CYP4F13 is the Major Enzyme for Conversion of α -Eleostearic Acid into *cis*-9, *trans*-11-Conjugated Linoleic Acid in Mouse Hepatic Microsomes

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Abstract: Our previous studies have shown that α -eleostearic acid (α -ESA; *cis*-9, *trans*-11, *trans*-13 (c9,t11,t13)-conjugated linolenic acid (CLnA)) is converted into c9,t11-conjugated linoleic acid (CLA) in rats. Furthermore, we have demonstrated that the conversion of α -ESA into CLA is a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzymatic reaction, which occurs mostly in the rat liver. However, the precise metabolic pathway and enzyme involved have not been identified yet. Therefore, in this study we aimed to determine the role of cytochrome P450 (CYP) in the conversion of α -ESA into c9,t11-CLA using an *in vitro* reconstitution system containing mouse hepatic microsomes, NADPH, and α -ESA. The CYP4 inhibitors, 17-ODYA and HET0016, performed the highest level of inhibition of CLA formation. Furthermore, the redox partner cytochrome P450 reductase (CPR) inhibitor, 2-chloroethyl ethyl sulfide (CEES), also demonstrated a high level of inhibition. Thus, these results indicate that the NADPH-dependent CPR/CYP4 system is responsible for CLA formation. In a correlation analysis between the specific activity of CLA formation and *Cyp4* family gene expression in tissues, *Cyp4a14* and *Cyp4f13* demonstrated the best correlations. However, the CYP4F substrate prostaglandin A₁ (PGA₁) exhibited the strongest inhibitory effect on CLA formation, while the CYP4A and CYP4B1 substrate lauric acid had no inhibitory effect. Therefore, we conclude that the CYP4F13 enzyme is the major enzyme involved in CLA formation. This pathway is a novel pathway for endogenous CLA synthesis, and this study provides insight into the potential application of CLnA in functional foods.

Key words: cytochrome P450, conjugated linolenic acid, conjugated linoleic acid, α -eleostearic acid, conversion

1 Introduction

Conjugated linoleic acid (CLA; C18:2) is a collective term for geometric and positional isomers of linoleic acid (*cis*9,*cis*12-18:2) with two conjugated double bonds. There are 56 different isoforms of CLA as a result of the various arrangement of double bonds, including positional (6,8; 7,9; 8,10; 9,11; 10,12; 11,13; 12,14; and 13,15), and geometric (*cis/cis*; *cis/trans*; *trans/trans*; or *trans/cis*) variations¹⁾. Several of them, and in particular, *cis*-9,*trans*-11 (c9,t11)- and t10,c12-CLA were reported to have various beneficial effects on physiological functions, such as anti-obesity and anticarcinogenic activities, immune enhancement, bone formation improvement, and lipid metabolism regulation in *in vivo* and *in vitro* studies^{1,2)}. Based on these benefits, CLA-rich foods are considered as functional foods. CLA is commonly found in dairy products and ruminant meat, which are the main sources of CLA for humans. However,

CLA is naturally present in very small amounts in these foodstuffs which typically contain fat in the range from 2.9 to 8.9 mg/g³⁾. As the CLA intake from dietary sources is far from the intended effective dosage due to the low content of CLA in nature⁴⁾, it is necessary to supplement the human diet with CLA. Generally, the CLA in supplements is not derived from natural foods but prepared by chemically altering the linoleic acid found in vegetable oils. It is important to note, however, that the balance of the different CLA isoforms is heavily distorted in supplements because they contain types of CLA isoforms that are never found in large amounts in nature. The c9,t11-CLA is believed to be the most common natural form of CLA comprising 80 to 90% of the total CLA in food products from ruminants, whereas t10,c12-CLA is present at a level of 3-5%, and other isomers are present in very small amounts^{5,6)}. Most of the commercially available CLA sup-

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plements contain almost equal amounts of the two major isomers c9,t11-CLA (40.85–41.1 %) and t10,c12-CLA (43.5–44.9 %), and levels of other CLA isomers (4.6%–10%)⁷⁾. However, it has been reported that mice fed the highly purified t10,c12-CLA isomer had adverse side effects such as insulin resistance, robust hyperinsulinemia, and massive liver steatosis^{8–10)}. Furthermore, insulin resistance has also been detected in obese men treated with purified t10,c12-CLA¹¹⁾, which raises concerns about the safety of dietary supplements containing the t10,c12-CLA isomer. On the other hand, c9,t11-CLA, which was found to improve the increased insulin resistance caused by t10,c12-CLA^{9, 12)}, is considered safer due to fewer reported side effects. For these reasons, CLA supplements do not provide the same health effects as CLA from natural foods.

In contrast to the generally low content of CLA in nature, the content of conjugated linolenic acid (CLnA; C18:3) with three conjugated double bonds in seed oil from certain plants can comprise up to ~80% of the total lipid content. For example, α -eleostearic acid (α -ESA; c9,t11,t13-CLnA) comprises up to 60% (wt/wt) of the total lipid content of bitter melon seed oil and 76% of the total lipid content of tung seed oil, whereas punicic acid (PA; c9,t10,c13-CLnA) comprises up to 74.5% of the total lipid content of pomegranate seed oil, and jacaric acid (JA; c8,t10,c12-CLnA) is found at a concentration of 15.9% in jacaranda seed oil. We are particularly interested in these CLnA-rich seed oils, because CLnA is the only conjugated fatty acid that can be prepared from natural sources in bulk¹³⁾. Our previous studies have also shown that CLnA has a stronger anti-tumor effect than CLA *in vitro* and *in vivo*. Moreover, α -ESA has been reported to have a strong antiangiogenic effect, a new confirmed physiological effect of α -ESA^{14–17)}. We first reported that α -ESA was converted into c9,t11-CLA in 1% α -ESA-fed rats. The structure of c9,t11-CLA was determined using gas chromatography-electron impact/mass spectrometry (GC-EI-MS) and ¹³C-NMR. Furthermore, we have demonstrated that this conversion is a $\Delta 13$ saturation, an NADPH-dependent enzymatic reaction occurring mostly in the rat liver^{18, 19)}. Moreover, we observed similar conversions of CLnA into CLA in PA- and JA-fed rats, where PA was converted into c9,t11-CLA and JA was converted into c8,t10-CLA^{13, 20)}. These conversions were also confirmed in mice and humans in other reports^{21–23)}.

In ruminants, CLA is formed as an intermediate during the ruminal bio-hydrogenation of linoleic acid to stearic acid (18:0) by *B. fibrisolvens* and other rumen bacteria²⁴⁾. Another pathway involves $\Delta 9$ -desaturase-dependent endogenous CLA synthesis from vaccenic acid (t11-18:1), another intermediate in ruminal biohydrogenation^{5, 25)}. Although the major source of CLA in humans comes from dietary intake, endogenous synthesis of CLA has also been reported in humans and other non-ruminants^{26–28)}. Accordingly, the conversion of CLnA into CLA could be a novel

pathway for endogenous CLA synthesis. As CLnA can be prepared more easily than CLA, once the mechanism of this conversion is elucidated, it is expected that CLnA, especially α -ESA will be a new source for CLA synthesis or supplant CLA as a dietary supplement.

Our previous studies indicated that the NADPH-dependent enzyme involved in this conversion should be classified as part of the drug metabolism, but not as part of the β -oxidation enzyme group in the fatty acid metabolic pathway¹⁸⁾. However, the precise metabolic pathway and enzyme involved have not been identified yet. The superfamily of cytochrome P450 (CYP) enzymes is considered the major enzyme family responsible for the phase I metabolism (reduction, oxidation, or hydrolysis reactions) of numerous endogenous and exogenous compounds, such as drugs and other xenobiotics²⁹⁾. The *in vivo* reactions catalyzed by CYP enzymes require the cofactor NADPH as the electron source and the redox partner of cytochrome P450 reductase (CPR), which functions in the electron transfer from NADPH to CYP in microsomes³⁰⁾. Based on these considerations, we sought to elucidate whether CYPs play a role in the conversion of α -ESA into CLA. For this purpose, we aimed to determine the subcellular distribution of enzymatic activity in mouse liver by using an *in vitro* reconstitution system of enzymatic activity containing mouse hepatic microsomes, NADPH, and α -ESA to test the effect of various inhibitors and CYP-substrates on c9,t11-CLA formation. Moreover, we aimed to determine the enzymatic activities and *Cyp4* family gene expression levels in various tissues for correlational analysis.

2 Experimental Procedures

2.1 Materials

Tung oil and standard CLA oil were obtained from the Nisshin Oil Co. Ltd. NADPH, NADP⁺, NADH, and NAD⁺ were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Trimethylsilyldiazomethane (TMSN₂CH₃), trimethylolaminomethane acetate (Tris-actate), fluvoxamine, ticlopidine, montelukast, fluconazole, chlormethiazole, and ketoconazole were purchased from Tokyo Chemical Industry (Tokyo, Japan). Indomethacin, niflumic acid, 2-chloroethyl ethyl sulfide (CEES), 17-octadecynoic acid (17-ODYA), and HET0016 were purchased from Sigma-Aldrich (St. Louis, MO). Lauric acid, palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, prostaglandin A1, sulfaphenazole, and quinidine were obtained from Cayman Chemical Company (Ann Arbor, MI). All other chemicals (sucrose, EDTA·2Na·2H₂O, DTT, PMSF) were purchased from Wako Pure Chemical Industries, Ltd.

2.2 Methods

2.2.1 Animals

All animal experiments were conducted in accordance with the Regulations for Animal Experiments and Related Activities at Tohoku University (2018AgA-015)³¹. Institute of Cancer Research (ICR) mice, 13 weeks old, were obtained from CLEA Japan Inc. and were allowed to acclimate to the facility for 1 week with standard chow diet (CE-2, CLEA Japan) before the initiation of the experiments. At 14 weeks of age, the mice were sacrificed by decapitation after an overnight fast for 12 h and the following tissues were collected: liver, kidney, small intestine, pancreas, brain, heart, lung, spleen, epididymal white adipose tissue (eWAT), and brown adipose tissue (BAT). All tissues were stored at -80°C until use.

2.2.2 Enzymatic activity assay

To prepare the enzyme substrate α -ESA (free fatty acid form), tung oil was hydrolyzed by potassium hydroxide (KOH) as previously reported with a slight modification¹⁷. After bubbling with nitrogen gas for 15 s, 30 mg of tung oil in a glass test tube was saponified with 0.25 mL of 0.3 M KOH in 2.5 mL methanol at 40°C for 90 min. After cooling to room temperature, 2.5 mL of H_2O and 5 mL of hexane were added. The reaction mixture was vigorously vortexed for 2 min and centrifuged at $500\times g$ for 5 min to separate the organic layer from the aqueous layer. The top organic layer containing non-saponaceous material was removed, while the bottom aqueous layer was mixed with 1.5 mL of 6 M HCl and 5 mL of hexane to extract the fatty acids. The mixture was vigorously vortexed for 2 min and centrifuged at $500\times g$ for 5 min. The top organic layer was then concentrated by solvent evaporation under vacuum, dissolved in dimethyl sulfoxide (DMSO), and stored at -80°C until use.

For detecting enzymatic activity, each one of the frozen biological samples (a total of 10 tissue samples) was homogenized in 9 volumes (w/v) of chilled 0.01 M Tris-acetate sucrose buffer, pH 7.4, containing 0.01 M Tris-Acetate, 0.25 M Sucrose, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA)¹⁸, by using the bead-type homogenizer Micro Smash MS-100 (TOMY Seiko, Tokyo, Japan) at 3600 rpm for 30 sec $\times$ 3 times. To unify the enzymatic reaction time, the experiments were performed in a crushed-ice bath. A total of 450 μL of homogenate (10% w/v) was mixed with 10 μL of α -ESA substrate (5 mM in DMSO) and 40 μL of coenzyme NADPH (0.1 M in 0.9% NaCl), followed by incubation at 37°C for 30 min. The reaction was stopped by placing the sample in a crushed-ice bath and a known amount of the internal standard heptadecanoic acid (C17:0) was added in order to quantify the fatty acids. The lipids in the reaction mixture were extracted with chloroform-methanol-water (1:1:0.9 in volume) according to the Bligh & Dyer method. The free fatty acids in the lipid

extract were then catalyzed into methyl ester derivatives by the TMSN_2CH_3 method³² for gas chromatography (GC) analysis to determine the CLA concentration. The protein concentration was determined by the Pierce BCA protein assay kit (Thermo Scientific, Houston, TX, USA). The specific activity of the conversion of α -ESA into CLA was obtained after normalizing to the total protein using the following formula: specific activity (nmol/min/g protein) = CLA amounts (nmol) / time taken (min) / protein (g).

2.2.3 Gas chromatography analysis of fatty acids

The fatty acid methyl esters (FAMES) were analyzed by GC (GC-4000 Plus, GL Science Inc., Tokyo, Japan) equipped with a Supelcowax-10 fused silica capillary column (60 m $\times$ 0.32 mm $\times$ 0.25 μm film thickness, Supelco, Bellefonte, PA) and a flame ionization detector²⁰. Helium was used as a carrier gas at a constant pressure of 400 kPa. The temperatures of the injector and detector were 200°C and 250°C ¹³, respectively, and the oven temperature program was as follows: an initial temperature of 50°C was ramped to 220°C at a rate of $20^{\circ}\text{C}/\text{min}$ and held for 30 min, then ramped to 250°C at a rate of $20^{\circ}\text{C}/\text{min}$ and held for 20 min. Each peak was annotated by comparing the retention times with CLA FAMES standards and GLC reference standards of FAMES (Nu-Chek-Prep, Elysian, MN, USA).

2.2.4 Characterization of α -ESA saturase

In order to further understand the characteristics of the conversion of α -ESA into CLA, the liver homogenate, NADPH, and α -ESA in the reaction mixture, were gradually replaced by 0.01 M Tris-acetate sucrose buffer, 0.9% NaCl solution, and DMSO, respectively. After incubation at 37°C for 30 min, the specific activity of α -ESA conversion into CLA was measured, as explained above.

To further confirm whether other coenzymes could initiate the reaction, 40 μL of 0.1 M coenzyme (NADPH, NADP^+ , NADH, and NAD^+ were all dissolved in 0.9% NaCl solution) was added individually in the presence of the homogenate and α -ESA. Subsequently, the reaction mixture was processed as described above to measure the specific activity of α -ESA conversion into CLA. Experiments with heated liver homogenate (incubations conducted at 60°C for 30 min) were also performed to further assess the role of the homogenate in the conversion of α -ESA into CLA.

To elucidate the subcellular distribution of α -ESA saturase, the liver homogenate was centrifuged at $600\times g$, at 4°C for 10 min to collect the pellet containing intact nuclei and debris. The nuclear pellet was re-suspended into 1 volume of 0.01 M Tris-acetate sucrose buffer and the post-nuclear supernatant was again centrifuged at $8000\times g$, at 4°C for 20 min using an Optima L-100 XP Ultracentrifuge with a type 70.1 Ti fixed-angle rotor (Beckman Coulter Ltd., Fullerton, CA) to sediment mitochondria. The mitochondrial pellet was re-suspended into 1 volume of the 0.01 M Tris-acetate sucrose buffer and the post-mitochondrial supernatant was further ultracentrifuged at $105000\times$

g, at 4°C for 60 min to obtain microsomes³³). The microsomal pellet was also re-suspended into 1 volume of the same buffer and the cytosolic supernatant was retained. A total of 450 µL of each subcellular fraction, namely nuclear, mitochondrial, microsomal, and cytosolic, was subjected to an enzymatic activity assay and the amounts of CLA were determined by GC analysis.

2.2.5 Inhibitory effect of inhibitors and CYP-substrates

To evaluate the inhibitory effect of inhibitors (CYP-selective inhibitors, COX inhibitors, and CPR inhibitor) in CLA formation, liver microsomes (10 mg protein/mL) were pre-incubated with inhibitors or DMSO solvent, which was used as the control, at 37°C for 5 min. Then, the pre-incubated mixtures were immediately subjected to the CLA formation assay as explained above. The IC₅₀ (concentration of inhibitor required to cause a 50% inhibition of the original enzyme activity) was determined graphically from the plot of the logarithm of inhibitor concentration versus the percentage of CLA remaining (% of control) after inhibition using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). To determine the inhibitory effect of CYP-substrates on CLA formation, liver microsomes (10 mg protein/mL) were pre-mixed simultaneously with α-ESA and CYP-substrates (α-ESA: CYP-substrates = 1:1 or 1:4 in mole ratio) before the addition of NADPH, which initiated the reaction. Following this, the mixtures were incubated at 37°C for 30 min as described above for the enzymatic activity assay and the amounts of CLA were determined by GC analysis.

2.2.6 *Cyp4* family messenger RNA expression analysis

For the purification of high-quality RNA, the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used for liver, kidney, small intestine, pancreas and spleen samples, while the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used for brain, eWAT, and BAT samples. The RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used for total RNA isolation from cardiac tissue according to the protocol given by the manufacturer. The

concentration and purity of the isolated RNA was determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, MA, USA). Subsequently, reverse transcription of RNA to complementary DNA (cDNA) was performed with the PrimeScript® RT Master Mix (Perfect Real Time) Kit (Takara Bio Inc., Shiga, Japan)³⁴. Briefly, an aliquot volume of 1000 ng of RNA, 4 µL of 5× PrimeScript RT Master Mix (Perfect Real Time) and RNase-free distilled water up to 20 µL were mixed and incubated at 37°C for 10 min, and then at 85°C for 5 sec. Finally, 480 µL of RNase-free dH₂O was added to dilute the cDNA and the samples were stored at −20°C for subsequent analysis.

The cDNA was used for real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to analyze the expression levels of *Cyp4* family genes. The qRT-PCR reaction was prepared in a final volume of 20 µL containing 10 µL 2×TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio Inc., Shiga, Japan), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM) and 10 µL diluted cDNA. The gene-specific primers, purchased from Sigma-Aldrich (Merck KGaA), are shown in Table 1. PCR amplification was performed with a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, California, USA) and each biological sample was assayed in two technical replicates. The reactions were subjected to an initial 30 sec denaturation at 95°C. To verify the specificity of the amplification reaction, a melting curve analysis was performed in the range of 60°C to 95°C, 0.5°C per 5 sec increments after thermo-cycling for each reaction³⁵. The threshold cycle (Ct) value, representing the PCR cycle at which an increase in reporter fluorescence signal significantly above the background fluorescence can first be detected, was also determined. The expression levels of the *Cyp4* family genes (10 genes total) in each biological sample was normalized to tissue weight, and shown as fold changes relative to the corresponding *Cyp4* transcripts in the liver.

Table 1 List of primer sequences used for real-time PCR.

Gene name	Accession Number	Forward Primer (5' -3')	Reverse Primer (5' -3')
<i>Cyp4a10</i>	NM_010011	GCTACTCAAGGCTTTCCAGCAG	CCAGAACCATCTAGGAAAGGCAC
<i>Cyp4a12a/b</i>	NM_017396/ NM_172306	CTCATTCCTGCCCTTCTCAG	GGTATGGGGATTGGGACTCT
<i>Cyp4a14</i>	NM_007822	CAAGACCCTCCAGCATTTC	GAGCTCCTTGTCCTTCAGATGGT
<i>Cyp4b1</i>	NM_007823	CACCTGGACTTCCTCGACAT	TCATCCCACTGGAAGGAGTC
<i>Cyp4f13</i>	NM_130882	CATCTTGGATTCTAGCCCGAA	GAAGCAACGAAGGCGACTG
<i>Cyp4f14</i>	NM_022434	ACTGGCTTATGGGTACGCTG	ACCCACCAAACGAGTCAATTC
<i>Cyp4f15</i>	NM_134127	CATGACATGGCTGGGTCCTA	GAGGCATTGAGAACAGATCGA
<i>Cyp4f16</i>	NM_024442	CCTTGCCCTGGACTTACTCATT	GTAACCAGCTGCATGCCTTC
<i>Cyp4f17</i>	NM_001101445	AAAACATTTCCAGGGAAGAGC	GGAGACGGCAGTAGTTGTCATA
<i>Cyp4f18</i>	NM_024444	AGAGCCTGGTGCGAACCTT	TGGAATATGCGGATGACTGG

2.3 Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) and were analyzed with one-way analysis of variance (ANOVA), followed by Tukey-Kramer test for comparisons among more than two groups. A P value of <0.05 was considered statistically significant. For correlation studies, the specific activity of CLA formation was normalized to the corresponding tissue weight. The correlations between the tissue specific activity and the relative expression level of *Cyp4* family genes were described using Spearman's rank correlation coefficients (r_s). The strength of correlation was ranked as follows: for absolute values of r_s , 0.01–0.19 was regarded as negligible, 0.20–0.29 as weak, 0.30–0.39 as moderate, 0.40–0.69 as strong, and ≥ 0.70 as very strong³⁶⁾.

3 Results

3.1 Characterization of α -ESA saturase *in vitro*

To characterize the conversion of α -ESA into CLA, liver homogenate, α -ESA, and NADPH in the *in vitro* reconstitution system were replaced by their corresponding solvents. However, CLA formation was not detected in the absence of any one of the ingredients (Fig. S1). Only the liver homogenate could convert α -ESA into CLA in the presence of NADPH, indicating that the converting process was an enzyme-mediated metabolic process. To further confirm whether other coenzymes could initiate the reaction, either NADP, NADH, or NAD was added to the *in vitro* reconstitution system instead of NADPH. However, in the presence of NADP, NADH, or NAD the CLA level was below the detectable limit due to a high preference for NADPH (Fig. S2). Furthermore, the heated liver homogenate incubated with α -ESA and NADPH also showed no activity (Fig. S2), demonstrating that the metabolism of α -ESA into CLA occurs through an NADPH-dependent enzymatic reaction. In the α -ESA saturase subcellular localization experiment in the liver homogenate, the enzymatic activity was the highest in the microsomal fraction, followed by the mitochondrial fraction, lower in the nuclear fraction, and absent in the cytosolic fraction (Fig. 1). Therefore, it was confirmed that α -ESA saturase required NADPH as the source of electrons and was abundant in microsomes, which contain the major CYP drug-metabolizing enzymes. For these reasons hepatic microsomes were used in the next experiments.

3.2 The effect of inhibitors on CLA formation

To explore whether the CYP enzymes were involved in CLA formation, the various CYP-specific inhibitors were selected to validate their inhibitory effect on CLA formation in hepatic microsomes. With the exception of fluconazole (CYP2C19 inhibitor) and ketoconazole (CYP3A4 inhibitor), all the other CYP-specific inhibitors, namely

Fig. 1 The specific enzymatic activity of CLA formation in different subcellular fractions separated from mouse liver homogenate. (A) Procedures for ultracentrifugal subcellular fractionation of liver homogenate. Nuclei, mitochondria and microsomes were re-suspended into 1 volume of homogenizing buffer. (B) GCs of free fatty acids from one replicate of a representative experiment in the subcellular fractions. (C) Bar graph analysis of the specific activity of CLA formation in subcellular fractions. ^{a,b,c,d} Values without a common letter are significantly different ($p < 0.05$). Data are presented as Mean $\pm$ SD, $n = 3$. ND, not detected. CLA, conjugated linoleic acid; GCs, gas chromatographs.

Fig. 2 Inhibitory effect of inhibitors in the conversion of α -ESA into CLA in hepatic microsomes. The hepatic microsomes were pre-incubated with inhibitors or DMSO for 5 min as described in the Materials and Methods sections. Cytochrome P450 (CYP) enzymatic activities were inhibited by the corresponding CYP-specific inhibitors (A-J) and the leukotriene B_4 12-hydroxydehydrogenase/15-ketoprostaglandin Delta 13-reductase (LTB $_4$ 12-HD/PGR) enzyme was inhibited by the cyclooxygenase (COX) inhibitors indomethacin and niflumic acid (K, L). The NADPH-cytochrome P450 reductase (CPR) activity was inhibited by CEES (M). Data are expressed as percentage of the control (no inhibitor), $n=3$. IC_{50} values are listed in Table 2. CEES, 2-chloroethyl ethyl sulfide; DMSO, dimethyl sulfoxide; α -ESA, α -eleostearic acid; CLA, conjugated linoleic acid; NADPH, nicotinamide adenine dinucleotide phosphate.

Table 2 Experimentally determined inhibition rate of inhibitors (1 mM and 0.1 mM) in the conversion of α -ESA into CLA and IC₅₀ values.

Inhibitors	Enzymes	1 mM	0.1 mM	IC ₅₀
		Inhibition ratio (%)		(mM)
Fluvoxamine	CYP1A2	18.1 ± 4.4 ^{bc}	− 0.2 ± 2.9 ^c	3.5 ± 0.6 ^b
Ticlopidine	CYP2B6	27.4 ± 5.1 ^b	− 0.2 ± 5.3 ^c	2.9 ± 0.5 ^{bc}
Montelukast	CYP2C8	20.2 ± 3.4 ^{bc}	1.0 ± 3.4 ^c	4.2 ± 0.7 ^b
Sulfaphenazole	CYP2C9	18.6 ± 2.4 ^{bc}	4.4 ± 2.8 ^c	3.8 ± 0.4 ^b
Fluconazole	CYP2C19	6.7 ± 5.7 ^{cd}	1.0 ± 3.4 ^c	—
Quinidine	CYP2D6	− 1.6 ± 2.5 ^d	0.5 ± 4.1 ^c	8.6 ± 0.6 ^a
Chlormethiazole	CYP2E1	9.9 ± 3.9 ^c	− 1.7 ± 2.7 ^c	5.3 ± 1.6 ^b
Ketoconazole	CYP3A4	6.7 ± 4.6 ^{cd}	1.6 ± 5.5 ^c	—
17-ODYA	CYP4A/B1/F	100 ± 0 ^a	20.2 ± 2.2 ^b	0.16 ± 0.02 ^d
HET0016	CYP4A/F	100 ± 0 ^a	53.2 ± 2.7 ^a	0.085 ± 0.099 ^c
Indomethacin	COX-1/2	3.9 ± 2.5 ^{cd}	0.8 ± 1.7 ^c	—
Niflumic acid	COX-2	11.6 ± 4.4 ^c	− 1.3 ± 4.6 ^c	—
CEES	CPR	28.2 ± 3.6 ^b	− 1.2 ± 1.8 ^c	2.2 ± 0.3 ^c

Data are presented as Mean ± SD, n=3.^{a,b,c,d} Values in a column without a common superscript letter are significantly different ($p < 0.05$).

fluvoxamine (CYP1A2 inhibitor), ticlopidine (CYP2B6 inhibitor), montelukast (CYP2C8 inhibitor), sulfaphenazole (CYP2C9 inhibitor), quinidine (CYP2D6 inhibitor), chlormethiazole (CYP2E1 inhibitor), 17-ODYA (CYP4A/B1/F inhibitor), and HET0016 (CYP4A/B1/F inhibitor), inhibited CLA formation at different concentrations. Furthermore, the activity of leukotriene B₄ 12-hydroxydehydrogenase/15-ketoprostaglandin Delta 13-reductase (LTB₄ 12-HD/PGR), which was thought to be involved in the conversion of α -ESA into CLA, could be inhibited by cyclooxygenase (COX) inhibitors, such as indomethacin and niflumic acid. However, the results demonstrated that either indomethacin or niflumic acid had a slight inhibitory effect on CLA formation at a high concentration (Fig. 2, Table 2). Conversely, the inhibitor CEES of cytochrome P450 reductase (CPR) required for electron transfer from NADPH to CYP significantly inhibited CLA formation with an IC₅₀ value of 2.2 ± 0.3 mM (Table 2). On the other hand, 17-ODYA and HET0016 showed a significantly higher inhibitory level than all the other inhibitors. Specifically, CLA formation was completely inhibited by 17-ODYA and HET0016 at a concentration of 1 mM. The inhibition ratio of 17-ODYA was about half that of HET0016 at 0.1 mM (20.2 ± 2.2 % versus 53.2 ± 2.7 %), and the IC₅₀ value of 17-ODYA was about two-fold that of HET0016 (0.16 ± 0.02 mM versus 0.085 ± 0.099 mM), but still significantly lower than that of other CYP1-3 inhibitors. These results thus indicate that the NADPH-dependent CPR/CYP4 electron transport system contribute to the conversion of α -ESA into CLA.

3.3 Difference in enzymatic activities and *Cyp4* mRNA expression in various tissues

By comparing the specific activity of CLA formation and the expression level of *Cyp4* family genes in tissues, we investigated the specific CYP4 enzyme involved in the conversion of α -ESA into CLA. The specific enzymatic activity of CLA formation was determined in various tissue homogenates (in a total of 10 tissues), and the activity levels from the most to the least active tissues were as follows: liver, kidney, small intestine, and pancreas, while activity was non-detected in other tissues (Fig. 3A). In addition, the mRNA expression levels of a total of 10 *Cyp4* family genes were examined to determine the tissue distribution patterns of these genes. The results of the relative mRNA expression level analysis illustrated that *Cyp4* family genes exhibited highly different tissue-divergent distribution patterns (Fig. 3B). Furthermore, *Cyp4a* subfamily isoforms were almost exclusively found in the liver and kidney. The highest expression of *Cyp4a10* and *Cyp4a12a/b* were found in the kidney, and the highest *Cyp4a14* mRNA expression was detected in the liver, while *Cyp4b1* was expressed almost entirely in kidney. *Cyp4f13* was expressed ubiquitously in various tissues with the highest expression in the liver followed by kidney, BAT, and small intestine. *Cyp4f14* and *4f15* were primarily expressed in the liver, and fairly low amounts were observed in the small intestine, brain, and kidney. *Cyp4f16*, *4f17*, and *4f18* were all expressed ubiquitously like *Cyp4f13* across tissues. *Cyp4f16* mRNA was high in both the kidney and small intestine, while *Cyp4f17* mRNA was highest in the liver and

Fig. 3 Comparison of the specific activity of CLA formation and the expression level of *Cyp4* family genes in various tissues. (A) The specific activity of α -ESA conversion into CLA in various tissues. The specific activity was normalized to tissue weight: specific activity (nmol/min /g tissue) = CLA amounts (nmol) / time taken (min) / tissue weight (g). (B) The relative expression level of *Cyp4* family genes in each tissue. The gene expression level was normalized to tissue weight and shown as fold changes relative to the corresponding *Cyp4* transcripts in the liver. Fourteen weeks old male mice were used for this experiment. Data are presented as Mean $\pm$ SD, n = 6. CLA, conjugated linoleic acid; α -ESA, α -eleostearic acid; eWAT, epididymal white adipose tissue; BAT, brown adipose tissue.

Fig. 4 Spearman's rank correlations between the specific activity of conjugated linoleic acid (CLA) formation and the relative expression level of *Cyp4* family genes in various tissues. Data points = 60.

kidney and slightly lower in BAT, eWAT, the spleen, and brain. *Cyp4f18* mRNA was most highly expressed in the spleen. These extensive analysis of *Cyp4* mRNA expression levels provides valuable information about the specific CYP4 enzymes that may be involved in CLA formation in various tissues.

3.4 Correlation analysis

The observed disparity between the tissue-divergent expression patterns of *Cyp4* genes and the alterations in the specific activities of CLA formation in tissues pointed to the necessity of a correlation study, which was used to confirm the specific CYP4 enzymes in the conversion of α -ESA into CLA. The results are displayed as the specific activity of CLA formation in 10 tissues from 6 male ICR mice versus the relative expression levels of each of the *Cyp4* family genes (Fig. 4). There were no significant correlations between the specific activities and the expression levels of the *Cyp4a10*, *4a12a/b*, *4b1*, or *4f18* genes. The best correlations were found in *Cyp4a14* and *4f13* (r_s values of 0.8611 and 0.8610, respectively) with a very high statistical significance ($p < 0.0001$). *Cyp4f14* and *4f15* had very strong positive correlations with a very high statistical significance. A moderate and a strong statistically significant positive correlation were detected in *Cyp4f16* and *Cyp4f17*, respectively. Therefore, the specific activity of CLA formation showed the most similar tissue-distribution pattern with the gene expression patterns of *Cyp4a14* and *Cyp4f13*. These results confirmed that CYP4 enzymes are involved in CLA formation, with CYP4F13 and CYP4A14 being the most relevant enzymes.

3.5 The effect of CYP-substrates on CLA formation

To further investigate the role of the most relevant

enzymes, CYP4F13 and CYP4A14, in the conversion of α -ESA into CLA, the liver microsomes were incubated simultaneously with CYP-substrates, α -ESA, and NADPH to test whether the CYP-substrates inhibit CLA formation. The results show that the yield of CLA formation varied in relation to the mole ratios of α -ESA and CYP-substrates (Figs. S3 and S4). The CLA formation was significantly inhibited by prostaglandin A_1 (PGA₁) even at a low concentration (Table 3 and Fig. S3), while lauric acid showed no inhibition of CLA formation based on the comparison between CYP-substrate groups (Table 3). The other CYP-substrates were proved to have a significant inhibitory effect only at a high concentration. PGA₁, the substrate of CYP4F, appeared to be the most potent substrate, while lauric acid, the substrate of CYP4A and CYP4B1, was the least potent substrate. Taken together, these results suggested that the α -ESA saturase that could convert α -ESA into CLA belongs to the CYP4F subfamily of enzymes, rather than the CYP4A or CYP4B subfamily of enzymes. Furthermore, the results of the correlation analysis described above confirmed that CYP4F13 is most suitable candidate enzyme among CYP4F subfamily involved in the conversion of α -ESA into CLA.

4 Discussion

In this study, we demonstrated that the conversion of α -ESA into CLA occurs through an NADPH-dependent enzymatic reaction. Furthermore, the highest specific activity of CLA formation was detected in the liver, followed by the kidney, small intestine, and pancreas (Figs. S1-2 and Fig. 3A). These results are consistent with an earlier study conducted with rats by our laboratory¹⁸. Moreover, we de-

Table 3 Inhibitory effect of various CYP-substrates in the conversion of α -ESA into CLA.

<div>CLA (% of control)</div> <div>Substrates</div>	<div>Mole Ratio</div> <div>(α-ESA : CYP-Substrate)</div>		Relevant CYP Enzymes
	1:1	1:4	
DMSO#	100.0 $\pm$ 10.1 ^a	100.0 $\pm$ 1.3 ^a	
Lauric Acid (C12:0)	98.2 $\pm$ 3.0 ^a	90.8 $\pm$ 3.4 ^{ab}	CYP4A, CYP4B1
Palmitic Acid (C16:0)	91.2 $\pm$ 1.8 ^a	78.9 $\pm$ 1.0 ^{bc}	CYP1-3, CYP4F
Stearic Acid (C18:0)	93.4 $\pm$ 7.1 ^a	78.5 $\pm$ 3.8 ^{bc}	CYP1-3, CYP4F
Oleic Acid (C18:1)	88.8 $\pm$ 4.3 ^a	77.3 $\pm$ 8.7 ^c	CYP1-3, CYP4F
Linoleic Acid (C18:2)	92.4 $\pm$ 3.2 ^a	74.7 $\pm$ 3.0 ^c	CYP1-3, CYP4F
Linolenic Acid (C18:3)	96.8 $\pm$ 3.4 ^a	76.6 $\pm$ 4.9 ^c	CYP1-3, CYP4F
Arachidonic Acid (C20:4)	98.9 $\pm$ 3.0 ^a	76.4 $\pm$ 2.1 ^c	CYP4A10, CYP4A12A/B, CYP4F
Prostaglandin A ₁ (C ₂₀ H ₃₂ O ₄)	87.8 $\pm$ 7.3 ^b	73.6 $\pm$ 4.8 ^c	CYP4F

Notes: #, all the substrates were dissolved in DMSO which is also taken as a control. ^{a,b,c} Values in a column without a common superscript letter are significantly different ($p < 0.05$). Data are presented as Mean $\pm$ SD, n=3.

terminated the subcellular distribution of α -ESA saturase in the liver. Our results demonstrate that α -ESA saturase was abundant in microsomes, but absent in the cytosol. Liver microsomes contain the major drug-metabolizing enzymes CYP and UDP-glucuronosyltransferase (UGT), along with other enzymes that contribute to drug metabolism³⁷⁾, which support our hypothesis that α -ESA saturase should be classified as an enzyme for drug metabolism. To test this hypothesis, CYP-specific inhibitors were selected to validate the effect of inhibitors on CLA formation in hepatic microsomes. The results showed that the inhibitors of CYP 1³⁸⁾, 2^{39, 40)}, and 3⁴¹⁾ family enzymes had minimal or modest inhibitory activities. However, CYP1-3 contain major xenobiotic-metabolizing enzymes responsible for the metabolism of the majority of drugs and other xenobiotics^{42, 43)}, while CYP4 enzymes typically metabolize fatty acids^{29, 44)}. According to this observation, CYP1-3 are unlikely to play a role in the metabolism of α -ESA, which is a type of conjugated triene fatty acid, and the slight inhibitory effect of CYP1-3 inhibitors could be due to a low specificity^{43, 45)}. In contrast, CYP4 inhibitors, 17-ODYA and HET0016^{44, 46)}, showed the highest level of inhibition (Fig. 2), which also indicated that CYP4 enzymes are involved in CLA formation. In addition, the CPR inhibitor of CEES⁴⁷⁾ also showed a significant inhibition of CLA formation. All these findings suggested that the CPR/CYP4 electron transport system is involved in the conversion of α -ESA into CLA.

We also noticed that the conversion of α -ESA into CLA is similar to that in the metabolic pathway of eicosanoids, where leukotriene B₄ (LTB₄), prostaglandin E₂ (PGE₂), and lipoxin A₄ (LXA₄) are the dominant eicosanoids. In the LTB₄ metabolic pathway, LTB₄ is oxidized by LTB₄ 12-HD/PGR to 12-oxo-LTB₄, and then the double bonds at C10-C11 and C14-15 are reduced to 10,11,14,15-tetrahydro-12-oxo-LTB₄ by an unknown reductase(s). Additionally, PGE₂ and LXA₄ are oxidized to 15-oxo-prostaglandin E₂ (15-oxo-PGE₂) and 15-oxo-lipoxin A₄ (15-oxo-LXA₄), respectively; LTB₄ 12-HD/PGR subsequently reduces 15-oxo-PGE₂ to 13,14-dihydro-15-oxo-PGE₂ and 15-oxo-LXA₄ to 13,14-dihydro-15-oxo-LXA₄ in the presence of NADPH⁴⁸⁾. In addition, Clish *et al.*^{49, 50)} have reported that LTB₄ 12-HD/PGR is a member of the zinc-independent medium chain dehydrogenase/reductase family, which exhibits high reductase activity toward double bonds in several xenobiotics. Therefore, we speculated that the conversion of α -ESA into CLA proceeds through the LTB₄ metabolic pathway, in which the double bond of α -ESA is saturated by the unknown reductase(s), or the PGE₂ and LXA₄ pathways, in which the double bond is directly reduced by LTB₄ 12-HD/PGR. However, our study showed that the LTB₄ 12-HD/PGR inhibitors, indomethacin and niflumic acid⁴⁹⁾, caused only a slight inhibitory effect on CLA formation, thereby indicating that LTB₄ 12-HD/PGR does not contribute to CLA formation. Moreover, Itoh *et al.*⁵¹⁾ have reported that LTB₄ 12-HD/PGR from

male Wistar rat liver was predominantly localized in the cytosolic fraction, and was absent in the microsomal fraction. In contrast, the α -ESA saturase activity was highest in the microsomal fraction and absent in the cytosolic fraction in this study (Fig. 1). This discrepancy also suggests that LTB₄ 12-HD/PGR is unlikely to play a role in the conversion of α -ESA into CLA.

In our previous studies, we have shown that α -ESA and PA were converted into c9,t11-CLA, JA was converted into c8,t10-CLA in rats, and the conversion ratio of α -ESA was higher than that of PA and JA. These results indicate that the double bond distal to the carboxyl group in the carbon chain of the conjugated triene acid is selectively saturated in this reaction and the variety in the conversion ratios may be attributed to the substrate-specificity of the saturase. Furthermore, these findings suggested that CYP-substrates could be utilized to determine which specific CYP4 enzyme catalyzed α -ESA metabolism. Lauric acid, a specific substrate of CYP4A enzymes⁴⁴⁾, also preferentially catalyzed by the rabbit CYP4B1 enzyme⁵²⁾ but not the CYP4F enzymes, had no inhibitory effect on CLA formation. Moreover, there were no statistically significant differences in the inhibitory effects between palmitic acid (C16:0), C18 carbon fatty acids (non-conjugated fatty acids with the same number of carbon atoms as α -ESA but with a variety in the number of double bond), and arachidonic acid (C20:4). This finding suggests that the number of carbon atoms and the non-conjugated double bond have no effect on the substrate-specific effects of α -ESA saturase. In addition, we found no inhibitory effect from the other CYP4F substrates at low concentrations except for PGA₁, which is one of the "classical" substrates of CYP4F enzymes, since other CYP4F-substrates could also be catalyzed by other CYP family enzymes besides CYP4F.

Taken together, these findings led us to select CYP4F isoforms as α -ESA saturase, consistent with previous reports showing that CYP4As metabolize intermediate-chain fatty acids (fatty acids with C10 to 16 carbon chain)²⁹⁾, while CYP4Fs catalyze long-chain fatty acids (C16 to 26)⁵³⁾. However, we could not determine the specific CYP4F enzyme involved due to a lack of a selective marker substrate for the activity of individual CYP4F enzymes in the study of the effect of CYP-substrates on CLA formation, although PGA₁ could be used as a nonselective marker substrate for CYP4F enzymes. Nonetheless, the correlation analysis showed that *Cyp4a14* and *4f13* had the best correlation with a very high statistical significance. After a comprehensive evaluation of these findings, we concluded that CYP4F13 was the primary enzyme involved in CLA formation. Conversely, CYP4B1 was predominantly expressed in extrahepatic tissues and has been reported to preferentially metabolize short-chain fatty acids (approximately C7 to 10)⁵²⁾, suggesting that CYP4B1 does not contribute to CLA formation. This is also verified by the results

of the correlation study.

CYP enzymes almost always act as monooxygenases, or mixed-function oxidases by inserting one atomic oxygen into the substrate. The stoichiometry of the oxidation reaction can be written as: $R-H + NADPH + O_2 \rightarrow ROH + H_2O + NADP^+$ ⁵⁴⁾. The CYP4 family plays a major role in the metabolism of fatty acids, in most cases through oxidation of fatty acids, including epoxidation and hydroxylation. Unlike the well-established oxidation reaction, the reduction reaction catalyzed by CYP has not been characterized in detail. The stoichiometry is written as: $R = X + NADPH + H^+ \rightarrow RH - XH + NADP^+$ ⁵⁵⁾. This reaction is mostly seen when the substrates contain quinones, azo-, halogenated, nitro-, N-hydroxy-, and hydroperoxide functional groups. Amunom *et al.*^{55, 56)} have reported that α, β -unsaturated aldehydes (9-anthracene aldehyde and 4-hydroxy-trans-2-nonenal) are reduced/hydrogenated to their corresponding carboxylic acid by several human and murine CYP enzymes. They also identified that this CYP-dependent reduction occurred in the presence and absence of molecular oxygen. Furthermore, replacement of the normal ambient air with carbon monoxide did not affect the reaction but significantly inhibited CYP-dependent oxidation. We also found that there was no significant difference in the specific activity of CLA formation in tissues determined under ambient air conditions and under nitrogen flow (weak anaerobic) conditions (data not shown). The CLA formation reaction would be more similar to CYP-dependent reduction rather than CYP-dependent oxidation in terms of molecular oxygen requirement. Although, to our knowledge, there have been no previously documented reports that unsaturated fatty acids can be reduced/hydrogenated by CYP, the precedent aldehyde group reduction suggests that there may be other functional groups, such as conjugated double bonds in fatty acids, which also can be reduced by CYP.

Mice have 102 CYP enzymes and many of the recently identified CYP enzymes are still considered "orphans" with no known functions⁵⁷⁾. In particular, the physiological and metabolic functions of the CYP4F subfamily have not been elucidated, only the catalytic activities of CYP4F14 and 4F18 are known, while other enzymes remain to be characterized⁵⁸⁾. From our results we inferred that CYP4F13 is the major enzyme responsible for the conversion of α -ESA into CLA, indicating that there may be a novel reduction reaction pathway for fatty acid metabolism in the liver, besides epoxidation and hydroxylation by CYP. This reduction reaction may be a unique function of CYP4Fs which remains to be fully understood. Thus, it will be of great interest to further examine the possible metabolic and functional effects of CYP4F *in vivo* at the molecular and physiological levels to extend our knowledge of CYP4F functions. As part of future research efforts, we intend to isolate and purify the CYP4F13 enzyme from mouse liver

to provide direct evidence of the α -ESA to CLA conversion reaction.

5 Conclusion

In summary, to our knowledge, the present study would be the first to characterize CYP4F13 as the major enzyme responsible for the conversion of α -ESA into CLA. Whether it plays a role in the conversion of other CLnA, such as PA and JA, into CLA remains to be explored.

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Supporting Information

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