

Bioconversion and Metabolic Fate of the *n*-1 Polyunsaturated Fatty Acids, 6,9,12,15-Hexadecatetraenoic (C16:4 *n*-1) and 8,11,14,17-Octadecatetraenoic (C18:4 *n*-1) Acids, in HepG2 Cells

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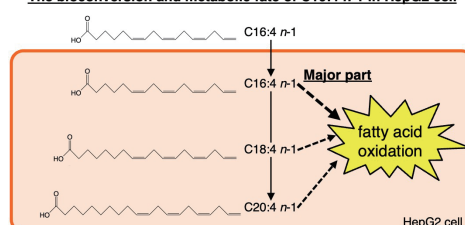
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Abstract: Fish oil contains not only major fatty acids with double bonds at the *n*-3, *n*-6, *n*-7, and *n*-9 positions but also those with a double bond at the *n*-1 position, such as 6,9,12,15-hexadecatetraenoic acid (C16:4 *n*-1; HDTA). However, intracellular bioconversion and metabolic fate of *n*-1 polyunsaturated fatty acids (PUFA) remain unclear. Therefore, in this study, we aimed to assess the intracellular bioconversion and metabolic fate of HDTA and its metabolite, 8,11,14,17-octadecatetraenoic acid (C18:4 *n*-1; ODTA), using HepG2 cells. Based on the results of cell viability and cytotoxicity assays for HDTA and ODTA, the concentration of each fatty acid supplemented in the experiments was set at 10 μ M. HepG2 cell culture with HDTA revealed C20:4 *n*-1 as a new HDTA metabolite, along with previously reported ODTA. Our findings suggest that the HDTA taken up by HepG2 cells undergoes elongation to form ODTA and C20:4 *n*-1. Following supplementation with HDTA, ODTA, and 5,8,11,14,17-eicosapentaenoic acid (C20:5 *n*-3; EPA), fatty acids disappeared from the culture medium within 24 h. Notably, the total relative level of HDTA and its metabolites, including ODTA and C20:4 *n*-1 in HDTA- and ODTA-supplemented cells were significantly lower than the total relative level of EPA and its metabolites, including 7,10,13,16,19-docosapentaenoic acid (C22:5 *n*-3), C24:6 *n*-3, and 4,7,10,13,16,19-docosahexaenoic acid (C22:6 *n*-3) in the EPA-supplemented cells. Except for a portion that was intracellularly elongated, most HDTA was taken up by HepG2 cells and may undergo rapid fatty acid β -oxidation. However, RNA-sequencing and real-time polymerase chain reaction analysis revealed no significant changes in fatty acid β -oxidation-related gene expression levels in HDTA-supplemented cells. Collectively, these results provide novel insights into the intracellular bioconversion mechanisms and metabolic fate of HDTA and ODTA in HepG2 cells, suggesting that the metabolic fate of *n*-1 PUFA is distinct from that of common PUFA.

The bioconversion and metabolic fate of C16:4 *n*-1 in HepG2 cell



Key words: *n*-1 polyunsaturated fatty acids, hexadecatetraenoic acid, octadecatetraenoic acid, HepG2

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1 Introduction

Unsaturated fatty acids, primarily found in edible oils, contain double bonds at the *n*-3, *n*-6, *n*-7, and *n*-9 positions. Several studies have investigated their metabolic mechanisms and biological activities¹⁾. For example, palmitoleic acid, an *n*-7 fatty acid, reprograms the gut microbiota and ameliorates gut inflammatory diseases²⁾. Additionally, oleic acid, an *n*-9 fatty acid, increases the high-density lipoprotein cholesterol levels in the blood³⁾. Biological activities of *n*-3 polyunsaturated fatty acids (PUFA), such as α -linolenic acid, 5,8,11,14,17-eicosapentaenoic acid (C20:5 *n*-3; EPA), and 4,7,10,13,16,19-docosahexaenoic acid (C22:6 *n*-3; DHA), and *n*-6 PUFA, such as linoleic and arachidonic acids, particularly in relation to their anti-inflammatory effects and roles in lipid metabolism, have been extensively studied⁴⁻⁶⁾. In addition to EPA and DHA, 4,7,10,13-hexadecatetraenoic acid (C16:4 *n*-3) is another minor *n*-3 PUFA found in fish oil. C16:4 *n*-3 inhibits the production of leukotrienes B4 and C4 and 5-hydroxyeicosatetraenoic acid in mouse mast cells⁷⁾ and neutralizes the anti-tumor activity of platinum-based chemotherapy in various mouse models⁸⁾. Biological regulatory functions of these fatty acids vary depending on the lipid class into which they are incorporated⁹⁾.

Fatty acids with double bonds at positions other than *n*-3, *n*-6, *n*-7, and *n*-9 have also been identified in edible oils. For example, *n*-1 PUFA, which contains a double bond at the *n*-1 position, has been detected in sardine oil at a concentration of approximately 2–3% in the form of 6,9,12,15-hexadecatetraenoic acid (C16:4 *n*-1; HDTA)¹⁰⁾. However, to date, specific metabolic mechanisms and biological activities of HDTA have not been reported due to the challenges in isolating this compound. We previously purified and isolated HDTA via preparative-scale high-performance liquid chromatography and showed that HDTA-ethyl ester (EE) exerts stronger serum triacylglycerol-lowering effects than EPA-EE in mice¹¹⁾. Additionally, we identified 8,11,14,17-octadecatetraenoic acid (C18:4 *n*-1; ODTA) as an HDTA metabolite; however, HDTA metabolites were accumulated only in trace amounts (<1% of total fatty acids) in the blood and major organs. Although our findings indicated that HDTA follows a metabolic fate distinct from that of common PUFA, the underlying mechanisms remain ambiguous. Elucidation of the intracellular bioconversion and metabolic fate of *n*-1 PUFA in fish oil can provide valuable insights to improve human health. Therefore, in this study, we investigated the intracellular bioconversion and metabolic fate of HDTA and ODTA compared to those of EPA (control) using HepG2 cells, a liver-derived cell line commonly used to study lipid metabolism (Fig. 1).

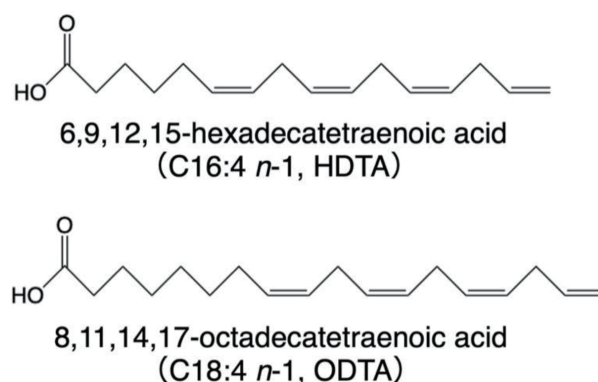


Fig. 1 Chemical structures of 6,9,12,15-hexadecatetraenoic (HDTA) and 8,11,14,17-octadecatetraenoic (ODTA) acids.

2 Materials and Methods

2.1 Materials

HDTA-EE (98.0%), ODTA (97.6%), and EPA (98.5%) were obtained from Bizen Chemical Co. Ltd. (Akaiwa, Japan). HDTA-EE was hydrolyzed under alkaline conditions to generate free and purified HDTA. HepG2 human hepatoma cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). Dulbecco's modified Eagle's medium was purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Fetal bovine serum was obtained from HyClone Laboratories, Inc. (Logan, UT, USA). All other materials were of reagent grade and purchased from Merck KGaA (Darmstadt, Germany) and Nacalai Tesque, Inc. (Kyoto, Japan).

2.2 Cell culture and cytotoxicity assays

HepG2 cells were cultured in the Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin–streptomycin and at 37°C in an atmosphere of 5% CO₂ and 95% air.

Cell viability and cytotoxicity were assessed via water-soluble tetrazolium salt (WST)-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) and lactate dehydrogenase (LDH) assay (Cytotoxicity LDH Assay Kit-WST; Dojindo Laboratories), respectively, following the manufacturer's instructions. HDTA, ODTA, and EPA were dissolved in ethanol and supplemented at concentrations of 0.5, 1, 5, 10, 25, and 50 μ M for 24 h (ethanol concentration in the medium = 0.1%). A blank solution was added to achieve a final ethanol concentration of 0.1%.

2.3 HDTA metabolite identification

HepG2 cells were cultured in 100-mm dishes at a density of 2×10^5 cells/dish and incubated for 24 h. After further incubation with HDTA (10 and 100 μ M) for 24 h, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS). Total lipids were extracted from the cells using the chloroform/methanol extraction

method, as previously described¹²⁾. HDTA metabolites were identified via gas chromatography–mass spectrometry (GCMS; GCMS-QP2010/PARVUM2; Shimadzu Co., Kyoto, Japan) using the Omegawax column (30 m × 0.25 mm × 0.25 μm; Merck KGaA) after fatty acid methylation with boron–trifluoride–methanol, as described in our previous report¹³⁾. GCMS analysis was performed under the following conditions: Column temperature = 120–240°C at a rate of 2°C/min, interface temperature = 240°C, injection temperature = 240°C, carrier gas = 1 mL/min helium, ionization voltage = 70 eV, and scan range = *m/z* 50–350 amu. MS spectra of the peaks exclusively detected for HDTA were acquired via comparison with those of the peaks observed for the control (0.1% ethanol). Then, MS spectra were compared with a library (The LipidWeb; <https://lipidmaps.org/resources/lipidweb/index.php?page=ms/methylesters.htm>) to identify the chemical structures.

2.4 Analyses of cell and medium fatty acid compositions

HepG2 cells were cultured in 100-mm dishes at a density of 2×10^5 cells/dish and incubated for 24 h, followed by incubation with 10 μM EPA, HDTA, and ODTA for 0, 3, 6, and 24 h. Then, the medium was collected, and the cells were washed with PBS. Total lipids were extracted from the medium and cells using the chloroform/methanol extraction method¹²⁾. Fatty acid methyl ester (FAME) was prepared as described in section 2.3.

HDTA and ODTA levels in the medium were measured using a GC system (GC-2014; Shimadzu Co.) with the Omegawax column. GC analysis was performed under the following conditions: Column temperature = 120–240°C at a rate of 2°C/min, injection temperature = 250°C, detector temperature = 260°C, and carrier gas = 1 mL/min helium.

Cell fatty acid composition was measured using the GC method described above. Each FAME was identified using a standard FAME mixture (Supelco 37 Component FAME Mix; Merck KGaA). Heptadecanoic acid was used as an internal standard to quantify the total fatty acids.

2.5 Analyses of the fatty acid compositions of the neutral and polar lipid fractions of cells

HepG2 cells were cultured in 100-mm dishes at a density of 2×10^5 cells/dish and incubated for 24 h. After incubation with 10 μM EPA and HDTA for 0 and 24 h, the medium was removed, and the cells were washed with PBS. Total lipids were extracted from the cells using the chloroform/methanol extraction method¹²⁾ and applied to the Sep-Pak silica cartridge (Waters Co., Milford, MA, USA) to separate the neutral and polar lipid fractions. Subsequently, the neutral lipid fraction was eluted with chloroform, whereas the polar lipid fraction was eluted with methanol. The two fractions were further used to prepare FAME, and EPA and HDTA metabolite levels were determined using the GC method described in section 2.4.

2.6 RNA-sequencing

HepG2 cells were cultured in 100-mm dishes at a density of 2×10^5 cells/dish and incubated for 24 h. The cells were further incubated with 10 μM EPA, HDTA, and ODTA for 6 h. Total RNA was isolated and purified using the RNAiso Plus reagent (Takara Bio Inc., Kusatsu, Japan), according to the manufacturer's protocol. RNA-sequencing was performed using a next-generation sequencing system (DNB-SEQ-G400RS; MGI Tech Co., Ltd., Shenzhen, China) conducted by Genome-Lead Inc. (Takamatsu, Japan).

After quality trimming with Trim Galore, the reads were mapped to the reference genome (GRCh38 Ensembl release 108) using STAR aligner¹⁴⁾. StringTie¹⁵⁾ and SQANTI3¹⁶⁾ were used to assemble and annotate the novel transcripts, respectively. Transcript-level quantification of all samples was performed using Salmon¹⁷⁾, and isoform-SwitchAnalyzer¹⁸⁾ was used for additional quality filtering based on the salmon mapping results. Gene expression levels were estimated using the median read count of all gene transcripts. Additionally, RUVSeq package¹⁹⁾ was used to correct for batch effects at the gene expression level. Differential expression analysis was conducted using DESeq2²⁰⁾, with differentially expressed genes (DEGs) defined by a false discovery rate or *p* value < 0.05 and an absolute log₂ fold-change > 0.5.

2.7 mRNA expression analysis

HepG2 cells were cultured in 100-mm dishes at a density of 2×10^5 cells/dish and incubated for 24 h, followed by incubation with 10 μM HDTA for 0, 3, and 12 h. Total RNA was isolated and purified using the RNAiso Plus reagent (Takara Bio Inc.). Concentration and purity of total RNA were determined by measuring the absorbance at 260 and 280 nm via UV spectroscopy (UV-1800; Shimadzu Co.) using the Hellma TrayCell (Hellma Analytics, Müllheim, Germany). Subsequently, cDNA was synthesized using the GoScript Reverse Transcription System (Promega Co., Madison, WI, USA), and mRNA expression levels were quantified using the Thermal Cycler Dice Real Time System (Takara Bio Inc.) and GoTaq qPCR Master Mix (Promega Co.). All primers were designed using Primer3Plus (<http://primer3plus.com/>). The primer sequences used for acetyl-CoA carboxylase α, acetyl-CoA oxidase 1 (ACOX), carnitine palmitoyltransferase (CPT)-1α, fatty acid synthase, and glyceraldehyde 3-phosphate dehydrogenase detection are listed in Table S1. Gene expression levels were normalized to the glyceraldehyde 3-phosphate dehydrogenase levels and expressed as fold-changes relative to the control.

2.8 Statistical analyses

Data are represented as the mean ± standard error of the mean. All experiments were conducted with at least three replicates. Differences among multiple groups were ana-

lyzed via analysis of variance, followed by Dunnett's multiple-comparison test. Differences between two groups were analyzed via Student's *t*-test. Statistical significance was set at $p < 0.05$. All analyses were conducted using the GraphPad Prism version 7 software (GraphPad Software, San Diego, CA, USA).

3 Results and Discussion

3.1 Cell viability and cytotoxicity

To determine the optimal concentration of each fatty acid (EPA, HDTA, and ODTA) to be added to the culture medium of HepG2 cells, cell viability and cytotoxicity were assessed via WST-8 and LDH release assays, respectively (Fig. 2). Upon incubation with 0.5, 1, 5, 10, 25, and 50 μM of HDTA, ODTA, and EPA, only 25 μM HDTA and 50 μM HDTA and ODTA significantly decreased the HepG2 cell viability compared to untreated samples (Figs. 2A and 2B). Furthermore, 25 μM and 50 μM HDTA and ODTA significantly increased the cytotoxicity compared to the untreated samples (Figs. 2D and 2E). Notably, EPA did not affect the cell viability and cytotoxicity at any tested concentration (up to 50 μM). This finding is consistent with that of a previous study revealing no change in HepG2 cell viability after 24-h treatment with 50 μM EPA and DHA (Figs. 2C and 2F)^{21, 22}. These results suggest that the *n*-1 PUFA, HDTA and ODTA, are more cytotoxic than the representative *n*-3 PUFA, EPA. The highest safe concentration that

did not cause any cytotoxicity was 10 μM for both HDTA and ODTA. Accordingly, the concentration of each fatty acid used in subsequent experiments was set at 10 μM .

3.2 Identification of HDTA metabolites

To identify the specific HDTA metabolites, HepG2 cells were treated with 10 and 100 μM HDTA for 24 h. FAME was prepared from the total lipids in HepG2 cells and analyzed via GCMS. Chromatograms of HDTA-treated and untreated samples were compared, and the peaks detected only in the HDTA-treated samples were identified as HDTA metabolites (Fig. 3). HDTA metabolite peaks with same retention times (RT) were observed in the chromatograms of both 10 and 100 μM HDTA treatments. Chromatogram of the 100 μM HDTA treatment, which provided sufficient mass spectra, is shown in Fig. 4.

Comparison of the total ion chromatograms of the HDTA-treated and untreated samples (control) revealed five peaks only in the HDTA-treated samples (Fig. 3A). Enlarged views of the five peaks are shown in Figs. 3B–3E. Peaks 1 and 2 were identified as HDTA (C16:4 *n*-1) and ODTA (C18:4 *n*-1; Figs. 3B and 3C), respectively, by matching the RT and mass spectra (Figs. 4A and 4B) with those of the HDTA-methyl ester (ME) and ODTA-ME standards. The mass spectrum (MS) of peak 4, for which sufficient MS data were obtained, is shown in Fig. 4C. The MS of peak 4 exhibited a molecular ion peak at m/z 318 and fragment ions at m/z 250 and 287. This spectrum is very similar to the previously reported spectrum of C20:4 *n*-1-

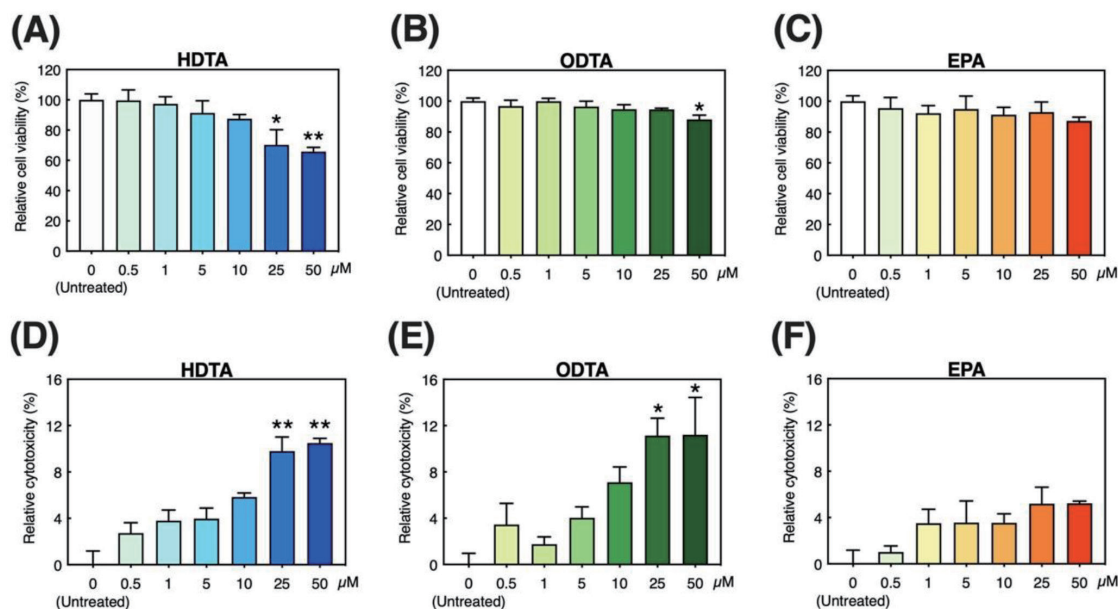


Fig. 2 Cell viability and cytotoxicity analyses of HDTA, ODTA, and EPA via WST-8 (A–C) and LDH release (D–F) assays. Data are represented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. 0 (untreated) using Dunnett's multiple-comparison test. EPA, 5,8,11,14,17-eicosapentaenoic acid; HDTA, 6,9,12,15-hexadecatetraenoic acid; LDH, lactate dehydrogenase; ODTA, 8,11,14,17-octadecatetraenoic acid; SEM, standard error of the mean; WST, water-soluble tetrazolium salt.

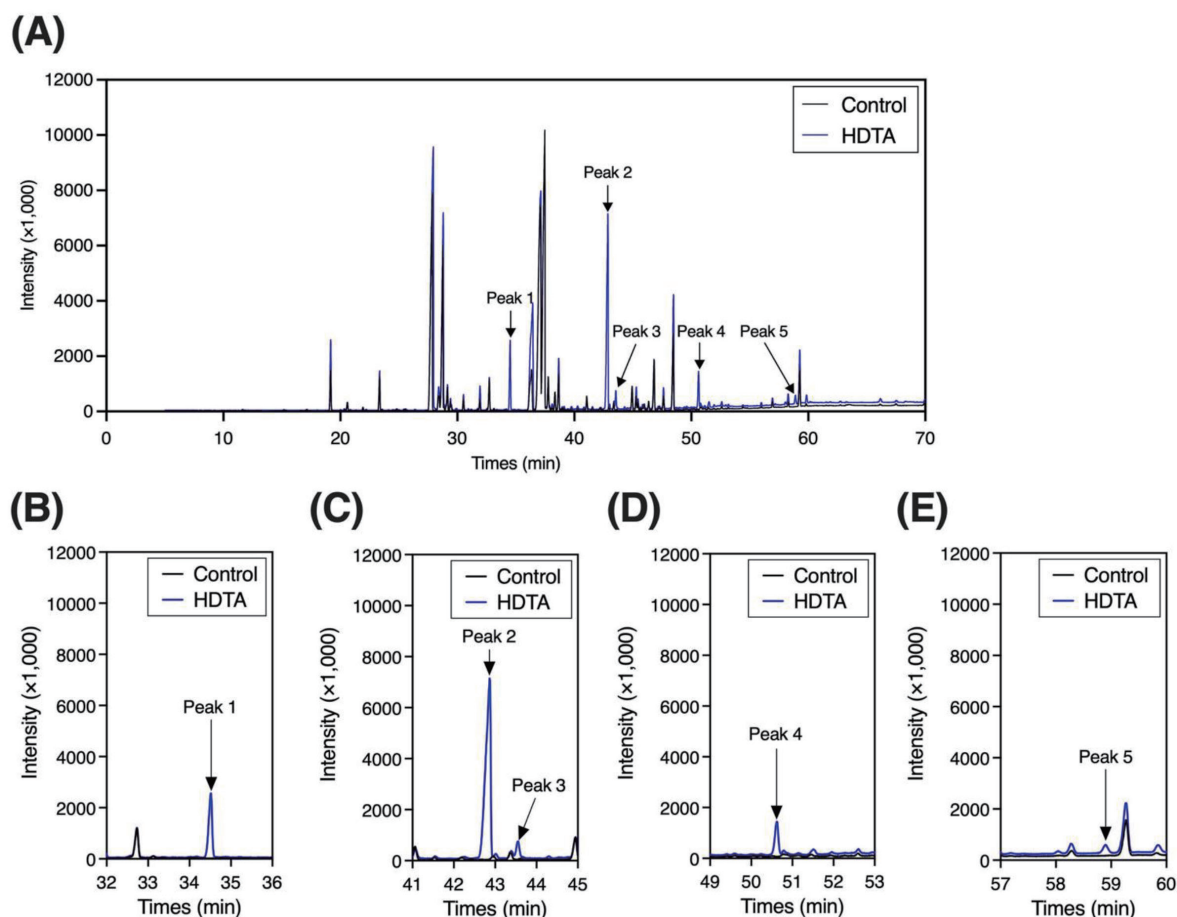


Fig. 3 Total ion chromatogram obtained via GCMS analysis of the cellular lipids in ethanol- and HDTA-supplemented HepG2 cells. Enlarged views of the chromatogram showing the retention times of 32–36 (B), 41–45 (C), 49–53 (D), and 57–60 (E) min. Mass spectra of peaks 1, 2, and 4 are shown in Fig. 4. GCMS, gas chromatography–mass spectrometry; HDTA, 6,9,12,15-hexadecatetraenoic acid; ODTA, 8,11,14,17-octadecatetraenoic acid.

ME²³). Based on these findings, the fatty acid corresponding to peak 4 was identified as C20:4 *n*-1.

Peaks 3 and 5 could not be identified due to insufficient MS data, with no molecular ion peaks. Peak 3 may be as C18:5 *n*-1 based on its RT and known fatty acid biosynthetic pathway. Elongation markedly increases, whereas the addition of a double bond slightly increases the RT of fatty acids. Accordingly, peak 3 was considered to be C18:5 *n*-1 produced by the action of Δ 6-desaturase on C18:4 *n*-1. Peak 5 could not be assigned to any specific compound based on its RT and known fatty acid biosynthetic pathway. Therefore, further analysis via high-resolution MS is necessary to determine the chemical structures of peaks 3 and 5.

3.3 Time-dependent changes in fatty acid levels in the medium and cells

Next, HDTA and ODTA levels in the medium were measured to determine whether they are taken up by the cells (Fig. 5). HDTA and ODTA in the medium disappeared with increasing incubation time, with neither fatty acid being

detected after 24 h. Additionally, EPA was not detected in the culture medium 24 h after addition. These findings suggest that EPA, HDTA, and ODTA are taken up by the cells within 24 h of addition.

Addition of free fatty acids, such as palmitic and oleic acids, promotes triacylglycerol accumulation in HepG2 cells^{24,25}. To evaluate the effects of EPA, HDTA, and ODTA on triacylglycerol accumulation in HepG2 cells, total fatty acid content of the cells was measured. Notably, no change in total fatty acid content was observed 24 h after the supplementation of HDTA, EPA, and ODTA compared to that before the supplementation of each fatty acid. Palmitic and oleic acids are typically used at 0.2–1.0 mM to induce triacylglycerol accumulation in HepG2 cells. As the supplemented concentration of each fatty acid in this study was 10 μ M, triacylglycerol accumulation was not observed in HepG2 cells.

Time-dependent changes in metabolite levels in EPA-, HDTA-, and ODTA-supplemented cells are shown in Fig. 6. In HDTA- and ODTA-supplemented cells, relative ODTA

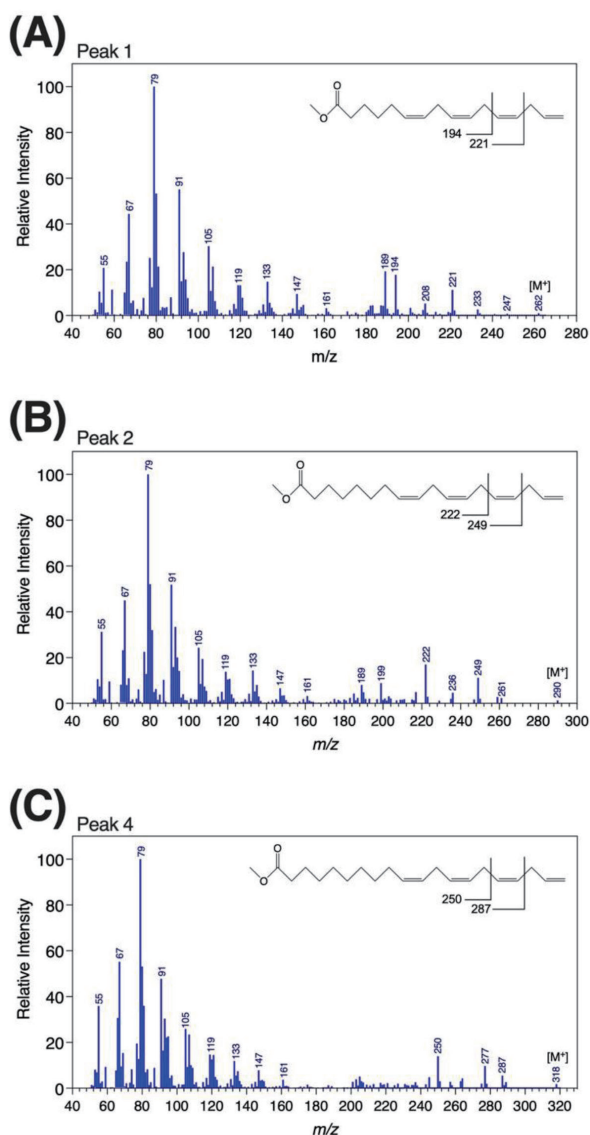


Fig. 4 Mass spectrum obtained via GCMS analysis of the cellular lipids in HDTA-supplemented HepG2 cells. Mass spectra of peaks 1 (A), 2 (B), and 4 (C) are shown. The peaks at $\geq m/z$ 180 were magnified ten times. Total ion chromatogram is shown in Fig. 3. GCMS, gas chromatography–mass spectrometry; HDTA, 6,9,12,15-hexadecatetraenoic acid.

and C20:4 *n*-1 levels first increased up to 6 h after supplementation and then remained stable until 24 h (Figs. 6A and 6B). Specifically, relative HDTA, ODTA, and C20:4 *n*-1 levels 6 h after HDTA supplementation were 0.2, 1.2, and 0.2 mol%, respectively. Relative HDTA level 24 h after HDTA supplementation was 0.03 mol%. A previous study detected ODTA, but not HDTA, at relative level of 1.0 mol% in the liver of HDTA-treated mice¹¹. Here, relative ODTA and C20:4 *n*-1 levels 6 h after ODTA supplementation were 2.5 mol% and 0.4 mol%, respectively. Notably,

the total relative levels of HDTA metabolites, including ODTA and C20:4 *n*-1, in ODTA-supplemented cells were significantly higher than those of HDTA and its metabolites in HDTA-supplemented cells 3, 6, and 24 h after supplementation (Fig. S2) when tested using Student's *t*-test. Figure 5 illustrates the time-dependent decrease in HDTA and ODTA contents in the medium. Figure 6 illustrates the relative levels of ODTA in HepG2 cells supplemented with HDTA and ODTA, which remained unchanged from 6 h to 24 h. The HDTA and ODTA levels in the medium decreased 6 h after supplementation with HDTA and ODTA, which may have reduced the uptake of HDTA and ODTA into the cells, resulting in a balance between fatty acid uptake and β -oxidation in the cells, and thus maintaining the relative levels of ODTA and C20:4 *n*-1.

Relative levels of EPA, 7,10,13,16,19-docosapentaenoic acid (C22:5 *n*-3; DPA), C24:6 *n*-3, and DHA 24 h after EPA supplementation were 7.5, 2.1, 1.0, and 1.2 mol%, respectively (Fig. 6C). Moreover, relative levels of EPA, DPA, and C24:6 *n*-3 increased up to 6 h after supplementation, whereas those of DHA remained unchanged. The total relative level of EPA and its metabolites, including DPA, C24:6 *n*-3, and DHA in EPA-supplemented cells reached 11.8 mol% after 24 h of supplementation. This total relative level of EPA and its metabolites significantly exceeded the total relative levels of HDTA and its metabolites, including ODTA and C20:4 *n*-1 in HDTA- and ODTA-supplemented cells. These results suggest that both HDTA and ODTA could be more rapidly β -oxidized than EPA when taken up into cells. When the cell was supplemented with HDTA and ODTA, HDTA hardly accumulated in the cells; however, ODTA accumulated in the cells (Figs. 6A and 6B). Perhaps ODTA is less susceptible to β -oxidation than HDTA in HepG2 cells, leading to a certain degree of accumulation in the cells. Hydroxy fatty acids (HFAs), such as 10-hydroxy-*cis*-12-octadecenoic and 10-hydroxy-octadecanoic acids, are less retained than linoleic acid in CHO-K1 (MKN74 stomach cancer) cells²⁶, possibly due to peroxisomal β -oxidation. Similar mechanisms possibly prevent the intracellular retention of HDTA and ODTA in HepG2 cells.

3.4 Metabolite levels in the neutral and polar lipid fractions of cells

To identify the lipid fractions containing the EPA and HDTA metabolites, these metabolite levels were measured in the neutral and polar lipid fractions of EPA- and HDTA-supplemented cells for 24 h (Fig. 7). Following HDTA supplementation, ODTA and C20:4 *n*-1 were detected in both the neutral and polar lipid fractions of cells (Figs. 7A and 7C). Interestingly, HDTA was present in the neutral lipid fraction, but not in the polar lipid fraction. Following EPA supplementation, EPA and EPA metabolites were detected in both the neutral and polar lipid fractions (Figs. 7B and 7D). A previous study detected HFAs in the phospholipid

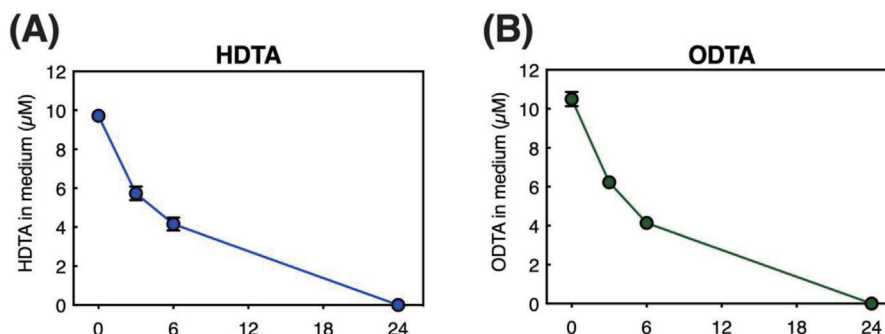


Fig. 5 Time-dependent changes in HDTA (A) and ODTA (B) levels in the culture media of HDTA- and ODTA-supplemented HepG2 cells. Data are represented as the mean $\pm$ SEM ($n = 3$). HDTA, 6,9,12,15-hexadecatetraenoic acid; ODTA, 8,11,14,17-octadecatetraenoic acid; SEM, standard error of the mean.

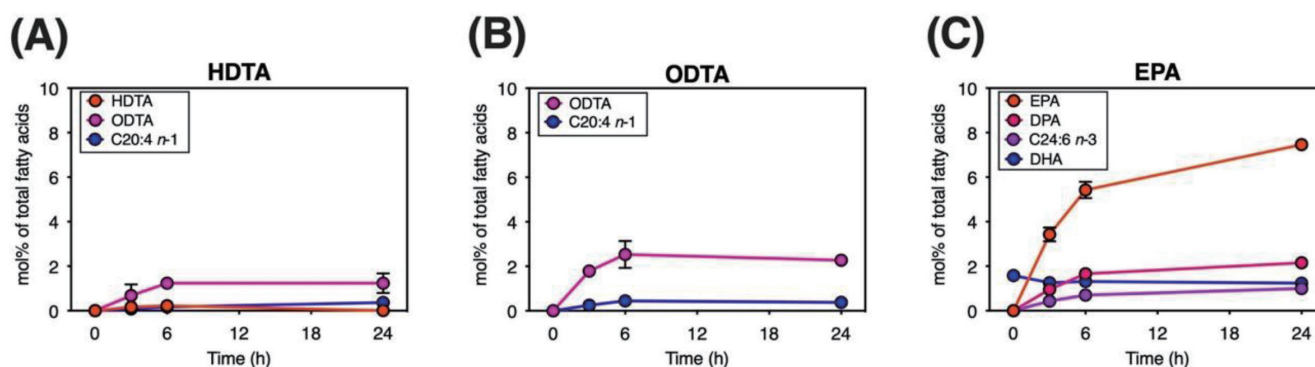


Fig. 6 Time-dependent changes in metabolites levels in HDTA (A)-, ODTA (B)-, and EPA (C)-supplemented HepG2 cells. Data are represented as the mean $\pm$ SEM ($n = 3$). DHA, 4,7,10,13,16,19-docosahexaenoic acid; DPA, 7,10,13,16,19-docosapentaenoic acid; EPA, 5,8,11,14,17-eicosapentaenoic acid; HDTA, 6,9,12,15-hexadecatetraenoic acid; ODTA, 8,11,14,17-octadecatetraenoic acid; SEM, standard error of the mean.

fraction, but not in the triacylglycerol and free fatty acid fractions, of CHO-K1 cells²⁶). Free fatty acids are transported by binding proteins, such as fatty acid-binding proteins, which play a critical role in determining the metabolic fate of fatty acids in cells²⁷). Therefore, fatty acid-binding proteins are possibly involved in the mechanisms preventing the acylation of HDTA in the polar lipid fraction; however, the specific mechanisms warrant further investigation.

3.5 Fatty acid metabolism-related gene expression levels

After 24-h fatty acid treatment, the total relative level of EPA and its metabolites, including DPA, C24:6 *n*-3, and DHA was 11.8 mol% in EPA-supplemented cells, whereas that of HDTA and its metabolites, including ODTA and C20:4 *n*-1 was 1.6 mol% in HDTA-supplemented cells (Figs. 6A and 6B). These results suggest that some HDTA is elongated to ODTA and C20:4 *n*-1, whereas most of it is degraded more rapidly than other fatty acids in the cells. To elucidate the underlying mechanisms, RNA-sequencing was performed to comprehensively assess the gene expression changes in HepG2 cells supplemented with EPA, HDTA, and ODTA for 6 h. Three DEGs were identified by

comparing the HDTA- and EPA-supplemented groups, whereas 25 were identified by comparing the ODTA- and EPA-supplemented groups (Table S1). However, these DEGs were not associated with fatty acid β -oxidation in the cells (Table S2).

Fatty acids are intracellularly degraded via β -oxidation, with *ACOX* and *CPT-1 α* serving as the key rate-limiting enzymes in this pathway²⁸). *ACOX* catalyzes the first step in peroxisomal β -oxidation, whereas *CPT-1 α* catalyzes the transport of fatty acids across the mitochondrial inner membrane. Acetyl-CoA carboxylase α and fatty acid synthase are the rate-limiting enzymes for fatty acid synthesis²⁹). As HDTA is rapidly degraded via β -oxidation in cells, temporal changes in the mRNA expression levels of genes associated with fatty acid β -oxidation and synthesis were measured in the HepG2 cells with or without HDTA supplementation for 3, 6, and 12 h (Fig. S1). Notably, no significant differences in the expression levels of genes associated with fatty acid β -oxidation and synthesis were observed after 3, 6, and 12 h of HDTA supplementation. The measured fatty acid concentrations in cells (Fig. 6) indicated that HDTA and ODTA underwent β -oxidation in the cells; however, no upregulation of fatty acid β -oxidation-related

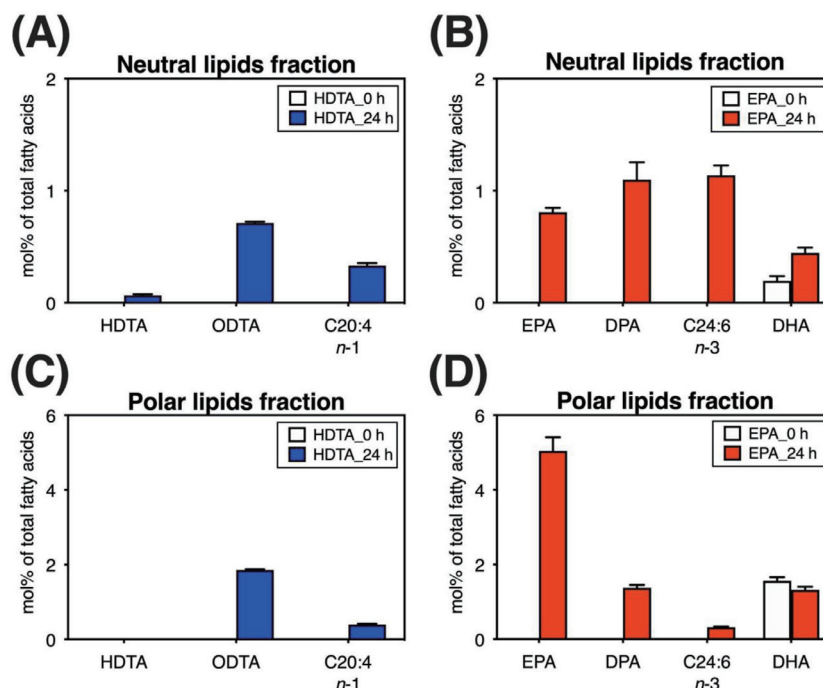


Fig. 7 Changes in metabolite levels in the neutral and polar lipid fractions of HepG2 cells before and 24 h after EPA and HDTA supplementation. Neutral (A) and polar (C) lipid fractions of HDTA-supplemented cells. Neutral (B) and polar (D) lipid fractions of EPA-supplemented cells. Data are represented as the mean $\pm$ SEM ($n=3$). DHA, 4,7,10,13,16,19-docosahexaenoic acid; DPA, 7,10,13,16,19-docosahexaenoic acid; EPA, 5,8,11,14,17-eicosapentaenoic acid; HDTA, 6,9,12,15-hexadecatetraenoic acid; ODTA, 8,11,14,17-octadecatetraenoic acid; SEM, standard error of the mean.

genes was observed at the transcriptional level. As the supplemented concentration of fatty acids was low ($10 \mu\text{M}$), β -oxidation possibly occurred without any changes in the fatty acid β -oxidation-related gene levels. However, whether HDTA and ODTA undergo fatty acid β -oxidation could not be confirmed, warranting further examination.

4 Conclusion

In summary, this study evaluated the bioconversion and metabolic fate of *n*-1 PUFA, including HDTA and ODTA, using HepG2 cells. HDTA elongated to ODTA and C20:4 *n*-1 in HepG2 cells. However, the total relative level of HDTA and its metabolites, including ODTA and C20:4 *n*-1 accumulated in the cells were substantially lower than those of EPA and its metabolites, including DPA, C24:6 *n*-3, and DHA, suggesting that only a portion of HDTA taken up by the cells undergoes intracellular elongation and accumulates as HDTA metabolites including ODTA and C20:4 *n*-1, whereas most of it undergoes rapid β -oxidation. Overall, our results provide novel insights into the intracellular bioconversion mechanisms and metabolic fate of *n*-1 PUFA in HepG2 cells.

Author Contributions

Conceptualization and design: KS, RH, ToT, TaT, NB, YoM, and KF. Analysis and interpretation: KS, HN, RH, ToT, ZW, MO, and YuM. SK, KF. Data collection: KS, HN, RH, ToT, TaT, NB, YoM, ZW, MO, YuM. SK, and KF. Writing the manuscript: KS, RH, and KF. Critical revision of the manuscript: All authors. Final approval of the manuscript: All authors. Statistical analysis: KS, RH, ZW, and MO. Funding: RH and KF. Supervision: KS, RH, and KF.

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Availability of Data and Materials

The data and materials presented in this study are available on request from the corresponding author.

Conflict of Interest

Toshifumi Tanizaki, Tadahiro Tsushima, Naomichi Baba, and Yoshihisa Misawa are employees of Bizen Chemical Co., Ltd. (Akaiwa, Japan), which supplied HDTA, ODTA, and EPA for this study. The other authors declare no conflicts of interest.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess25120

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