable roles in adipocytes differentiation through the regulation of Peroxisome proliferator-activated receptor γ. *J. Biol. Chem.* 2002, *277*, 16906–16912.

[27] Vellosillo, T., Martínez, M., López, M. A., Vicente, J. *et al.*, Oxylipin produced by the 9-lipoxygenase pathway in arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. *Plant Cell* 2007, *19*, 831–846.

[28] Moya-Camarena, S. Y., Vanden Heuvel, J. P., Blanchard, S. G., Leesnitzer, L. A., Belury, M. A., Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARα. *J. Lipid Res.* 1999, *40*, 1426–1433.

[29] Di Mascio, P., Kaiser, S., Sies, H., Lycopene as the most efficient biological carotenoids singlet oxygen quencher. *Arch. Biochem. Biophys*. 1989, *274*, 532–538.

[30] Rao, A. V., Agarwal, S., Role of antioxidant lycopene in cancer and heart disease. *J. Am. Coll. Nutr.* 2000, *19*, 563–569.

[31] Kim, G. Y., Kim, J. H., Ahn, S. C., Lee, H. J. *et al.*, Lycopene suppresses the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mutagen-activated protein kinases and nuclear factor-kappa B. *Immunology* 2004, *113*, 203–211.

[32] Reifen, R., Nissenkorn, A., Matas, Z., Bujanover, Y., 5-ASA and lycopene decrease the oxidative stress and inflammation induced by iron in rats with colitis. *J. Gastroenterol.* 2004, *39*, 514–519.

[33] Joo, Y. E., Karrasch, T., Mühlbauer, M., Allard, B. *et al.*, Tomato lycopene extract prevents lipopolysaccharide-induced NF-kB signaling but worsens dextran sulfate sodium-induced colitis in NF-kB^{EGFP} mice. *PLos ONE* 2009, *4*, e4562.

[34] Aizawa, K., Matsumoto, T., Inakuma, T., Ishijima, T. *et al.*, Administration of tomato and paprika beverages modifies hepatic glucose and lipid metabolism in mice: a DNA microarray analysis. *J. Agric. Food Chem.* 2009, *57*, 10964–10971.

[35] Ibrahim, H. S., Ahmed, L. A., El-din, M. M., The functional role of tomato products on lipid profile and liver function in adult rats. *J. Med. Food* 2008, *11*, 551–559.

[36] Hsu, M. H., Savas, U., Griffin, K. J., Johnson, E. F., Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor α in HepG2 cells. *J. Biol. Chem.* 2001, *276*, 27950–27958.

[37] Watts, G. F., Dimmitt, S. B., Fibrate, dyslipoproteinaemia and cardiovascular disease. *Curr. Opin. Lipidol.* 1999, *10*, 561–574.

[38] Remick, J., Weintraub, H., Setton, R., Offenbacher, J. *et al.*, Fibrate therapy: an update. *Cardiol. Rev.* 2008, *16*, 129–141.

[39] Fruchart, J. C., Peroxisome proliferator-activated receptor-alpha (PPARα): at the crossroads of obesity, diabetes and cardiovascular disease. *Atherosclerosis* 2009, *205*, 1–8.

[40] Vollenweider, S., Weber, H., Stolz, S., Chételat, A., Farmer, E. E., Fatty acid ketodiens and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased *Arabidopsis* leaves. *Plant J.* 2000, *24*, 467–476.

[41] Forman, B. M., Jasmine, C., Roland, M. E., Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Proc. Natl. Acad. Sci. USA.* 1997, *94*, 4312–4317.

[42] Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J. *et al.*, Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. *Proc. Natl. Acad. Sci. USA.* 1997, *94*, 4318–4323.

[43] Belury, M. A., Kempa-Steczko, A., Conjugated linoleic acid modulates hepatic lipid composition in mice. *Lipids* 1997, *32*, 199–204.

[44] Gudbrandsen, O. A., Rodríguez, E., Wergedahl, H., Mørk, S. *et al.*, Trans-10, cis-12- conjugated linoleic acid reduces hepatic triacylglycerol content and leptin mRNA level in adipose tissue in obese Zucker fa/fa rats. *Br. J. Nutr.* 2009, *102*, 803–815.

[45] Houseknecht, K. L., Vanden Heuvel, J. P., Moya-camarena, S. Y., Portocarrero, C. P. *et al.*, Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat. *Biochem. Biophys. Res. Commun.* 1998, *244*, 678–682.

