Ximenynic Acid Regulation of n-3 PUFA Content in Liver and Brain

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Keywords

Ximenynic acid · Sandalwood seed oil ·
Fatty acid metabolism · Fatty acid desaturase ·
Stearoyl-CoA desaturase

Abstract

Background/Aims: Ximenynic acid is a rare conjugated enyne fatty acid found primarily in plants in the Santalaceae family. It has been reported that sandalwood seed oil (SWSO) affects fatty acid metabolism in animal studies; however, the effects of pure ximenynic acid remain unclear. The present study aimed to study the impact of SWSO and ximenynic acid on n-3 fatty acid metabolism in the liver and brain.

Methods: Thirty C57BL/6 male mice aged 4 weeks were fed SWSO (1.0 mL/20 g bodyweight), olive oil (OO), or a combination of SWSO and OO ($n = 10/\text{group}$) for 8 weeks. Liver and brain fatty acid compositions were determined using gas chromatography. HepG2 cells were treated with up to 150 μM ximenynic acid and oleic acid for 48–72 h. The expression and abundance of genes and proteins relevant to n-3 fatty acid metabolism pathways were investigated. **Results:** The intake of SWSO in mice elevated the levels of total n-3 fatty acids and decreased total n-9 fatty acids in the liver ($p < 0.05$) compared with the OO group. In contrast, total

n-3 fatty acids were significantly decreased in the brain ($p < 0.05$). HepG2 cells treated with ximenynic acid for 48 h showed significant reductions in n-9 fatty acids and docosahexaenoic acid (C22:6n-3) ($p < 0.05$) compared with HepG2 cells treated with oleic acid. In HepG2 cells, stearoyl-CoA desaturase (SCD) and fatty acid desaturase 2 (FADS2) gene expression, as well as FADS2 protein expression, were significantly down-regulated after a 72-h incubation with 150 μM of ximenynic acid compared with the vehicle ($p < 0.05$). **Conclusion:** Ximenynic acid may regulate fatty acid metabolism by suppressing the expression of key enzymes of lipid metabolism. In contrast, SWSO, which has a high level of C18:3n-3, positively affected n-3 fatty acid synthesis in mouse liver compared to pure ximenynic acid. We hypothesize that a high level of precursor C18:3n-3 in SWSO promotes the endogenous synthesis of C22:6n-3 despite the presence of ximenynic acid.

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Introduction

Ximenynic acid [*trans*-11-octadecen-9-ynoic acid, 18:2 (9a, 11t)] is a natural conjugated enyne fatty acid that is primarily found in the seed oils of plants in the Santa-

laceae family [1]. Sandalwood seed kernels are a valuable part of the traditional Australian aboriginal diet [2] and have been used as a topical treatment for skin lesions [3] and an oral treatment for rheumatoid arthritis [4]. The seed contains a high amount of drying fixed oil (50–60%), which is characterized by a high percentage of unusual acetylenic fatty acids such as *trans*-ximenynic acid [5]. Acetylenic fatty acids have been proven to inhibit specific enzymes involved in prostaglandin synthesis and fatty acid metabolism [6–8]. Recently, acetylenic fatty acids have generated considerable interest due to their potential bioactivity as lysozyme inhibitors and their involvement in fatty acid metabolism [9].

Previous reports have displayed that the fatty acid compositions of mouse liver and adipose tissue were markedly altered following the consumption of sandalwood seed oil (SWSO) [10, 11]. Specifically, ximenynic acid metabolism was studied in mice fed a diet enriched with SWSO (15% SWSO, 5.2% [w/w] fat) [10]. Interestingly, mice consuming SWSO showed markedly lower levels of arachidonic acid in the liver (C20:4n-6) and higher levels of docosahexaenoic acid (C22:6n-3). Similarly, another study showed that rats consuming a diet enriched with SWSO (8% SWSO, the fat percentage in the diet was not mentioned) had increases in total n-3 polyunsaturated fatty acids (PUFA), C22:6n-3, and the n-6:n-3 PUFA ratio [11]. Together, these previous studies suggest that ximenynic acid in SWSO may regulate n-3 fatty acid synthesis.

α -Linolenic acid (C18:3n-3) is an essential fatty acid that must be ingested from the diet. C18:3n-3 is the precursor fatty acid for longer-chain (20- and 22-carbon) n-3 PUFA [12]. This is relevant given that C22:6n-3 is critically essential for brain and retinal development [13]. C22:6n-3 can be obtained directly from the consumption of marine and animal food but can also be endogenously synthesized from C18:3n-3 by several desaturation and elongation reactions [14]. Fatty acid desaturase 2 (FADS2) is the rate-limiting desaturase for the synthesis of C22:6n-3 from C18:3n-3 [15]. It is demonstrated that n-3 PUFA can prevent and improve many chronic diseases, such as cardiovascular diseases [16], type 2 diabetes [17, 18], malignant tumors [19], Alzheimer disease [20], and inflammatory diseases [21].

The fatty acid composition of SWSO revealed that C18:1n-9 is the predominant fatty acid (~52% of total fatty acids), followed by ximenynic acid (~31% of total fatty acids) [22], while other fatty acids were present as minor components. Differences in fatty acid profiles of SWSO have been observed; however, the variation dem-

Table 1. Composition of basic feed for mice

Nutritional constituents	Content
Water, %	10
Crude protein, %	20
Crude fat, %	4.7
Crude fiber, %	3.3
Crude ash, %	6
Nitrogen-free extract, %	54
Calcium, %	1.1
Total phosphorus, %	0.73
Calories, kcal/100 g	348

onstrated by different authors is minimal and most likely insignificant. The goal of the present study was to investigate the effect of both SWSO and pure ximenynic acid on n-3 fatty acid metabolism using both in vivo (mice) and in vitro (HepG2) models.

Materials and Methods

Reagents and Fatty Acids

Ximenynic acid (98%) was extracted according to a previously published method [5]. C18:1n-9 and oil-red-O solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fatty acid methyl ester (FAME) standards were purchased from Sigma-Aldrich. The cDNA synthesis kit was acquired from Roche Diagnostics GmbH (Mannheim, Germany). The total RNA extraction kit was bought from Omega Bio-Tek (Norcross, GA, USA). The FADS2 antibody (CAT#: ab72189) was purchased from Abcam Biotechnology (Cambridge, MA, USA), and GAPDH antibody (CAT#: 2118s) and anti-rabbit IgG, HRP-linked antibody (CAT#: 7074) were purchased from Cell Signaling Technology (Danvers, MA, USA). SsoFast™ EvaGreen® Supermix was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All reagents were of analytical or HPLC grade.

Animals and Diets

Four-week-old C57BL/6 male mice were obtained from the Laboratory Animal Center, Zhejiang University (Hangzhou, China). Mice were housed in a specific pathogen-free animal laboratory under constant temperature and humidity, and with a 12-h dark:12-h light cycle. Mice had ad libitum access to water and a basic feed (SLaCCaS, Shanghai, China), which was comprised of 57.3% carbohydrates, 20% proteins, and 4.7% fat (Table 1). After 7 days of adaptation to the basic feed, mice were divided into 3 groups ($n = 10/\text{group}$): olive oil (OO), SWSO, or a 1:1 mixture of OO and SWSO (SO). Because SWSO contains a high amount of ximenynic acid, which is highly oxidizable, oils were administered by oral gavage (1 mL/20 g body weight) once every 2 days for 8 weeks. This ensured that we could control the energy and fat intake of mice. At the end of the 8-week feeding period, mice were fasted overnight and then sacrificed after anesthesia (10% chloral hy-

drate). Tissue samples were collected from anesthetized mice and stored at -80°C until analysis.

Cell Culture

The human liver HepG2 cell line was available from the Type Culture Collection of the Chinese Academy of Sciences (TCCAS, Shanghai, China). Cells were cultured in complete culture medium that was changed every 2 days until cells reached 80% confluence. Cells were serum starved for 24 h with fatty acid-free media prior to treatment with several different concentrations of ximenynic acid or C18:1n-9 (0–150 μM) for 48–72 h. The fatty acids used for in vitro treatments were first dissolved in DMSO, then in fatty acid-free bovine serum albumin (BSA) with a molar ratio of 4:1, and later dissolved in fatty acid-free medium. We previously ran a toxicity test [8] and found that the IC_{50} for ximenynic acid in HepG2 cells was about 194.2 μM after 72 h. Therefore, the concentrations employed in the present study were deemed nontoxic.

Complete culture medium was composed of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, CA, USA), 10% fetal bovine serum (FBS; Biowest, Nuaille, France), antibiotics (100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin), 0.11 g/L sodium pyruvate, 4 mM L-glutamine, and 10 mM HEPES (Corning Inc., New York, NY, USA). Fatty acid-free medium contained 1% insulin-transferrin-selenium (Invitrogen, CA, USA), 100 mg/mL fatty acid-free BSA (MP Biomedicals, CA, USA), antibiotics (100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin), 0.11 g/L sodium pyruvate, 4 mM L-glutamine, and 10 mM HEPES (Corning Inc.). The vehicle medium was fatty acid-free medium supplemented with DMSO and BSA with the same concentration of the experimental medium.

Oil-Red-O Staining

Cells were seeded in 24-well plates with 20,000 cells/well and incubated overnight. The next day, the cell monolayer was washed with phosphate-buffered saline (PBS) and treated with fatty acids. After incubation for 48 h, cells were gently washed with PBS and fixed with 10% formaldehyde for 30 min. Cells were gently rinsed again to eliminate formaldehyde and stained with oil-red-O working solution for 30 min. Excess oil-red-O solution was removed by sequential rinses in PBS. Images were acquired by an inverted microscope (Olympus, Tokyo, Japan).

Fatty Acid Composition Analysis

Cells were washed twice with ice-cold PBS, scraped on ice into a glass tube, and then centrifuged (2,000 rpm, 10 min, 4°C). After removing the supernatant, 5 mL of a chloroform-methanol (2:1 v/v) solution containing 0.005% butylated hydroxyl toluene (BHT) was added. The solution was vortexed vigorously and then kept at -20°C for 24 h. The solution was filtered into a new glass tube and then dried under nitrogen in a 50°C water bath. Next, 4 mL of sulfuric acid in methanol (0.9 mol/L) and 1 mL methylbenzene were added, and the samples were methylated at 70°C for 2 h. Subsequently, 2 mL hexane and 1 mL saline were added to each sample prior to centrifugation (2,000 rpm, 10 min, 4°C); the upper phase was removed, placed in a new glass tube, and then washed with distilled water and dried with anhydrous sodium sulfate. FAME derivatives were purified with a SupelcleanTM LC-Si SPE Tube (Sigma-Aldrich). The FAME cleaning solution was then dried under nitrogen and resuspended in 50 μL hexane.

Table 2. Primer sequences for quantitative RT-PCR

Primers	Primer sequences
FADS1	Forward 5'-GCAAAGACCCAGACATCAAC-3'
	Reverse 5'-GAAGCGGACGTAGAAGGTAATC-3'
FADS2	Forward 5'-CCTTTGTCCTTGCTACCTCTC-3'
	Reverse 5'-TCCACTTGGGTTTTCTGTAGAC-3'
SCD	Forward 5'-GACCTAGAAGCTGAGAACTGG-3'
	Reverse 5'-GCAAGAAAGTGGCAACGAAC-3'
ELOVL2	Forward 5'-CTGCGGATCATGGAACATCT-3'
	Reverse 5'-TGTTACCCAGCCATATTGAGAG-3'
ELOVL5	Forward 5'-ACCCACATTTATCTGCTCTGTC-3'
	Reverse 5'-ATTTGCCTTCCATACTCTCG-3'
ELOVL6	Forward 5'-GGAAGCAGACAGGAGAACAC-3'
	Reverse 5'-CATACAGAGCAGAAAACAGGAAAG-3'
RPLP0	Forward 5'-GAAACTCTGCATTCTCGCTTC-3'
	Reverse 5'-GGTGAATCCGTCTCCACAG-3'

RPLP0 is the reference gene.

Liver and brain tissue samples (100 mg) were ground on dry ice, transferred to tubes with 5 mL chloroform-methanol (2:1 v/v) containing 0.005% BHT and kept at -20°C for 24 h. The isolation of FAME from mouse liver and brain samples used the same protocol as that detailed above for cells.

FAME were analyzed using a GC2014 capillary GC/FID gas chromatograph (Shimadzu, Kyoto, Japan) with a DB-23 capillary column (60 m $\times$ 0.25 mm i.d., 0.25- μm film thickness; Agilent Technologies, Palo Alto, CA, USA). Data were analyzed using a N2010 chromatography data system (Zhida Information Technologies, Inc., Hangzhou, China). Fatty acid data are reported as a percentage of total fatty acids.

Quantitative Real-Time PCR

Total RNA was extracted from cultured cells using the HP total RNA kit according to the manufacturer’s protocol (Omega Bio-Tek). RNA purity and concentration were determined using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Next, cDNA was transcribed from 1 μg total RNA by reverse transcriptase according to the manufacturer’s instructions (transcriptor first-strand cDNA synthesis kit; Roche, Mannheim, Germany). Real-Time-PCR was performed using the SsoFastTM EvaGreen[®] Supermix and a CFX96TM real-time PCR detection system. Relative gene expression data were determined using Bio-Rad CFX Manager 1.5 software and the $2^{-\Delta\Delta\text{C}_\text{T}}$ method. RPLP0 was used as the housekeeping gene. Primer sequences are listed in Table 2.

Western Blotting Analysis

Cultured cells were washed twice with ice-cold PBS and scraped into a tube with cell lysis buffer containing PMSF (phenylmethanesulfonyl fluoride; Beyotime, Haimen, China) on ice. The cell protein supernatant was obtained by centrifugation (14,000 rpm, 10 min, 4°C). Protein concentration was determined with a bicin-

Table 3. Fatty acid composition of experimental oils

Fatty acid	SWSO	OO	SO
C16:0	3.62	10.04	7.01
C18:0		2.91	2.19
C18:1n-9	57.82	79.60	68.76
C18:2n-6	1.21	5.57	3.58
C18:3n-3	4.15	0.61	2.29
C20:0		0.35	0.20
C20:1n-9		0.21	0.31
C20:3n-6	0.23		
Ximenynic acid	32.92		15.66
C22:0		0.09	

SWSO, sandalwood seed oil; OO, olive oil; SO, SWSO + OO.

chonic acid protein assay (Beyotime). Protein lysates (20 µg) were boiled with loading buffer for 5 min and then separated by SDS-PAGE. Protein was transferred to PVDF (polyvinylidene difluoride) membranes and then blocked with 5% skim milk in a PBST solution. Primary antibodies FADS2 (dilution: 1:700) and GAPDH (dilution: 1:1,000) were incubated with the above membranes for 1 h under agitation and then washed with PBST solution for 15 min 3 times under agitation. Following primary antibody incubation, the secondary antibody anti-rabbit IgG, HRP-linked antibody (dilution: 1:1,000) was added to the PVDF membranes for 1 h and washed with PBST solution as above. Immunoreactive bands were detected by electrochemiluminescent detection reagent, and the data were analyzed with Quantity One software (Bio-Rad).

Statistical Analysis

Data were analyzed using independent-sample *t* tests or one-way analysis of variance (ANOVA) using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as means ± SD. A value of *p* < 0.05 was regarded as statistically significant.

Results

Effects of SWSO on Mouse Hepatic Fatty Acid Composition

SWSO (*Santalum spicatum* R.Br.) contains high levels of C18:1n-9 (~58%) and ximenynic acid (~33%). Consequently, we chose to use OO (~80% C18:1n-9) as the control oil. The SO treatment consisted of homogeneous mixture of an equal amount of SWSO and OO. The fatty acid compositions of the experimental oils are shown in Table 3.

There were no significant differences in individual saturated fatty acids (SFA) or total SFA in the livers between any of the experimental groups (Table 4). Intriguingly,

Table 4. Effects of different oil feeds on total fatty acid composition in mouse liver

Fatty acid	OO	SO	SWSO
C14:0	0.21±0.05 ^a	0.2±0.09 ^a	0.17±0.03 ^a
C15:0	0.16±0.04 ^a	0.19±0.12 ^a	0.1±0.09 ^a
C16:0	18.44±1.46 ^a	17.83±1.19 ^a	19.14±1.45 ^a
C17:0	0.17±0.03 ^a	0.17±0.04 ^a	0.19±0.03 ^a
C18:0	5.44±1.12 ^a	5.28±0.76 ^a	5.32±1.04 ^a
C20:0	0.08±0.07 ^a	0.08±0.06 ^a	0.06±0.09 ^a
C22:0	0.15±0.03 ^a	0.17±0.03 ^a	0.16±0.05 ^a
C24:0	0.14±0.05 ^a	0.13±0.06 ^a	0.17±0.15 ^a
Total SFA	24.8±2.63 ^a	24.05±1.88 ^a	25.31±2.41 ^a
C16:1n-7	1.05±0.21 ^b	1.37±0.26 ^a	1.62±0.33 ^a
C17:1n-7	0.48±0.2 ^a	0.78±0.51 ^a	0.15±0.03 ^b
C18:1n-9	21.82±2.23 ^a	20.99±3.01 ^{ab}	18.76±1.68 ^b
C20:1n-9	0.27±0.05 ^a	0.25±0.03 ^a	0.24±0.02 ^a
C22:1n-9	1.11±0.4 ^a	1.03±0.36 ^a	1.26±0.39 ^a
C24:1n-9	0.28±0.06 ^a	0.25±0.08 ^a	0.23±0.11 ^a
Total n-9 MUFA	23.45±1.94 ^a	22.53±2.81 ^{a,b}	20.52±1.44 ^b
Total MUFA	24.97±2.06 ^a	24.67±2.94 ^a	22.29±1.63 ^b
C18:2n-6	26.26±1.8 ^a	25.67±1.93 ^a	25.11±1.99 ^a
C18:3n-6	0.43±0.12 ^{a,b}	0.44±0.08 ^a	0.34±0.07 ^b
C20:2n-6	0.15±0.06 ^b	0.16±0.03 ^b	0.23±0.04 ^a
C20:3n-6	0.74±0.11 ^c	1.08±0.21 ^b	1.39±0.23 ^a
C20:4n-6	8.26±0.89 ^a	7.98±1.39 ^a	8.26±1.17 ^a
C22:4n-6	0.15±0.08 ^a	0.11±0.03 ^b	0.09±0.02 ^b
Total n-6 PUFA	35.99±1.02 ^a	35.43±1.61 ^a	35.41±1.24 ^a
C18:3n-3	1.03±0.21 ^a	1.1±0.2 ^a	1.15±0.28 ^a
C20:5n-3	1.18±0.21 ^a	1.01±0.19 ^a	0.94±0.22 ^a
C22:5n-3	0.71±0.1 ^a	0.87±0.11 ^a	0.92±0.09 ^b
C22:6n-3	11.25±1.21 ^b	12.34±1.43 ^{a,b}	13.1±0.88 ^a
Total n-3 PUFA	14.17±1.34 ^a	15.31±1.35 ^{a,b}	16.12±0.96 ^b
Ximenynic acid	nd	0.6±0.19 ^b	0.85±0.22 ^a
n-6/n-3	2.56±0.19 ^b	2.33±0.19 ^a	2.2±0.17 ^a

Values represent mean % ± SD. *n* = 10. For each fatty acid, values sharing the same superscript letter indicate no significant difference, as determined by one-way analysis of variance (*p* < 0.05). nd, not detected; SWSO, sandalwood seed oil; OO, olive oil; SO, SWSO + OO.

the livers of mice in the SWSO group had significantly higher levels of C16:1n-7 (*p* < 0.05), but lower levels of C18:1n-9 (*p* < 0.05), total n-9 monounsaturated fatty acids (MUFA) (*p* < 0.05) and total MUFA (*p* < 0.05) compared to the OO group. Other MUFA, such as C20:1n-9, C22:1n-9, and C24:1n-9, were not different between the SWSO and the OO group.

For PUFA, C20:2n-6 and C20:3n-6 were significantly higher in the SWSO group than the OO group (*p* < 0.05), while C22:4n-6 was markedly lower. There were no significant differences in total n-6 PUFA between any of the experimental groups.

While C18:3n-3 and C20:5n-3 were similar between the SWSO group and the OO group, levels of C22:5n-3, C22:6n-3, and total n-3 PUFA were significantly higher compared to the OO group ($p < 0.05$). The n-6:n-3 ratio was lower in the SWSO group than the OO group. It is possible that the n-3 PUFA level was in response to the concentration of SWSO after feeding mice (Table 4).

Effects of SWSO on Mouse Brain Fatty Acid

Composition

The fatty acid composition of mouse brain was not the same as that observed in hepatic tissue following the experimental treatments (Table 5). Compared to the OO and the SO group, there were no significant differences in the SWSO group in most SFA and MUFA, total n-9 MUFA, and total MUFA. C20:3n-6 was significantly higher in the SWSO group than the OO group ($p < 0.05$), but there was no significant difference in most n-6 PUFA and total n-6 PUFA levels between the SWSO and the OO group. C22:5n-3 was significantly higher in the SWSO group than the OO group ($p < 0.05$). Total n-3 PUFA in the SWSO group was markedly lower than in the OO and the SO group ($p < 0.05$), and the n-6:n-3 ratio was higher than in the OO group.

Free Fatty Acid Uptake by HepG2 Cells

It has previously been reported that ximenynic acid can be taken up by the liver (and many other tissues) of mice and rats [7, 8], but little is known about its uptake in human tissue. HepG2 cells were treated with oleic acid or ximenynic acid for 48 h at different concentrations (ranging from 0 to 150 μM) in fatty acid-free conditions. As shown in Figure 1, the number of intracellular lipid droplets increased in accordance with fatty acid concentration compared to the vehicle, especially in ximenynic acid-treated cells. This suggests that ximenynic acid was taken up by HepG2 cells and may regulate triglyceride synthesis in human liver cells.

Effects of Ximenynic Acid on HepG2 Fatty Acid

Composition

After a 48-h incubation with either ximenynic acid or oleic acid, the lipid composition of HepG2 cells was analyzed by gas chromatography (Table 6). As expected, the relative levels of cellular ximenynic acid and oleic acid increased in accordance with increasing concentrations. Compared to the vehicle, C14:0, C16:0, C18:0, and total SFA levels were significantly reduced with increasing concentrations of both ximenynic acid and oleic acid ($p < 0.05$). C18:1n-9, C20:1n-9, total n-9 MUFA, and total

Table 5. Effects of different oil feeds on total fatty acid composition in mouse brain

Fatty acids	OO	SO	SWSO
C14:0	0.15±0.04 ^a	0.11±0.03 ^a	0.11±0.02 ^a
C15:0	0.07±0.02 ^b	0.05±0.01 ^{ab}	0.04±0.03 ^a
C16:0	17±1.15 ^a	16.53±1.47 ^a	17.24±1.69 ^a
C17:0	0.14±0.02 ^a	0.12±0.02 ^a	0.13±0.01 ^a
C18:0	16.98±0.95 ^a	16.42±1.07 ^a	17.83±1.52 ^a
C20:0	0.38±0.16 ^a	0.34±0.16 ^a	0.42±0.17 ^a
C22:0	0.41±0.16 ^a	0.41±0.24 ^a	0.43±0.12 ^a
C24:0	0.7±0.3 ^a	0.56±0.22 ^a	0.76±0.25 ^a
Total SFA	35.82±1.59 ^a	34.54±2.16 ^a	36.96±2.87 ^a
C16:1n-7	0.48±0.05 ^a	0.52±0.04 ^a	0.51±0.08 ^a
C17:1n-7	3.19±0.32 ^a	3.42±0.54 ^a	3.2±0.34 ^a
C18:1n-9	18.7±2.48 ^a	17.98±2.91 ^a	18.92±2.02 ^a
C20:1n-9	2.23±0.95 ^a	2.1±1.13 ^a	2.23±0.79 ^a
C22:1n-9	2.13±0.56 ^a	1.97±0.63 ^a	2.27±0.68 ^a
C24:1n-9	1.95±0.81 ^a	1.87±0.8 ^a	2.28±0.72 ^a
Total n-9 MUFA	25.02±4.63 ^a	23.93±4.77 ^a	25.7±3.21 ^a
Total MUFA	28.69±4.67 ^a	27.86±4.99 ^a	29.4±3.39 ^a
C18:2n-6	0.95±0.09 ^a	0.82±0.08 ^a	0.93±0.34 ^a
C20:2n-6	0.24±0.11 ^a	0.24±0.11 ^a	0.2±0.08 ^a
C20:3n-6	0.55±0.04 ^a	0.62±0.05 ^b	0.63±0.04 ^b
C20:4n-6	10.2±2.09 ^a	10.65±2.3 ^a	9.58±1.21 ^a
C22:4n-6	2.55±0.27 ^a	2.65±0.42 ^a	2.43±0.23 ^a
Total n-6 PUFA	14.49±2.17 ^a	14.98±2.53 ^a	13.77±1.18 ^a
C20:5n-3	0.11±0.06 ^a	0.14±0.1 ^a	0.19±0.15 ^a
C22:5n-3	0.2±0.02 ^a	0.27±0.05 ^b	0.27±0.04 ^b
C22:6n-3	20.13±1.77 ^a	21.98±2.29 ^b	19.11±1.03 ^a
Total n-3 PUFA	23±1.95 ^b	22.39±2.31 ^b	19.57±0.95 ^a
Ximenynic acid	nd	0.13±0.04 ^a	0.19±0.05 ^b
n-6/n-3	0.63±0.05 ^a	0.67±0.06 ^{a, b}	0.70±0.05 ^b

Values represent mean % $\pm$ SD. $n = 10$. For each fatty acid, values sharing the same superscript letter indicate no significant difference (one-way analysis of variance) ($p < 0.05$). nd, not detected; SWSO, sandalwood seed oil; OO, olive oil; SO, SWSO + OO.

MUFA levels were reduced by ximenynic acid in a dose-dependent manner ($p < 0.05$) compared to the vehicle and oleic acid groups. In contrast, C20:1n-9, total n-9 MUFA, and total MUFA levels were markedly increased with oleic acid treatment compared to the vehicle ($p < 0.05$).

C18:2n-6, C20:4n-6, and total n-6 PUFA were significantly reduced with ximenynic acid and oleic acid compared to the vehicle in a dose-dependent manner ($p < 0.05$). However, there were no differences in these fatty acids when comparing cells treated with ximenynic acid or oleic acid at 150 μM .

Although the level of C22:5n-3, C22:6n-3, and total n-3 PUFA did not change compared with oleic acid and

Fig. 1. Oil-red-O staining of HepG2 cells after fatty acid treatment. OA, oleic acid; XA, ximenynic acid. $\times 400$.

ximenynic acid treatment (150 μM), these fatty acids were significantly lower compared to vehicle treatment ($p < 0.05$).

Effects of Ximenynic Acid on HepG2 Gene Expression

FADS1 (Δ^5 desaturase) and FADS2 (Δ^6 desaturase) are key rate-limiting enzymes for the synthesis of long-chain n-3 PUFA. Stearoyl-CoA desaturase (SCD, Δ^9 desaturase) is the rate-limiting enzyme that catalyzes MUFA synthesis. To analyze the effects of ximenynic acid on fatty acid synthesis pathways in HepG2 cells, we examined the expression of 6 key genes by quantitative PCR. The results showed that treating HepG2 cells for 72 h with 150 μM ximenynic acid significantly inhibited the expression of *SCD* and *FADS2* compared to the vehicle ($p < 0.05$) but did not affect the expression of *ELOVL2*, *ELOVL5*, *ELOVL6*, or *FADS1* (Fig. 2). Interestingly, oleic acid dramatically suppressed the expression of both *SCD* and *FADS1* compared to the vehicle ($p < 0.05$) (Fig. 2).

We also extracted total cellular protein and analyzed FADS2 protein expression by Western blotting. The results showed that the expression of FADS2 protein was also influenced by ximenynic acid in a dose-dependent manner, while oleic acid had no effect (Fig. 3).

Discussion/Conclusion

Ximenynic acid is a rare fatty acid that is abundant in SWSO; however, its effect on fatty acid metabolism is poorly studied. Therefore, the current study aimed to examine the effects of both SWSO and ximenynic acid on n-3 fatty acid metabolism in both mouse and human liver cells (Fig. 4). The major components of SWSO are oleic acid and ximenynic acid; therefore, using OO (which contains $\sim 80\%$ oleic acid) as a control is ideal for determining the effects of ximenynic acid on fatty acid metabolism in mice. Our results show that SWSO significantly elevated the levels of total n-3 PUFA and decreased total n-9 MUFA in the mouse liver compared to mice treated with OO. Interestingly, total n-3 PUFA in the mouse brain was significantly reduced ($p < 0.05$). To investigate these effects further, we performed an in vitro study using HepG2 liver cells.

The liver is an essential organ for fatty acid metabolism and is sensitive to changes in dietary lipid content [23]. Compared with the OO control group, mice treated with SWSO had lower total n-9 MUFA and higher n-3 PUFA, especially C22:6n-3. This difference may be explained by

Table 6. Effects of ximenynic acid on total fatty acid composition of HepG2 cells

Fatty acids	Vehicle	X50	X100	X150	O50	O100	O150
C14:0	6.20±1.83 ^a	3.56±0.78 ^b	3.19±0.47 ^{b, c}	1.87±0.37 ^{c, d}	3.73±0.35 ^b	1.65±0.36 ^d	1.14±0.15 ^d
C16:0	24.16±1.3 ^a	14.82±3.34 ^b	10.63±1.01 ^c	5.82±1.54 ^d	13.38±.83 ^{b, c}	6.47±0.40 ^d	4.36±0.47 ^d
C18:0	6.48±1.8 ^a	2.88±0.82 ^b	1.84±0.24 ^{bc}	1.15±0.17 ^c	3.01±0.08 ^b	1.99±0.07 ^{b, c}	1.56±0.36 ^{b, c}
Total SFA	36.83±1.26 ^a	21.26±4.58 ^b	15.67±1.28 ^c	8.84±2.05 ^d	20.12±1.10 ^b	10.12±0.74 ^d	7.06±0.97 ^d
C16:1n-7	14.5±4.35 ^a	8.54±1.12 ^b	7.34±0.61 ^b	4.30±0.72 ^c	2.75±0.16 ^{cd}	0.72±0.13 ^d	0.34±0.03 ^d
C18:1n-9	23.27±1.44 ^d	15.33±1.62 ^e	11.09±0.51 ^f	6.11±1.08 ^g	59.45±0.83 ^c	71.91±1.26 ^b	76.65±1.46 ^a
C20:1n-9	1.2±0.20 ^c	0.81±0.03 ^d	0.64±0.08 ^d	0.43±0.08 ^d	5.67±0.06 ^b	8.11±0.41 ^a	8.36±0.28 ^a
C22:1n-9	2.04±0.50 ^a	1.56±0.47 ^{a, b}	1.65±0.34 ^a	0.98±0.04 ^b	2.14±0.016 ^a	1.74±0.14 ^a	1.70±0.44 ^a
C24:1n-9	3.06±0.80 ^a	1.47±0.40 ^{b, c}	1.02±0.03 ^{c, d}	0.59±0.01 ^d	2.04±0.05 ^b	1.15±0.03 ^{c, d}	0.69±0.16 ^d
Total n-9	29.57±1.03 ^d	19.17±2.30 ^e	14.40±0.30 ^f	8.11±1.03 ^g	69.30±0.83 ^c	82.92±1.76 ^b	87.39±1.47 ^a
Total MUFA	44.06±5.18 ^c	27.71±3.39 ^d	21.74±0.81 ^e	12.41±1.73 ^f	72.05±0.68 ^b	83.65±1.63 ^a	87.74±1.49 ^a
C18:2n-6	4.84±1.38 ^a	3.47±0.07 ^b	2.44±0.03 ^c	1.62±0.07 ^{cd}	1.83±0.01 ^{c, d}	1.30±0.22 ^d	1.03±0.15 ^d
C20:4n-6	4.45±2.11 ^a	3.50±0.35 ^{ab}	2.60±0.25 ^{bc}	1.50±0.27 ^c	1.86±0.15 ^c	1.44±0.30 ^c	1.20±0.32 ^c
Total n-6	9.29±3.49 ^a	6.98±0.42 ^{ab}	5.04±0.27 ^{bc}	3.12±0.20 ^{cd}	3.70±0.14 ^{c, d}	2.74±0.52 ^{c, d}	2.24±0.46 ^d
C22:5n-3	1.57±0.15 ^a	1.39±0.11 ^a	0.93±0.04 ^b	0.63±0.04 ^{cd}	0.96±0.12 ^b	0.82±0.23 ^{b, c}	0.57±0.06 ^d
C22:6n-3	1.87±0.34 ^a	1.33±0.29 ^b	0.73±0.05 ^c	0.36±0.05 ^d	0.51±0.01 ^{c, d}	0.35±0.07 ^d	0.39±0.17 ^d
Ximenynic acid	nd	27.07±4.60 ^c	44.36±1.46 ^b	65.17±2.75 ^a	nd	nd	nd
Total n-3	3.44±0.47 ^a	2.72±0.39 ^b	1.66±0.09 ^c	0.99±0.05 ^d	1.47±0.12 ^{c, d}	1.16±0.31 ^{c, d}	0.95±0.23 ^d
Total PUFA	12.73±3.96 ^d	36.76±5.38 ^c	51.06±1.79 ^b	69.28±2.58 ^a	5.17±0.25 ^e	3.90±0.82 ^e	3.19±0.69 ^e
n-6/n-3	2.64±0.61 ^{a, b}	2.59±0.25 ^{a, b}	3.03±0.14 ^{a, b}	3.15±0.13 ^a	2.52±0.10 ^{a, b}	2.39±0.21 ^b	2.36±0.08 ^b

Values are mean % ± SD ($n = 3$). For each fatty acid, values sharing the same superscript letter indicate no significant difference, as determined by one-way analysis of variance ($p < 0.05$). nd, not detected; X, ximenynic acid; O, oleic acid.

the fact that ximenynic acid can impact n-9 MUFA synthesis directly or that the oleic acid levels in OO were higher than in SWSO. The higher content of C22:6n-3 and n-3 PUFA may be due to the ability of SWSO to activate n-3 PUFA synthesis enzymes or simply related to the fact that C18:3n-3 levels (a C22:6n-3 precursor) are higher in SWSO than OO.

n-3 fatty acids are essential for brain development and function from infants to elderly adults. A previous study reported that mice fed SWSO showed no detectable levels of ximenynic acid in the brain [10]. The present study indicated that SWSO may have positive effects on n-3 PUFA levels in the mouse liver compared with OO, but the levels of these same fatty acids are down-regulated in the brain. In HepG2 cells, C22:6n-3 was significantly decreased, while *SCD*, *FADS1*, and *FADS2* gene expression, as well as *FADS2* protein expression, was significantly down-regulated following a 72-h incubation with 150 μ M of ximenynic acid compared with vehicle. Further, we found ximenynic acid to be present at very low levels in the mouse brain following gavage with SWSO and SO, indicating that ximenynic acid can penetrate the blood-brain barrier and enter brain tissue, which might inhibit the formation of

n-3 fatty acids. However, these low levels probably have little influence on the overall fatty acid concentration in the mouse brain. In contrast, our results may suggest that ximenynic acid might prevent the brain from obtaining n-3 PUFA critical for brain development.

FADS1 and *FADS2* are key enzymes for endogenous PUFA synthesis, and they encode Δ^5 and Δ^6 desaturase in humans, respectively [24]. C22:6n-3 concentration was markedly lower in HepG2 cells following ximenynic acid treatment compared to oleic acid treatment, and *FADS2* expression was also reduced. Moreover, *FADS2* protein expression corroborated *FADS2* gene expression results. Compared with oleic acid groups, total n-3 PUFA levels in ximenynic acid-treated cells were not affected by different concentrations. It might be because oleic acid also has a negative effect on n-3 fatty acid metabolism through *FADS1* inhibition. The present study is consistent with a previous study [10] that showed that SWSO had a progressive effect on C22:6n-3 levels in the mouse liver. However, our in vitro study did not support that ximenynic acid could enhance n-3 PUFA synthesis. Therefore, the increased levels of n-3 PUFA in the mouse liver might be due to other factors.

Fig. 2. a–f Expression of genes involved in fatty acid metabolism in HepG2 cells. Data were obtained from 3 different passages, and each passage was run in triplicate. Results are presented as means $\pm$ SD ($n = 3$ different passages). Bars sharing the same superscript letters are not significantly different ($p < 0.05$) from one another, as determined by one-way analysis of variance. X, ximenynic acid; O, oleic acid; vehicle, DMSO + BSA.

SCD is a crucial enzyme for the synthesis of MUFA from SFA. This enzyme especially catalyzes the dehydrogenation of SFA at the Δ^9 position [25]. Therefore, a reduction in SCD activity generally corresponds to a decrease in MUFA synthesis. The present in vitro study shows for the first time that n-9 MUFA, e.g., C18:1n-9, C20:1n-9, and C24:1n-9, were markedly and dose-dependent reduced by ximenynic acid. We also found that high treatment levels of ximenynic acid significantly sup-

pressed SCD gene expression. Together, this suggests that ximenynic acid may be involved in the regulation of n-9 MUFA synthesis. The similar structure between ximenynic acid and oleic acid may explain why SCD was inhibited.

In conclusion, ximenynic acid might affect n-3 PUFA metabolism by regulating the expression of key enzymes in the liver, such as FADS2. However, oleic acid might be in a position to suppress the expression

Fig. 3. a, b Effect of fatty acid treatment on FADS2 protein expression in HepG2 cells. Results are presented as means $\pm$ SD ($n = 3$). * $p < 0.05$ vs. the vehicle group (DMSO + BSA) (independent-sample t tests). X, ximenynic acid; O, oleic acid.

of Δ^5 desaturase FADS1 in HepG2 cells. SWSO has a positive impact on n-3 PUFA levels in the mouse liver but a negative one in the mouse brain. The different effects of SWSO on n-3 PUFA levels in the mouse brain and liver might be because the basic feed consumed by mice provided sufficient C18:3n-3 to support brain development. Therefore, the gavage experimental oils may have been completely metabolized by the liver. Although our in vitro study showed that ximenynic acid inhibited FADS2 expression in HepG2 cells, our results suggest that the high levels of C18:3n-3 in SWSO might overcome this ximenynic acid inhibition in vivo and promote the endogenous production of C22:6n-3 in the mouse liver.

Fig. 4. Proposed effect of sandalwood seed oil on n-3 fatty acid metabolism pathways.

Acknowledgment

This work was supported by the National Basic Research Program of China (973 Program: 2015CB553604); by the National Natural Science Foundation of China (NSFC: 81773433); and by the Key Scientific Research Projects in Shandong Province, China (2017YYSP007).

Statement of Ethics

This work was approved by the Ethics Committee of the College of Biosystems Engineering and Food Science, Zhejiang University.

Disclosure Statement

The authors have no conflicts of interest to report in respect of this work.

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