9-Oxo-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid activates peroxisome proliferator-activated receptor α in hepatocytes

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Short title: 9-oxo-OTA activates PPARα in hepatocytes

Abbreviations: 9-oxo-OTA, 9-oxo-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 9-oxo-ODA, 9-oxo-10,12-octadecadienoic acid; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EtOH, ethanol; HAD, *cis*-10-heptadecenoic acid; LC-MS, liquid chromatography–mass spectrometry; LNA, linoleic acid; ALA, α-linolenic acid; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; QTOFMS, quadrupole-time-of-flight MS; UPLC, ultra performance LC

Abstract

Peroxisome proliferator-activated receptor (PPAR)α is mainly expressed in the liver and plays an important role in the regulation of lipid metabolism. It has been reported that PPARα activation enhances fatty acid oxidation and reduces fat storage. Therefore, PPARα agonists are used to treat dyslipidemia. In the present study, we found that 9-oxo-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-oxo-OTA), which is α-linolenic acid (ALA) derivative, is present in tomato (*Solanum lycopersicum*) extract. We showed that 9-oxo-OTA activated PPARα and induced the mRNA expression of PPARα target genes in murine primary hepatocytes. These effects promoted fatty acid uptake and the secretion of β-hydroxybutyrate, which is one of the endogenous ketone bodies. We also demonstrated that these effects of 9-oxo-OTA were not observed in PPARα-knockout (KO) primary hepatocytes. To our knowledge, this is the first study to report that 9-oxo-OTA promotes fatty acid metabolism via PPARα activation and discuss its potential as a valuable food-derived compound for use in the management of dyslipidemia.

Key words: PPARα, oxylipin, hepatocyes, fatty acid metabolism, LC-MS.

Introduction

Dyslipidemia, which results from obesity, is a recognized risk factor for serious disorders, including arteriosclerosis, hyperlipidemia, hepatic steatosis, insulin resistance, and cardiovascular disease [1-4]. Therefore, avoidance of dyslipidemia is important to help prevent these lifestyle diseases.

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors [5-9] comprised of three isoforms PPARα, PPARβ/δ, and PPARγ. PPARα is very important in the regulation of lipid metabolism [5-7] and is expressed at high levels in the liver where it promotes β-oxidation, ketogenesis, and lipid transport [10, 11]. PPARα activation induces the expression of PPARα-regulated genes (e.g., *carnitine-O-palmitoyltransferase 1* [*CPT*] and *acyl-CoA oxidase* [*ACO*]) and activates β-oxidation [12-14]. These effects result in increased energy expenditure and reduced fat storage [15]. It has been reported that PPARα activation enhances fatty acid metabolism and decreases the levels of circulating and cellular lipids in obese diabetic patients [9, 16]. Therefore, regulation of PPARα activity is one of the important ways to manage chronic diseases related to lipid metabolism dysfunction.

Tomato (*Solanum lycopersicum*) is one of the most extensively consumed crops worldwide. Numerous studies have shown that dietary tomato intake is associated with a reduced risk of chronic diseases [17–20]. Recently, we reported that tomatoes contain the oxidized linoleic acid (LNA) derivatives 9-oxo-10,12-octadecadienoic acid (9-oxo-ODA) and 13-oxo-9,11-octadecadienoic acid (13-oxo-ODA), which can function as PPARα agonists [21,22]. Oxo-ODAs are oxylipins, which include fatty acid hydroperoxides, hydroxyl-, oxo-, epoxy, and keto fatty acids, divinyl ethers, aldehydes, and jasmonic acid [23]. Tomato is believed to contain not only oxo-ODAs but also

many other beneficial oxylipins that enhance lipid metabolism and suppress diseases such as arteriosclerosis, hyperlipidemia, and hepatic steatosis.

The aim of the present study was to identify the metabolite in tomato that enhances fatty acid metabolism and to determine its mechanism of action. In the present study, we showed that 9-oxo-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-oxo-OTA), which is a derivative of α-linolenic acid (ALA), is present in tomato fruit extract. We showed that 9-oxo-OTA activates PPARα and induces the mRNA expression of PPARα target genes, which promotes fatty acid metabolism in murine hepatocytes. To the best of our knowledge, this is the first study to report that 9-oxo-OTA induces fatty acid metabolism through PPARα activation, and our findings suggest that 9-oxo-OTA could be a valuable food-derived compound capable of improving dyslipidemia.

Materials and methods

Materials

Authentic 9-oxo-OTA was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Authentic *cis*-10-heptadecenoic acid (HDA), which was used as an internal standard, was purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from Sigma or Wako (Osaka, Japan). Buffers used were of high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS) grade. Tomatoes were obtained from a local market.

Analysis of 9-oxo-OTA by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS)

The levels of 9-oxo-OTA were assessed as previously described, using a LC-MS system [24]. Briefly, each freeze-dried tomato (10 mg) was homogenized in 1 mL of extraction solvent (99.5% EtOH containing 1 μg/mL HDA) with a mixer. After centrifugation (15,000 rpm, 10 min, 4°C), the supernatant was collected for use as an extract. The extract was filtered through a 0.2-μm-pore polyvinylidene difluoride (PVDF) membrane (Whatman, Brentford, UK), and the filtrate was used for LC-MS.

LC-MS was performed using a Waters Acquity UPLC system (Milford, MA) coupled to a Xevo QTOF-MS equipped with an electrospray source operating in negative ion mode (ESI negative mode), with a lock-spray interface for accurate mass measurements. Leucine enkephalin was employed as the lock-mass compound. It was infused directly into the MS at a flow rate of 30 μL/min and a concentration of 200 μg/mL (in 50% acetonitrile, 50% water, 0.1% formic acid). The capillary, sampling cone, and extraction cone voltages were set at 2700 V, 20 V, and 1 V, respectively. The source

and desolvation temperatures were 120°C and 450°C, respectively. The cone and desolvation gas flow rates were set at 50 and 800 L/h, respectively.

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% to 50% B for 0–4 min, 50% to 85% B for 4–14 min, 99% B for 14–17 min, and 30% B for 3 min, at a flow rate of 300 μL/min. Data were acquired with MassLynx software (Waters). External mass calibration was performed following the manufacturer's protocol.

Analysis of β-hydroxybutyrate by UPLC-QTOF-MS

The medium from a culture of primary hepatocytes (50 μL) was mixed with 50 μL of acetonitrile. After centrifugation (15,000 rpm, 10 min, 4°C), the supernatant was collected for use as an as an extract. The extract was filtered through a 0.2-μm-pore polyvinylidene difluoride (PVDF) membrane (Whatman), and the filtrates were used for LC-MS.

In the MS system, the capillary, sampling cone, and extraction cone voltages were set at 2600 V, 15 V, and 3 V, respectively (ESI negative mode). The source and desolvation temperatures were 120°C and 450°C, respectively. The cone and desolvation gas flow rates were set at 50 and 800 L/h, respectively.

We used a BEH Amide column (column size, 2.1 × 100 mm; particle size, 1.7 μm). The column temperature was set to 40°C. The buffer gradient consisted of 95% B for 0–4 min, 95% to 50% B for 4–10 min, 50% B for 10–16 min, and 95% B for 4 min, at a

flow rate of 300 μL/min. Data were acquired with MassLynx software (Waters).

Luciferase assay

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

Preparation of mouse primary hepatocytes

All the animal experiments were approved by the Kyoto University Animal Care Committee. C57BL/6J male mice (*wild type* and *PPARα*$^{-/-}$, free-feed) were anesthetized with intraperitoneal administration of pentobarbital, and the liver was perfused with liver perfusion medium (Life Technologies Japan Ltd.). Then, the liver was treated with liver digestion medium (Life Technologies Japan Ltd.). After filtration through a 100-μm nylon mesh filter, hepatocytes were isolated by repeated centrifugation at 50 × *g* for 3 min (3 times). The isolated hepatocytes were cultured in type-1 collagen-coated 12-well plates at a cell density of 2.0×10^5 cells/well. After a 5-h incubation at 37°C in 5% CO_2 in 20% serum DMEM (1.0g/L glucose), the hepatocytes were cultured in serum-free DMEM (1.0g/L glucose) with or without 9-oxo-OTA for 5 h (mRNA assay) or 8 h (LC-MS and RI assay), and then used for mRNA quantification and LC-MS

assays.

Quantification of mRNA expression levels

Total RNA was prepared from primary hepatocytes using Sepasol (Nacalai Tesque), according to the manufacturer's protocol. Using M-MLV reverse transcriptase (Life Technologies Japan Ltd.), total RNA was reverse transcribed. 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, 26]. The oligonucleotide primer sets for mouse *36B4* (internal control) and the PPARα target genes were designed using a PCR primer selection program on the website of the Virtual Genomic Center at GenBank and the sequences are shown in Table 1. All mRNA expression data are presented as ratios relative to the control in each experiment.

Measurement of ^{14}C-Palmitic acid uptake and $^{14}CO_2$ production in murine primary hepatocytes

These experiments were conducted as previously described with some modifications [27-29]. In the measurement of ^{14}C-palmitic acid uptake, the cells were washed with PBS (500 µL/well) and then incubated in assay buffer (^{14}C-palmitic acid, 2 µCi/well; fatty acid free BSA, 2.5%; and normal palmitic acid, 200 µM) for 3 min at room temperature (1 mL/well). The experiment was stopped by washing the cells 3 times with PBS (500 µL/well). The cells were lysed in 0.1% Triton-X solution (200 µL/well). The radioactivity in the cell lysate was counted for 1 min in 2 mL of scintillation solution to measure fatty acid uptake. In the measurement of $^{14}CO_2$ production, mice primary

hepatocytes were incubated in assay buffer (DMEM containing ^{14}C-palmitic acid, 2 μCi/well; glucose, 1.0g/L; L-carnitine, 200 μM ; fatty acid free BSA, 2.5%; and normal palmitic acid, 200 μM) for 8h. The medium was transferred to a 50-mL tube. An uncapped-2mL sample tube containing a piece of filter paper soaked in 0.1N NaOH was placed inside a 50-mL tube. After the tube was sealed, 200μL of 70% perchloric acid was added to the medium sample to release $^{14}CO_2$. The saturated filter paper containing trapped $^{14}CO_2$ was assessed for radioactivity in a liquid scintillation counter (LS6500, Beckman Coulter, CA, USA).

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). Differences between groups were compared with the Student's *t*-test (for two groups) and one-way analysis of variance (ANOVA) followed by Tukey's test. *P* values less than 0.05 were considered statistically significant.

Results

Identification of 9-oxo-OTA in tomato fruit extract

First, we explored the oxylipins, which are metabolized from ALA in plants (Fig. 1), in the tomato extract using LC-MS. The results showed that the extract of tomato fruit homogenate obtained under heating (37 °C, 30 min) contained 9-oxo-OTA (Fig. 2 A; Rt = 7.91 min, m/z = 291.20, Fig. 2 B, C). The 9-oxo-OTA peak was detected as an unfragmented deprotonation ion ($C_{18}H_{27}O_3^-$, $[M-H]^-$, Fig. 2 C) by electrospray negative ionization mass spectrometry. In the tandem mass spectrometry (MS/MS) data, we confirmed that the fragment ion detected in the tomato extract (m/z = 185, 125, and 121) matched the pure 9-oxo-OTA sample (Fig. 2D).

Quantitative analysis of the 9-oxo-OTA content in each part of the tomato fruit homogenate under liquid nitrogen (gelatinous tissue: low limit of quantification (LLOQ), sarcocarp: LLOQ, peel: LLOQ; Table 2) or heating (gelatinous tissue: approximately 0.46 μg/g of tissue weight, sarcocarp: 0.23 μg/g of tissue weight, peel: 0.62 μg/g of tissue weight; Table 2) revealed that the amount of 9-oxo-OTA in all tissues was increased by heat treatment.

9-oxo-OTA activated PPARα and induced the mRNA expression of PPARα target genes

9-oxo-OTA is analogous to 9-oxo-10,12-octadecadienoic acid (9-oxo-ODA, Fig. 1) and 13-oxo-9,11,15-octadecatrienoic acid (13-oxo-OTA, Fig. 1). In our previous studies showed that 9-oxo-ODA is involved in PPARα activation [22] and 13-oxo-OTA activates PPARγ [24]. Therefore, we hypothesized that 9-oxo-OTA activates PPARα and/or PPARγ. To determine the effect of 9-oxo-OTA on PPARα and PPARγ activity, we

first performed a luciferase ligand assay. In the PPARα activation level, 9-oxo-OTA was stronger than 13-oxo-OTA (Fig. 3 A). In the PPARγ activation level, 13-oxo-OTA was stronger than 9-oxo-OTA (Fig. 3 B). We also showed that 9-oxo-OTA increased luciferase activity in a time dependent manner (Fig. 3 C), and this luciferase activity was inhibited by PPARα antagonist (GW6471) treatment (Fig. 3 D). PPARα is expressed in liver where it promotes β-oxidation, ketogenesis, and lipid transport [10, 11] and important in the regulation of lipid metabolism [5-7]. To elucidate the contribution of 9-oxo-OTA to lipid metabolism in hepatocytes, murine hepatocytes were cultured in medium containing 9-oxo-OTA. In murine hepatocytes, we showed that the expression of PPARα mRNA was increased by 9-oxo-OTA treatment (approximately 1.8-fold, Fig. 4 A). Furthermore, the mRNA expression levels of PPARα target genes, such as *Cpt1a*, *Acs*, *Hmgcs2*, and *Cd36*, were increased by 9-oxo-OTA treatment (approximately 1.5-fold, 1.4-fold, 1.5-fold, and 1.4-fold, respectively, Fig. 4 B–E). We also demonstrated that the 9-oxo-OTA-mediated effect on PPARα target gene expression was not observed in PPARα-KO primary hepatocytes (Fig. 4 B–E). These findings suggest that 9-oxo-OTA promotes the mRNA expression of the genes involved in fatty acid oxidation via PPARα activation.

9-oxo-OTA promoted fatty acid oxidation in primary hepatocytes

PPARα is the major regulator of fatty acid metabolism [5-7], suggesting that 9-oxo-OTA induces fatty acid oxidation via PPARα activation. We investigated whether 9-oxo-OTA influences not only mRNA expression levels but also lipid metabolism. The results showed that ^{14}C-palmitic acid uptake was increased by 9-oxo-OTA treatment (approximately 1.4-fold, Fig. 5 A), suggesting enhanced fatty acid metabolism. We also

showed that $^{14}CO_2$ production was increased by 9-oxo-OTA treatment (approximately 1.5-fold, Fig. 5 B). β-Oxidation is one of the primary modes of fatty acid metabolism, which converts fatty acids to ketone bodies. β-hydroxybutyrate, which is one of the ketone bodies, is an important marker of fatty acid oxidation. We investigated whether 9-oxo-OTA effects the production of β-hydroxybutyrate in murine primary hepatocytes and demonstrated that β-hydroxybutyrate secretion was increased by 9-oxo-OTA treatment (approximately 1.5-fold, Fig. 5 C). The enhancement of fatty acid uptake and secretion of $^{14}CO_2$ and β-hydroxybutyrate induced by 9-oxo-OTA were not observed in PPARα-KO primary hepatocytes (Fig. 5). These findings suggest that 9-oxo-OTA promotes fatty acid metabolism in primary hepatocytes via a PPARα-dependent pathway.

Discussion

In the present study, we analyzed the metabolites in tomato extract by using LC-MS and identified 9-oxo-OTA. Our previous study showed that ALA, which is a 9-oxo-OTA precursor, was present in tomato extracts [30]. These data suggested that 9-oxo-OTA can be derived from ALA via a non-enzymatic and/or enzymatic reaction. It is possible that ALA is susceptible to oxidation at C9 via auto-oxidation and/or lipoxygenase is involved in the production of 9-oxo-OTA in tomato fruits. Our previous study also revealed that 9-oxo-ODA is localized primarily in the fruit peel [30]. However, here we showed that 9-oxo-OTA exists in not only peel but also in the gelatinous tissue and sarcocarp. Further examinations are necessary to elucidate the differences in the localization of 9-oxo-OTA and 9-oxo-ODA. We also revealed that the amount of 9-oxo-OTA in tomato extract is increased by heating. This finding suggested that heat processing increases the amount of 9-oxo-OTA in processed tomato foods. Tomato fruit contains not only 9-oxo-OTA but also many other compounds. Therefore, it is difficult that the effect of tomato on fatty acid oxidation is explained by only 9-oxo-OTA via PPARα dependent pathway. To evaluate 9-oxo-OTA *in vivo*, it is necessary to process selective breeding (tomato fruit having high 9-oxo-OTA content) and/or to improve processing condition (e.g. tomato juice having high 9-oxo-OTA content).

PPARα is expressed principally in the liver, and it is a key regulator of various aspects of lipid metabolism, including β-oxidation, ketogenesis, and lipid transport [10, 11]. PPARα agonists are used in the treatment of dyslipidemia [9, 16]. Although we previously reported that 9-oxo-ODA contributes to the activation of PPARα [21], little is known about the effect of 9-oxo-OTA on PPARα. The structure of 9-oxo-OTA is

277 similar to that of 9-oxo-OTA. Therefore, we hypothesized that 9-oxo-OTA also has the
278 ability to activate PPARα.

279 Here, we demonstrated for the first time that 9-oxo-OTA activates PPARα in
280 luciferase ligand assay. There is the possibility that 9-oxo-OTA activate PPARα both
281 directly and/or indirectly. In case of the direct action, 9-oxo-OTA acts as PPARα ligand.
282 In case of the indirect action, the metabolites of 9-oxo-OTA act as PPARα ligands. We
283 also showed that 9-oxo-OTA increased the mRNA expression of *Cpt1a*, *Acs*, *Hmgcs2*,
284 and *Cd36*, which are PPARα target genes that are involved in fatty acid metabolism
285 [12-14, 31-33, 35-36]. These effects of 9-oxo-OTA on the expression of PPARα target
286 genes were not observed in PPARα-KO primary hepatocytes. These findings suggested
287 that 9-oxo-OTA promotes fatty acid metabolism in murine hepatocytes via a
288 PPARα-dependent pathway. However, the expression of *Cd36* was trend toward
289 significance in PPARα-KO primary hepatocytes treated with 9-oxo-OTA. We confirmed
290 that 9-oxo-OTA also activates PPARγ in luciferase ligand assay. It is well known that
291 the expression of *Cd36* is induced by not only PPARα but also PPARγ activation
292 [34-36]. Therefore, there is the possibility that the effect of 9-oxo-OTA on PPARγ
293 activation contributes to trend toward increment of *Cd36* expression.

294 Our present study also showed that the effect of 9-oxo-OTA on fatty acid oxidation
295 occurs through alteration of not only mRNA expression levels but also metabolism.
296 Fatty acids are taken up by hepatocytes primarily via CD36 [32]. We showed that fatty
297 acid uptake was increased by 9-oxo-OTA treatment. This effect was not observed in
298 PPARα-KO primary hepatocytes, suggesting that 9-oxo-OTA promotes fatty acid uptake
299 via PPARα activation. We also showed that the mRNA expression level of *Hmgcs2*
300 (3-hydroxy-3-methylglutaryl-CoA synthase 2) was increased by 9-oxo-OTA treatment.

Hmgcs2 is the key enzyme involved in ketone body formation [33]. β-Hydroxybutyrate is a major component of ketone bodies and a marker of fatty acid oxidation. Therefore, we hypothesized that 9-oxo-OTA also promotes the production of β-hydroxybutyrate in murine hepatocytes. The LC-MS analysis revealed that β-hydroxybutyrate secretion from murine primary hepatocytes was increased by 9-oxo-OTA treatment. In the RI experiment using ^{14}C-palmitic acid, we showed that $^{14}CO_2$ production was increased by 9-oxo-OTA treatment. The increment of $^{14}CO_2$ production and β-hydroxybutyrate secretion suggest that 9-oxo-OTA promoted lipid metabolism in wild-type murine hepatocytes. In contrast, 9-oxo-OTA treatment decreased $^{14}CO_2$ production and β-hydroxybutyrate secretion in PPARα-KO primary hepatocytes. Our present study is focused on the effect of 9-oxo-OTA on PPARα activation and its downstream targets. But, there is the possibility of the effect of 9-oxo-OTA on other pathways including PPARγ activation. The previous study showed that acetoacetyl-CoA synthetase (AACS) is a PPARγ target gene [37]. AACS is a ketone body-specific ligase and might play an important role in the provision of acetyl-CoA for lipogenesis [38]. 9-oxo-OTA activates both PPARα and PPARγ. In the PPARα KO hepatocytes treated with 9-oxo-OTA, there is the possibility that fatty acid oxidation via PPARα dependent pathway disappeared and lipogenesis proceed via PPARγ dependent pathway. In this study, we showed that 9-oxo-OTA promotes fatty acid metabolism via PPARα activation and increases the expression of PPARα target genes in murine hepatocytes. Further examination is necessary to determine the effect of 9-oxo-OTA on human metabolism.

In conclusion, 9-oxo-OTA contained in tomato fruit activates PPARα and induces the mRNA expression of PPARα target genes. This promotes fatty acid oxidation in murine hepatocytes. These findings provide the first evidence that 9-oxo-OTA induces

fatty acid metabolism through a PPARα-dependent pathway and may contribute to the development of improved treatments for dyslipidemia patients. Our findings suggest that 9-oxo-OTA might be a valuable, food-derived compound for maintaining health.

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Figure legends

Fig. 1. Oxo-OTAs, Oxo-ODAs and their precursors (fatty acids).

Fig. 2. Identification of 9-oxo-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-oxo-OTA) by LC-MS. (A) The structure of 9-oxo-OTA. **(B)** Extracted ion chromatogram (m/z = 291.20) data and **(C)** full mass data for 9-oxo-OTA in an authentic sample and a tomato extract sample. **(D)** MS/MS data for 9-oxo-OTA in an authentic sample and a tomato extract sample.

Fig. 3. 9-oxo-OTA activated PPARα. (A) Effect of 9-oxo-OTA and 13-oxo-OTA (30μM respectively) on PPARα activity with a luciferase reporter assay. **(B)** Effect of 9-oxo-OTA and 13-oxo-OTA (10μM respectively) on PPARγ activity with a luciferase reporter assay. **(C)** Effect of 9-oxo-OTA on PPARα activity in a dose-dependent manner. **(D)** Evaluation of PPARα activity with a luciferase reporter assay in cells treated with 9-oxo-OTA (30 μM) and/or GW6471 (PPARα antagonist, 100 nM). Data are presented as mean ± SEM (n = 5). Symbol for $p < 0.05$ between the means of the 3 groups. OTA; 9-oxo-OTA, GW; GW6471.

Fig. 4. 9-oxo-OTA activated the expression of PPARα target genes in murine hepatocytes. Effect of 9-oxo-OTA (30 μM) on **(A)** *PPARα* expression levels in murine primary hepatocytes. Effect of 9-oxo-OTA (30 μM) on **(B)** *Cpt1a*, **(C)** *Acs*, **(D)** *Hmgcs2*, and **(E)** *Cd36* expression in wild-type (WT) or PPARα$^{-/-}$ (KO) primary hepatocytes. Data are presented as mean ± SEM (n = 5–6). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

Fig. 5. 9-oxo-OTA promoted fatty acid metabolism in murine hepatocytes. (A) The uptake of ^{14}C-palmitic acid and **(B)** $^{14}CO_2$ production in murine WT or KO primary hepatocytes treated with 9-oxo-OTA (30 μM). **(C)** The secretion of β-hydroxybutyrate from murine WT or KO primary hepatocytes treated with 9-oxo-OTA (30 μM). Data are presented as mean ± SEM (n = 3–5). *$p < 0.05$, **$p < 0.01$ vs. control.

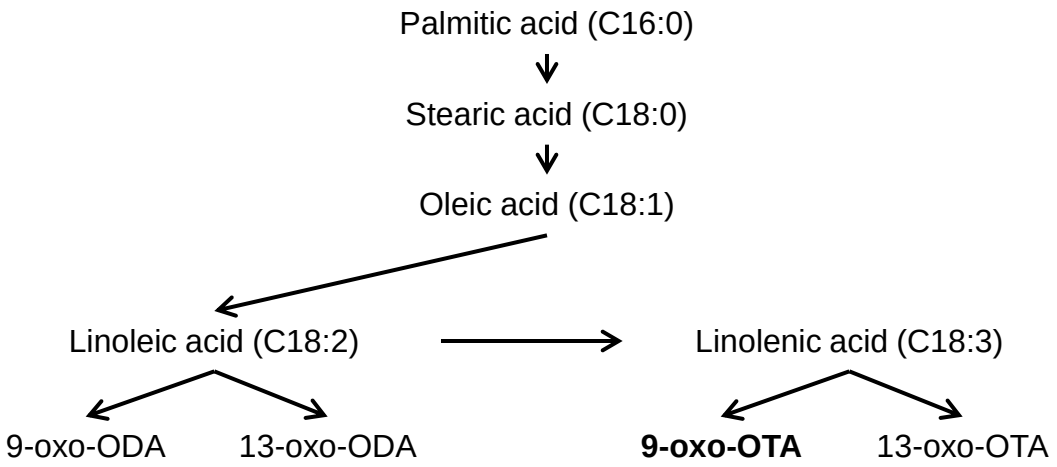

Figure 1. Takahashi H, et al.

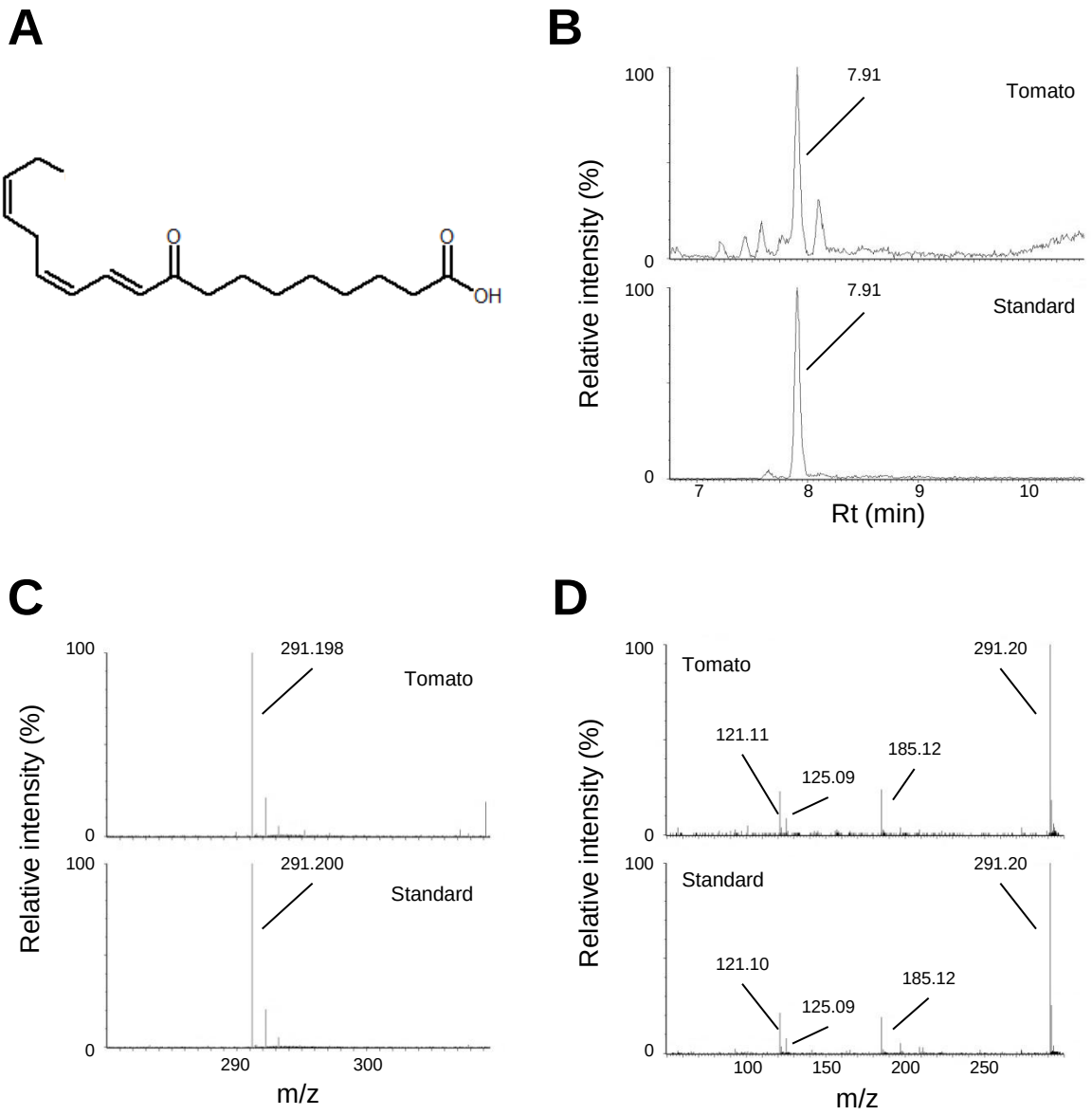

Figure 2. Takahashi H, et al.

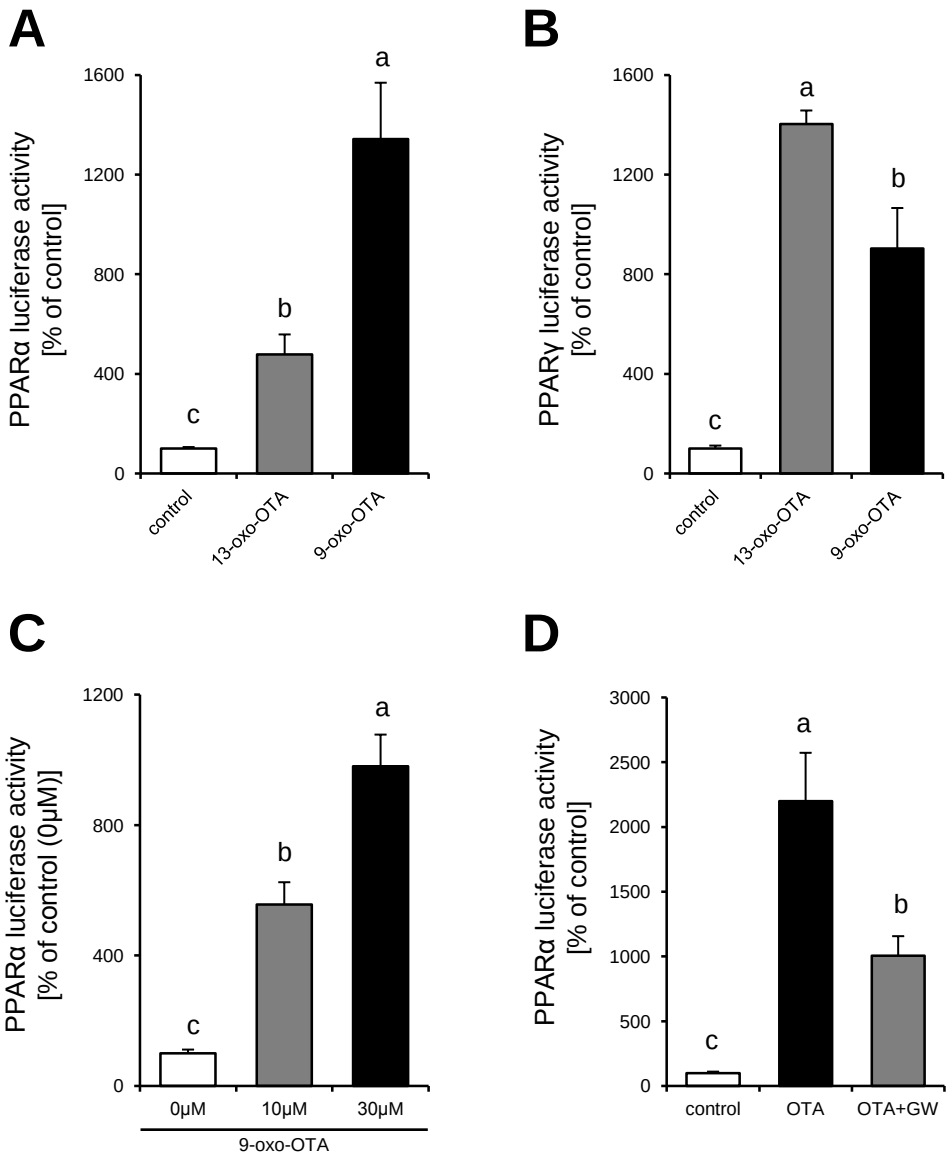

Figure 3. Takahashi H, et al.

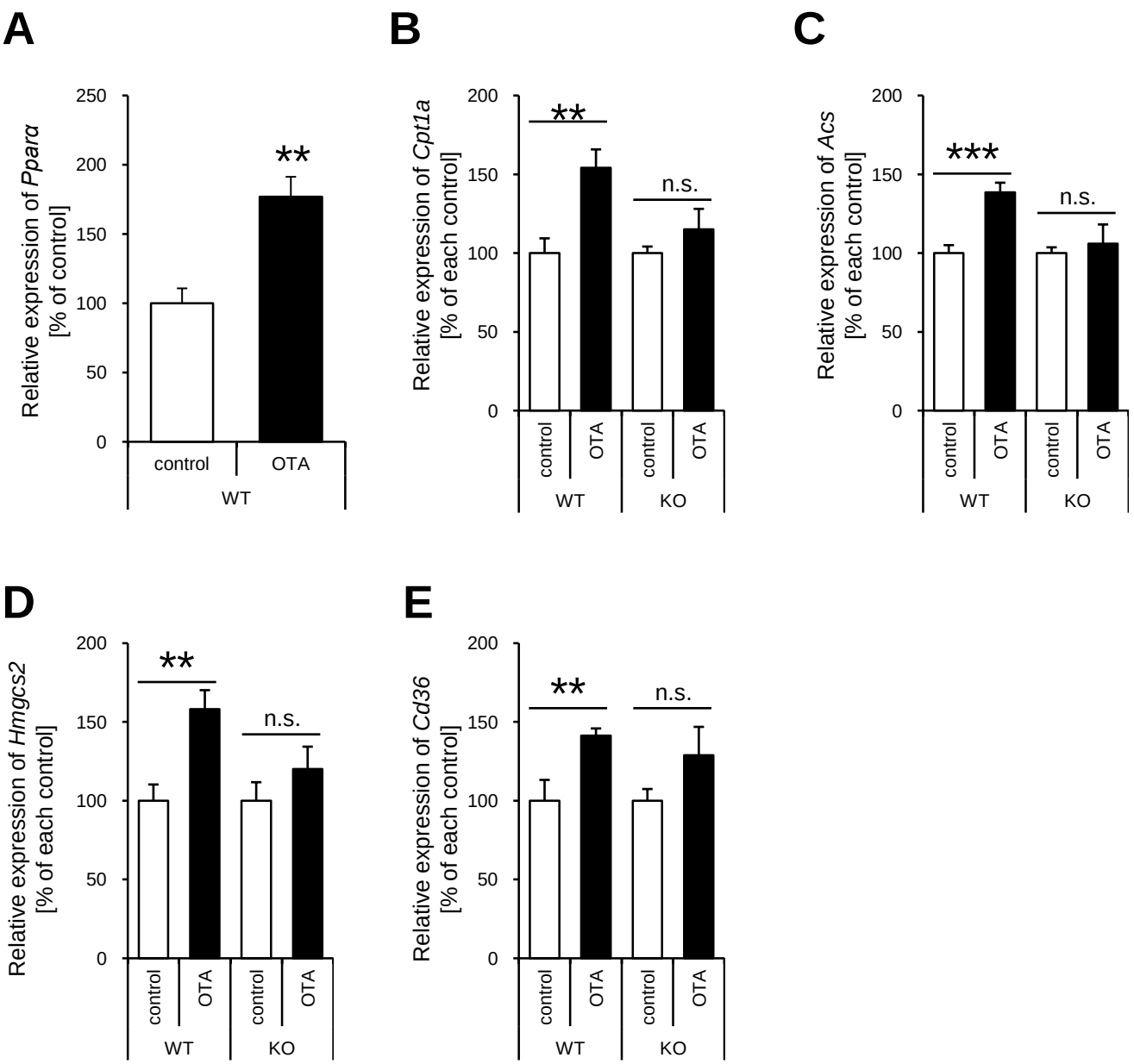

Figure 4. Takahashi H, et al.

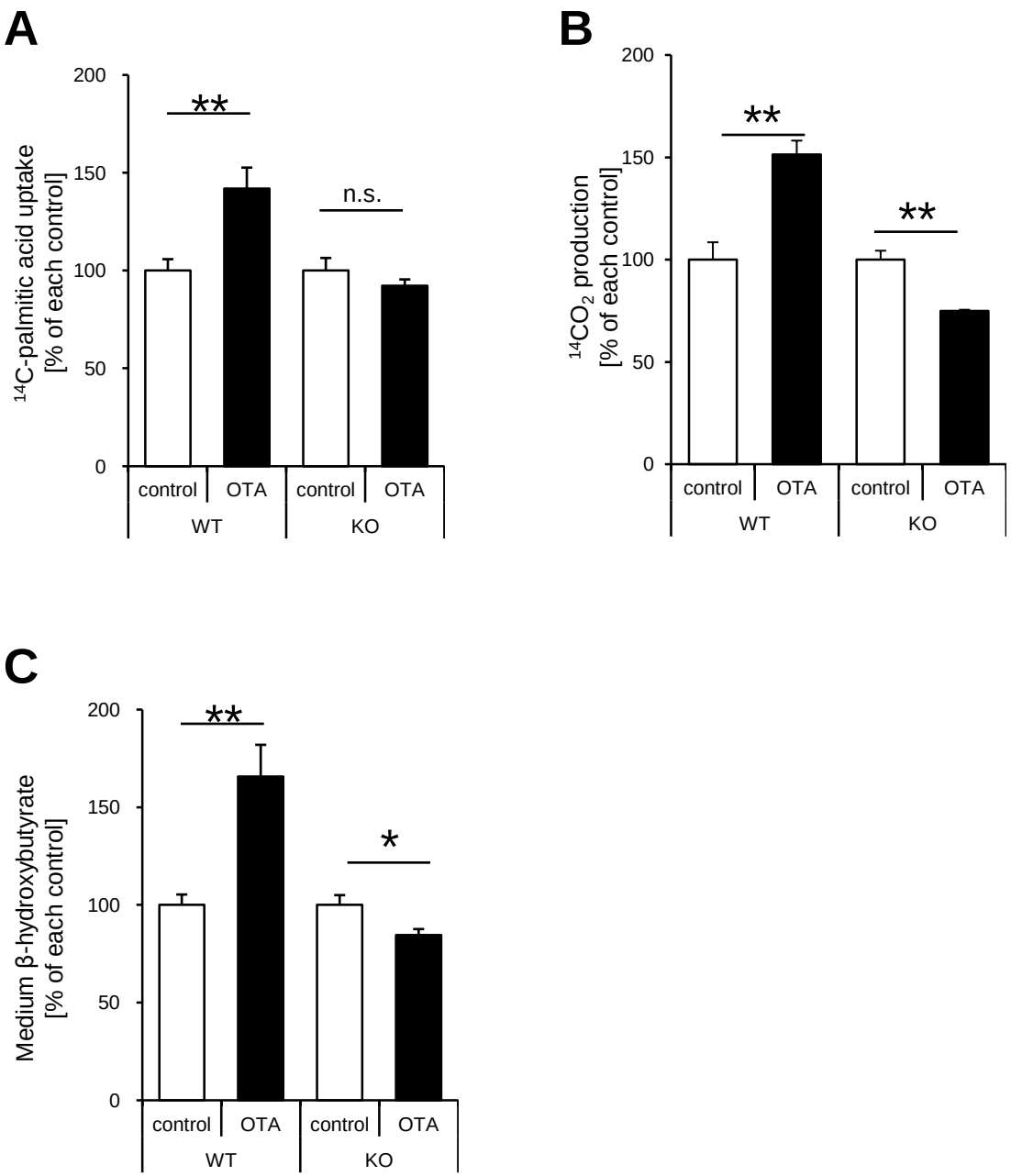

Figure 5. Takahashi H, et al.

Table 1. Oligonucleotide primers used for mRNA analysis.

gene	forward primer	reverse primer
Pparα	TCGCGTACGGCAATGGCTTT	TCTTCATCCCCAAGCGTAGGAGG
Cpt1a	CTCAGTGGGAGCGACTCTTCA	GGCCTCTGTGGTACACGACAA
Acs	ACATCCACGTGTATGAGTTCTACGC	AGTAGACGAAGTTCTCACGGTCGAT
Hmgcs2	AATCAGTGGAAGCAAGCTGGA	GTCCAGGGAGGCCTTCAAAA
Cd36	GATGTGGAACCCATAACTGGATTCAC	GGTCCCAGTCTATTTAGCCACAGT
36B4	TCCTTCTTCCAGGCTTTGGG	GACACCCTCCAGAAAGCGAG

Table 2. Quantitative analysis of 9-oxo-OTA in tomato fruit homogenate under liquid nitrogen or heating (at 37°C for 30 min; μg/g fresh weight).

tissue	liquid nitrogen	heating
gelatinous tissue	LLOQ	0.46±0.10
sarcocarp tissue	LLOQ	0.23±0.05
peel tissue	LLOQ	0.62±0.12

LLOQ: low limit of quantification. Data are presented as mean ± SEM (n = 3).