Metabolism of Natural Highly Unsaturated Fatty Acid, Tetracosahexaenoic Acid (24:6n-3), in C57BL/KsJ-db/db Mice

Naohiro Gotoh^{1*}, Koji Nagao², Hiroki Ishida¹, Kazusa Nakamitsu², Kazuaki Yoshinaga³, Toshiharu Nagai³, Fumiaki Beppu¹, Aya Yoshinaga-Kiriake¹, Hiroyuki Watanabe⁴, and Teruyoshi Yanagita⁵

¹ Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, JAPAN

² Department of Applied Biochemistry and Food Science, Saga University, 1 Honjo-machi, Saga-shi, Saga 840-8502, JAPAN

³ Tsukishima Foods Industry Co. Ltd. (3-17-9, Higashi Kasai, Edogawa-ku, Tokyo 134-8520, JAPAN

⁴ Department of Health Science, University of Kochi, 2751-1 Ike, Kochi-shi, Kochi 781-0111, JAPAN

⁵ Department of Health and Nutrition Science, Nishikyushu University, 4490-9 Ozaki, Kanzaki-machi, Kanzaki-shi, Saga 842-8585, JAPAN

Abstract: Tetracosahexaenoic acid (THA; 24:6n-3) is a natural, n-3 highly unsaturated fatty acid (n-3HUFA) that exists in fish, including Baltic herring (*Clupea harengus*) and the flathead flounder (*Hippoglossoides dubius*). In this study, natural n-3HUFAs, i.e. eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and THA were administrated to C57BL/KsJ-db/db mice for 4 weeks and the liver and serum lipid profiles, hepatic enzyme activity, expression of mRNA related to lipid metabolism, and adiponectin serum levels were then analyzed. The results showed that THA had the highest activity in suppressing hepatic triglyceride (TG) accumulation and increase in liver weight among the test groups. Furthermore, THA increased adiponectin levels in serum. These results indicate that THA is an excellent natural n-3HUFA that can suppress the development of metabolic syndromes and circulatory system diseases. The order of the n-3HUFA activity was THA > DHA > EPA in almost all the factors examined here. In a previous study of ours, the order was DHA > DPA > EPA, so the final order was summarized as THA > DHA > DPA > EPA. This order clearly translates to the rule that “the number of double bonds and carbon atoms in the n-3HUFA structure relates to their clinical functions”.

Key words: tetracosahexaenoic acid, n-3 polyunsaturated fatty acid, function, docosahexaenoic acid, eicosapentaenoic acid, C57BL/KsJ-db/db mice

1 INTRODUCTION

Metabolic syndrome is associated with an increased risk of developing diabetes and cardiovascular disease. It is also associated with the accumulation of triglyceride (TG) in the liver and increased levels of cholesterol (Ch) and TG in the blood. Both genetic and dietary factors are known to play a role in the onset of the syndrome¹⁻³. For example, the Japanese have a much lower rate of cardiovascular disease compared to the Europeans and Americans⁴. One of the reasons for this is thought to be the fact that fish has played a central role in the Japanese diet for a long time⁵. Fish oil possesses attributes that can suppress the development of cardiovascular diseases and could play a role in the

improvement of the pathogenic mechanism of metabolic syndrome^{6,7}. The European Society of Cardiology, the European Atherosclerosis Society, and the American Heart Association all promote diets low in calories and certain fats^{8,9}. High-quality fats are thought to promote good health, especially those found in fish, such as n-3 type fatty acids^{8,9}.

Fish oil contains many types of n-3 highly unsaturated fatty acids (n-3HUFA), including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). These fatty acids have the ability to decrease the amount of TG in the liver¹⁰, the levels of Ch and TG in the blood¹¹, accumulation of visceral fat¹², and to increase the levels of

* Correspondence to: Naohiro Gotoh, Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, JAPAN

E-mail: ngotoh@kaiyodai.ac.jp

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adiponectin in the blood¹³), among others. EPA has been used as a drug to decrease TG levels in the blood¹⁴. DHA also improves lipid metabolism, so much so that the Ministry of Health, Labor, and Welfare in Japan approved certain DHA-enriched foods as Food for Specified Health Use¹⁵. Docosapentaenoic acid (DPA, 22:5n-3) is also a well-known n-3HUFA that is found in seal oil¹⁶ and that consists of the same numbers of double bonds in EPA and carbon atoms in DHA. Specifically, the structure is an intermediate of EPA and DHA. The abilities of EPA, DPA, and DHA to improve the lipid profiles in the blood, lipid metabolism, adiponectin, etc., were investigated using C57BL/KsJ-*db/db* mouse¹⁷. Interestingly, our results for all the observed factors using DPA were between EPA and DHA. These results indicate that the clinical function derived from n-3HUFA relates to both the number of double bonds and carbon atoms. Particularly, the increased ability of adiponectin was the lowest in the EPA treatment group and the highest in DHA treatment group among the three n-3HUFAs; therefore, DHA was thought to be the strongest n-3HUFA for the suppression of metabolic syndrome and cardiovascular disease progress. DHA is an n-3HUFA synthesized from EPA by elongation and desaturation reactions in our body. EPA is elongated to form DPA at first; however, humans do not possess $\Delta 4$ desaturase that can form DHA from DPA directly¹⁸. Therefore, DPA is elongated again to form tetracosapentaenoic acid (24:5n-3) and desaturated by $\Delta 6$ desaturase. As a result, tetracosahexaenoic acid (THA, 24:6n-3) forms. THA is beta-oxidized to form DHA. THA is also one of the n-3HUFAs found in fishes such as Baltic herring (*Clupea harengus*) and the flathead flounder (*Hippoglossoides dubius*), among others^{19–21}. THA consists of 24 carbon atoms and 6 double bonds. Specifically, the number of carbon atoms in THA is two more compared to that in DHA. As indicated above, the health function benefits of n-3HUFA is related to the number of double bonds and carbon atoms in n-3HUFA. According to this rule, THA might act as the strongest n-3HUFA for the suppression of visceral fat accumulation, increasing adiponectin levels, improving the lipid profile in blood and liver, suppressing the development of metabolic syndrome, and suppressing the progress of atherosclerotic diseases. In fact, we already compared the lipid metabolism ability among these four kinds of n-3HUFA, using HepG2 cells²². The results showed that THA exerted the strongest suppression on the synthesis of triglyceride and cholesteryl ester (ChE), and the order of the suppression strength was THA > DHA > DPA > EPA, as we predicted previously.

Therefore, in this study, we examined the health-related functions of THA in C57BL/KsJ-*db/db* mice to confirm the results obtained from the HepG2 cells, and the number of double bonds and carbon atoms rule in n-3HUFA. This study could provide us with a basic understanding of the development of new medicines for treating metabolic syn-

drome.

2 MATERIALS AND METHODS

2.1 Chemicals and materials

All the AAA-type TGs such as tripalmitate (tri16:0), trioleate (tri18:1n-9), trilinoleate (tri18:2n-6), trilinolenate (tri18:3n-3), triEPA, triDHA, and triTHA were obtained from Tsukishima Foods Industry Co., Ltd (Tokyo, Japan). The purity of the fatty acids consisting of AAA-type TG was greater than 98%. 5,5'-Dithiobis (2-nitro-benzoic acid) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Beta-corn starch, cellulose, nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide, and reduced nicotinamide adenine dinucleotide phosphate were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Triton X-100, 6-phosphogluconate dehydrogenase, dithiothreitol, casein, *dl*-methionine, and choline bitartrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mineral mixture (AIN-76) and vitamin mixture (AIN-76) were purchased from Nosan Corporation (Tokyo, Japan). Sucrose was obtained from Mitsui Sugar Co., Ltd. (Tokyo, Japan). Silica gel (silicic acid 100 mesh) was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Palmitoyl CoA was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Glucose 6-phosphate was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). Acetyl CoA, *l*-malic acid, malonyl CoA, *l*-carnitine, flavin adenine dinucleotide (FAD), phosphatidylcholines, and phosphatidic acid were bought from Sigma-Aldrich, Japan K.K. (Tokyo, Japan). Other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Structures of EPA, DPA, DHA, and THA are compared in Fig. 1.

2.2 Animal test

All experiments were performed in accordance with the Saga University guidelines for animal use and care provided by the ethics committee (No. 22-042-1). Five-week-old male C57BL/6J mice and C57BL/KsJ-*db/db* mice were bought from Nippon Clea Co. (Tokyo, Japan). The mice were housed individually in plastic cages lined with sawdust in a temperature ($23 \pm 2^\circ\text{C}$) and light (12-h cycle) controlled environment. The mice were initially fed a com-

Fig. 1 The structure of n-3HUFAs.

mercial diet CE-2 (Nippon Clea Co.) for 1 week and then divided into four groups: Control group (control diet; $n = 5$), EPA-TG Group (1% [w/w] of trilinoleate in the control diet was replaced with triEPA; $n = 5$), DHA-TG group (1% of trilinoleate in control diet was replaced with triDHA; $n = 5$), and THA-TG group (1% of trilinoleate in control diet was replaced with triTHA; $n = 5$). TG comprised 9% of the control diet and consisted of tripalmitate (3%), trioleate (3%), trilenoleate (2.9%), and trilinolenate (0.1%). All the diets were prepared based on an AIN-76 composition. The detailed diet composition is shown in Table 1. Each group of C57BL/KsJ-*db/db* mice was given their diet for 4 weeks by pair-feeding. Furthermore, as the positive control group, a group of C57BL/6J mice was also fed a control diet for 4 weeks (Normal Control). All the mice were allowed free access to water. The weight and consumption amount of the feed for each mouse were measured every day. At the end of the feeding period, the mice were killed by withdrawing blood from the heart under isoflurane anesthesia after a 9-h starvation period. The liver, muscles, heart, kidney, testis, brain, epididymal fat, adrenal gland, and spleen were harvested. All the organs and tissues were preserved in liquid nitrogen. The withdrawn blood samples were centrifuged at $1750 \times g$ for 15 min at 4°C to collect the sera.

2.3 Serum analysis

Serum TG and cholesterol levels were analyzed using commercial enzyme assay kits (Wako Pure Chemical Indus-

tries, Ltd.). Insulin and adiponectin levels were measured using an Insulin ELISA kit (Shibayagi Co. Ltd., Gunma, Japan) and Mouse/Rat Adiponectin ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), respectively. Alanine aminotransferase (ALT) activity was analyzed using a Transaminase CII-test Wako kit (Wako Pure Chemical Industries, Ltd.).

2.4 Liver lipid analysis

Hepatic lipid was extracted from the liver by the method described by Folch *et al.*²³⁾. Hepatic concentrations of TG and phospholipid were measured according to the methods described by Fletcher *et al.*²⁴⁾ and Rouser *et al.*²⁵⁾, respectively. Hepatic cholesterol concentration was analyzed using the Cholesterol E-Test Wako (Wako Pure Chemical Industries, Ltd.).

2.5 Hepatic enzyme activity assay

Liver mitochondrial and cytosol fractions were prepared by homogenization and centrifugation. The protein concentration of each fraction was analyzed by the method of Lowry²⁶⁾. The enzyme activity of fatty acid synthase (FAS)²⁷⁾ and glucose 6-phosphate dehydrogenase (G6PDH)²⁸⁾ in each cellular fraction was measured by the method of Kelly. Malic enzyme (ME), carnitine palmitoyl transferase (CPT), peroxisomal β -oxidation, and phosphatidate phosphohydrolase (PAP) in each cellular fraction were assayed by the method of Ochoa *et al.*²⁹⁾, Markwell *et al.*³⁰⁾, Lazarow *et al.*³¹⁾, and Walton *et al.*³²⁾, respectively.

Table 1 Composition of the experimental diet (g/100 g).

Ingredient	Normal Control Group	Control Group	EPA-TG Group	DHA-TG Group	THA-TG Group
	(C57BL/6J)				
Casein	20.00	20.00	20.00	20.00	20.00
Corn starch	15.00	15.00	15.00	15.00	15.00
Cellulose	5.00	5.00	5.00	5.00	5.00
Mineral mixture (AIN-76)	3.50	3.50	3.50	3.50	3.50
Vitamin mixture (AIN-76)	1.00	1.00	1.00	1.00	1.00
DL-Methionine	0.30	0.30	0.30	0.30	0.30
Choline bitartrate	0.20	0.20	0.20	0.20	0.20
Tripalmitate (triPA)	3.00	3.00	3.00	3.00	3.00
Trioleate (triOA)	3.00	3.00	3.00	3.00	3.00
Trilinoleate (triLA)	2.90	2.90	1.90	1.90	1.90
Trilinolenate (triLnA)	0.10	0.10	0.10	0.10	0.10
TriEPA	—	—	1.00	—	—
TriDHA	—	—	—	1.00	—
TriTHA	—	—	—	—	1.00
Sucrose	46.00	46.00	46.00	46.00	46.00

2.6 Hepatic mRNA level assay

Total RNA was extracted from 100 mg of liver, using the RNeasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan). A TaqMan® Universal PCR Master Mix (Applied Biosystems, Tokyo, Japan), an Assays-on-Demand™, Gene Expression Product (Mm01304289_m1 for acetyl-CoA carboxylase 1 (ACC1), Mm01204659_m1 for acetyl-CoA carboxylase 2 (ACC2), Mm00662319_m1 for fatty acid synthase (FAS), Mm00517221_m1 for delta 6 desaturase (D6D), Mm00550338_m1 for sterol regulatory element binding protein -1 (SREBP1), Mm00441242_m1 for monocyte chemoattractant protein 1 (MCP1), Mm03928991_g1 for 18S rRNA (Applied Biosystems, Tokyo, Japan), and the TaqMan® MGB Gene Expression Kits (Applied Biosystems) for stearoyl-coenzyme A desaturase-1: SCD1 (forward primer, 5'-AGCCTGTTTCGTCAGCACCTT-3'; reverse primer, 5'-CACCCAGGGAAACCAGGAT-3'; and TaqMan® MGB probe, 5'-FAM-CACTCTGGTGTCTCAAC-MGB-3') were used for quantitative real-time RT-PCR analysis of ACC1, ACC2, FAS, D6D, SREBP1, MCP1, SCD1, and 18S rRNA expression in the liver. The amplification was performed on a real-time PCR system (ABI Prism 7000 Sequence Detection System; Applied Biosystems). The results were expressed as a relative value after normalization to the 18S RNA expression.

2.7 Analysis of fatty acid composition in organs and tissues

Lipids contained in the organs and tissues were also extracted by the method described by Folch *et al.*²³⁾. The extracted lipids were dried and weighed. Methyl esterification of the extracted lipids was performed using a boron trifluoride methanol solution. The methyl-esterified fatty acid was subjected to a gas chromatography-flame ionization detector (GC-FID) system (GC-14B, Shimadzu, Tokyo, Japan) equipped with a capillary column (Omegawax320, 30 m × 0.25 mm ID, SIGMA-ALDRICH Japan K.K., Tokyo, Japan) and a Chromatopac integrator (C-R6A; Shimadzu) to analyze the fatty acid composition and relative ratio. The temperature of the injection port and detector was 250°C and 260°C, respectively. The initial column temperature of 175°C was increased to 225°C at a rate of 1°C/min. Helium was used as the carrier gas at a flow rate of 32 cm/s. The fatty acid species were identified using the retention time of a fatty acid methyl ester standard solution (Supelco 37 Component FAME Mix, SIGMA-ALDRICH Japan K.K.). The relative contents of the respective fatty acids were calculated using a GC-FID chromatogram.

2.8 Statistical analyses

All values are expressed as means ± SE. Data were ana-

Table 2 Effect of experimental diet on growth parameters in C57BL/6J and C57BL/KsJ-*db/db* mice.

	Normal Control Group	Control Group	EPA-TG Group	DHA-TG Group	THA-TG Group
	(C57BL/6J)	(C57BL/KsJ- <i>db/db</i>)			
Initial body weight (g)	20.1 ± 0.2	28.8 ± 0.8 *	28.8 ± 0.5	28.8 ± 0.6	28.8 ± 0.4
Final body weight (g)	22.1 ± 0.2	37.0 ± 1.7 *	37.9 ± 0.6	36.3 ± 1.0	35.6 ± 1.5
Total weight gain (g)	2.00 ± 0.16	8.20 ± 1.46 *	9.10 ± 0.35	7.50 ± 0.61	6.84 ± 1.06
Total food intake (g)	78.2 ± 1.1	136 ± 5 *	136 ± 3	136 ± 4	135 ± 3
Food efficiency (mg gain/g intake)	25.6 ± 2.0	61.3 ± 12.5 *	66.9 ± 3.0	55.1 ± 4.0	49.9 ± 6.7
Liver weight (g /100 g body weight)	4.07 ± 0.07	5.46 ± 0.21 ^a	4.50 ± 0.13 ^b	4.62 ± 0.07 ^b	4.46 ± 0.11 ^b
Testis (g)	0.196 ± 0.012	0.148 ± 0.010 *	0.152 ± 0.005	0.135 ± 0.008	0.124 ± 0.010
Kidney (g)	0.277 ± 0.006	0.378 ± 0.005 *	0.382 ± 0.013	0.397 ± 0.011	0.393 ± 0.013
Adrenal gland (mg)	5.56 ± 0.68	6.52 ± 0.54	5.52 ± 0.40	5.02 ± 0.19	5.54 ± 0.71
Spleen (mg)	56.2 ± 0.6	34.0 ± 2.7 *	45.2 ± 1.7	36.8 ± 2.2	32.8 ± 0.2
Heart (g)	0.114 ± 0.002	0.121 ± 0.002 *	0.125 ± 0.002	0.114 ± 0.002	0.110 ± 0.003
Brain (g)	0.452 ± 0.004	0.393 ± 0.004 *	0.394 ± 0.003	0.380 ± 0.011	0.382 ± 0.005
Accumulated fat (white adipose tissue) weight (g/100 g body weight)					
Total	2.27 ± 0.19	12.0 ± 0.3 *	12.0 ± 0.3	11.2 ± 0.2	10.9 ± 0.6
Epididymal	1.17 ± 0.08	5.23 ± 0.06 *	5.10 ± 0.19	4.80 ± 0.10	5.00 ± 0.11
Perirenal	0.489 ± 0.034	2.98 ± 0.13 *	3.08 ± 0.12	2.95 ± 0.08	2.65 ± 0.12
Omental	0.607 ± 0.093	3.83 ± 0.20 *	3.87 ± 0.08	3.43 ± 0.11	3.82 ± 0.10

Each value represents mean ± SE (n = 5).

Different letters indicate significant difference at $p < 0.05$ among C57BL/KsJ-*db/db* groups.

* $p < 0.05$ (Normal Control vs Control group)

lyzed by one-way ANOVA, and all differences were inspected by Tukey-Kramer post-hoc test (Kaleida Graph, Synergy Software, Reading, PA). Differences between the Normal Control and the Control group in the animal test were evaluated by Student's *t*-test. The difference was considered significant when *P* was less than 0.05.

3 RESULTS

3.1 Weight change

Growth parameters such as body weight, food intake, organ weight, and tissue weight for each experimental diet group of C57BL/KsJ-*db/db* and C57BL/6J mice are shown in Table 2. The changes in the liver and white adipose

tissue (namely visceral fat) weights were strongly affected by n-3HUFA intake and the tendency of those weight increments to be affected by the numbers of double bonds and carbon atoms was observed. Particularly, the weight of the livers in the n-3HUFA groups decreased significantly compared to that in the control group. Comparison of the average weights of the liver and adipose tissue indicates that THA tends to have strong effect for the suppression of weight gain.

3.2 Lipid profile in liver

The lipid profiles in the liver among the test groups are compared in Table 3. Levels of TG, total cholesterol, and phospholipid of "mg/whole liver" significantly decreased in all n-3HUFA-administrated groups compared to those in

Table 3 Effect of experimental diet on hepatic lipids in C57BL/6J and C57BL/KsJ-*db/db* mice (mg/g liver).

	Normal Control Group (C57BL/6J)	Control group	EPA-TG Group	DHA-TG Group	THA-TG Group
		(C57BL/KsJ- <i>db/db</i>)			
Triglyceride (mg/g liver)	28.2 ± 0.9	180.0 ± 32.0 ^{*a}	126.0 ± 14.0 ^{ab}	91.7 ± 11.0 ^b	70.0 ± 15.7 ^b
Triglyceride (mg/whole liver)	25.4 ± 1.2	357 ± 83 ^{*a}	218 ± 33 ^{ab}	151 ± 13 ^{ab}	114 ± 29 ^b
Total cholesterol (mg/g liver)	3.41 ± 0.10	3.41 ± 0.16 [*]	3.20 ± 0.07	3.10 ± 0.09	3.17 ± 0.10
Total cholesterol (mg/whole liver)	3.06 ± 0.06	6.84 ± 0.38 ^{*a}	5.45 ± 0.22 ^b	5.21 ± 0.29 ^b	5.02 ± 0.21 ^b
Phospholipid (mg/g liver)	33.1 ± 0.2	27.7 ± 0.5 [*]	28.3 ± 0.9	25.9 ± 0.5	25.4 ± 1.1
Phospholipid (mg/whole liver)	29.7 ± 0.5	56.0 ± 3.7 ^{*a}	48.0 ± 1.8 ^{ab}	43.6 ± 2.5 ^b	40.5 ± 3.3 ^b

Each value represents mean ± SE (n = 5).

Different letters indicate significant difference at *p* < 0.05 among C57BL/KsJ-*db/db* groups.

* *p* < 0.05 (Normal Control vs Control group)

Table 4 Activities of hepatic triglyceride metabolism-related enzymes in C57BL/6J and C57BL/KsJ-*db/db* mice. (nmol/min/mg protein)

	Normal Control Group (C57BL/6J)	Control Group	EPA-TG Group	DHA-TG Group	THA-TG Group
		(C57BL/KsJ- <i>db/db</i>)			
Cytosol fraction					
FAS	7.42 ± 0.26	11.3 ± 1.8 ^{*a}	8.93 ± 0.69 ^{ab}	6.06 ± 0.55 ^b	4.82 ± 0.70 ^b
ME	100 ± 5	137 ± 19 ^a	114 ± 10 ^{ab}	80.8 ± 9.8 ^b	76.6 ± 8.2 ^b
G6PDH	7.65 ± 0.37	4.39 ± 0.66 ^{*a}	3.88 ± 0.27 ^{ab}	2.91 ± 0.09 ^b	2.95 ± 0.06 ^{ab}
Mitochondrial fraction					
CPT	8.00 ± 0.22	10.0 ± 0.3 [*]	10.2 ± 0.2	10.5 ± 0.4	11.0 ± 0.3
Peroxisomal β-oxidation	7.55 ± 0.29	13.1 ± 0.8 ^{*a}	10.2 ± 0.2 ^b	11.3 ± 0.6 ^{ab}	11.8 ± 0.5 ^{ab}
Microsome fraction					
PAP	16.4 ± 2.2	4.10 ± 0.70 [*]	4.05 ± 0.51	3.52 ± 0.29	5.38 ± 0.25

Each value represents mean ± SE (n = 5).

Different letters indicate significant difference at *p* < 0.05 among C57BL/KsJ-*db/db* groups.

* *p* < 0.05 (Normal Control vs Control group)

FAS, fatty acid synthase; ME, malic enzyme; G6PDH, glucose 6-phosphate dehydrogenase; CPT, carnitine palmitoyl transferase; PAP, phosphatidate phosphohydrolase

the control group, and their averages decreased in the order of EPA, DHA, and THA. Consequently, THA showed the strongest suppression effect. In contrast, only the level of TG significantly decreased compared to that in the control group when using the unit “mg/g liver.”

3.3 Hepatic enzyme activity assay

The activities of hepatic TG metabolism-related enzymes are shown in Table 4. Activity of FAS in the DHA-TG and THA-TG groups, ME treated with the DHA-TG and THA-TG groups, G6PDH treated with the DHA-TG group, and peroxisomal β -oxidation treated with the EPA-TG group decreased significantly compared to those in the control group.

3.4 Hepatic mRNA level assay

The mRNAs related to lipid metabolism are shown in Table 5. mRNA levels of ACC1 in the THA-TG group, FAS in the DHA-TG group, steroyl-CoA desaturase in the THA-TG group, delta 6 desaturase in the DHA-TG group, and MCP in the EPA-TG, DHA-TG, and THA-TG groups decreased significantly compared to those in the control group. No significant difference was detected in the mRNA levels of ACC2 and SREBP-1 between the n-3HUFA-administrated groups and the control group.

3.5 Lipid profile in serum

Serum parameters are shown in Table 6. Levels of TG in the EPA-TG group and ALP in the EPA-TG, DHA-TG, and

Table 5 Relative hepatic mRNA levels in C57BL/6J and C57BL/KsJ-*db/db* mice.

	Normal Control Group	Control Group	EPA-TG Group	DHA-TG Group	THA-TG Group
	(C57BL/6J)	(C57BL/KsJ- <i>db/db</i>)			
ACC1	17.6 $\pm$ 2.2	100 $\pm$ 7 ^{*a}	82.1 $\pm$ 9.1 ^{ab}	74.3 $\pm$ 4.0 ^{ab}	72.1 $\pm$ 3.3 ^b
ACC2	39.0 $\pm$ 4.4	100 $\pm$ 6 ^{*ab}	112 $\pm$ 10 ^a	92.8 $\pm$ 7.5 ^{ab}	73.4 $\pm$ 8.7 ^b
FAS	6.05 $\pm$ 0.64	100 $\pm$ 5 ^{*a}	83.2 $\pm$ 1.3 ^{ab}	73.3 $\pm$ 7.0 ^b	79.6 $\pm$ 6.6 ^{ab}
Steroyl-CoA desaturase	36.6 $\pm$ 5.9	100 $\pm$ 12 ^{*a}	118 $\pm$ 16 ^a	70.6 $\pm$ 9.0 ^{ab}	50.0 $\pm$ 8.2 ^b
Delta 6 desaturase	40.8 $\pm$ 3.3	100 $\pm$ 18 ^{*a}	83.3 $\pm$ 10.1 ^{ab}	39.1 $\pm$ 3.9 ^b	56.0 $\pm$ 15.9 ^{ab}
SREBP-1	60.9 $\pm$ 4.0	100 $\pm$ 12 [*]	124 $\pm$ 14	126 $\pm$ 12	127 $\pm$ 14
MCP1	13.0 $\pm$ 5.4	100 $\pm$ 11 ^{*a}	35.0 $\pm$ 5.8 ^b	23.3 $\pm$ 4.5 ^b	26.8 $\pm$ 6.2 ^b

Each value represents mean $\pm$ SE (n = 5).

Different letters indicate significant difference at $p < 0.05$ among C57BL/KsJ-*db/db* groups.

* $p < 0.05$ (Normal Control vs Control group)

ACC1, acetyl-CoA carboxylase 1; ACC2, acetyl-CoA carboxylase 2; FAS, fatty acid synthase; SREBP-1, sterol regulatory element binding protein-1; MCP1, monocyte chemotactic protein 1

Table 6 Effect of experimental diet on serum parameters in C57BL/6J and C57BL/KsJ-*db/db* mice.

	Normal Control Group	Control Group	EPA-TG Group	DHA-TG Group	THA-TG Group
	(C57BL/6J)	(C57BL/KsJ- <i>db/db</i>)			
Triglyceride (mg/dL)	62.0 $\pm$ 5.3	123.0 $\pm$ 15.0 ^{*a}	80.8 $\pm$ 3.0 ^b	83.5 $\pm$ 6.3 ^{ab}	87.7 $\pm$ 9.9 ^{ab}
Total cholesterol (mg/dL)	98.3 $\pm$ 2.1	174 $\pm$ 13 ^{*ab}	137 $\pm$ 5 ^a	203 $\pm$ 15 ^b	170 $\pm$ 8 ^{ab}
Glucose (mg/dL)	199 $\pm$ 16	578 $\pm$ 72 [*]	452 $\pm$ 30	492 $\pm$ 29	563 $\pm$ 50
Insulin (ng/mL)	0.690 $\pm$ 0.323	7.51 $\pm$ 0.73 ^{*a}	22.4 $\pm$ 5.4 ^b	18.4 $\pm$ 3.9 ^{ab}	10.6 $\pm$ 1.4 ^{ab}
Adiponectin (μ g/mL)	24.5 $\pm$ 1.8	13.3 $\pm$ 0.3 ^{*a}	18.9 $\pm$ 1.0 ^b	20.3 $\pm$ 1.1 ^b	20.0 $\pm$ 0.7 ^b
MCP1 (pg/mL)	71.0 $\pm$ 2.1	259 $\pm$ 10 [*]	243 $\pm$ 16	219 $\pm$ 14	270 $\pm$ 8
GPT $\cdot$ ALT (IU/L)	6.00 $\pm$ 0.43	30.0 $\pm$ 2.0 [*]	23.7 $\pm$ 3.0	25.0 $\pm$ 1.3	25.7 $\pm$ 0.4
ALP (IU/L)	61.4 $\pm$ 2.1	121 $\pm$ 4 ^{*a}	91.0 $\pm$ 4.4 ^b	90.6 $\pm$ 7.0 ^b	90.8 $\pm$ 5.9 ^b

Each value represents mean $\pm$ SE (n=5).

Different letters indicate significant difference at $p < 0.05$ among C57BL/KsJ-*db/db* groups.

* $p < 0.05$ (Normal Control vs Control group)

MCP1, monocyte chemotactic protein 1; GPT, glutamate pyruvate transaminase; ALT, alanine aminotransferase
ALP, alkaline phosphatase

THA-TG groups decreased significantly compared to those in the control group. In contrast, levels of insulin in the EPA-TG group and adiponectin in the EPA-TG, DHA-TG, and THA-TG groups increased significantly compared to those in the control group.

3.6 Fatty acid composition in organ and tissue

The fatty acid composition of the organs and tissues is summarized in Fig. 2. 24:6n-3 is characteristically found in the muscles, heart, testis, and spleen of the THA-TG group. Interestingly, the 22:6n-3 content in the liver, muscle, heart, kidney, testis, adrenal gland, and spleen of the THA group was almost the same as those in the DHA-TG group. The relative contents of 20:5n-3 and 22:5n-3 in the muscle, heart, kidney, adrenal gland, and spleen of the EPA-TG group were higher than those of the DHA-TG and THA-TG groups. The relative content of 20:4n-6 in the muscle, heart, kidney, and testis of the control group was characteristically high among C57BL/KsJ-*db/db* mice. Not much content of 18:3n-3 was found in any of the groups. Equally, there was a tendency for a lack of 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3, and 24:6n-3 accumulation in the epididymal fat of all the groups. The distribution of fatty acids in the epididymal fat or in the brain was similar among all groups of C57BL/KsJ-*db/db* mice and between the normal group (C57BL/6J mice) and the control group (C57BL/KsJ-*db/db* mice).

4 DISCUSSION

Metabolic syndrome is gaining attention and visceral fat accumulation and fatty liver are thought to be key elements of its mechanism¹⁻³⁾. This accumulation leads to dyschylia of adiponectin and causes insulin resistance, hyperlipidemia, diabetes, and hypertension along with the complications associated with these pathologies that can develop into atherosclerotic diseases³³⁾. Particularly, a series of events that include abnormal accumulation of visceral fat, dyschylia of adiponectin, and the development of insulin resistance occur at the center of this pathogenic mechanism, where the first two steps have been most recently studied. We already showed that n-3HUFAs can suppress the accumulation of lipids in HepG2 cells according to the number of carbon atoms and double bonds in the n-3HUFA structure³⁴⁾. This could not only disconnect this series of events in metabolic syndrome, where cardiovascular disease and diabetes occur downstream, but also improve the development of cardiovascular disease and insulin sensitivity as a result. In this study, the hypothesis³⁴⁾ was confirmed using C57BL/KsJ-*db/db* mice. C57BL/KsJ-*db/db* mice carry a G-to-T point mutation at the leptin receptor-coding gene and therefore lack the ability to bind leptin, which leads to an excessive appetite. This has been used as a model

animal for the evaluation of obesity, hyperlipidemia, and hyperglycemia, among other conditions. The comparison of growth parameters such as body weight, food intake, organ weight, and tissue weight for each experimental diet group showed that n-3HUFAs significantly affected only weight changes in the liver (Table 2). The weight of the white adipose tissue (namely visceral fat) was also affected by n-3HUFA intake; however, significant differences were not detected and only a tendency affected by the numbers of double bonds and carbon atoms was observed. An accumulation of hepatic lipid would mean that a decrease in liver weight is due to the suppression of TG accumulation (Table 3). Because only TG expressed with "mg/g liver" in n-3HUFAs decreased significantly compared to that in the control group, in spite of all the factors expressed with "mg/whole liver" in n-3HUFAs decreased significantly compared to that in the control group. These results suggest that the amount of TG per cell decreased; however, the amounts of total Ch and phospholipids per cell did not differ between the control and n-3HUFA groups. As a result of a significant decrease in TG accumulation in the cell, the total weight of the liver would have decreased significantly, and all the factors expressed as "mg/whole liver" in n-3HUFAs also decreased significantly compared to that in the control group. Consequently, it was thought that the significant decrease in the weight of the liver was caused by the suppression of TG accumulation by n-3HUFAs. The suppression strength tends to be THA > DHA > EPA according to the average values and this order is the same as was confirmed with the HepG2 cell culture test²²⁾. The accumulation of fat in the liver is also associated with metabolic syndrome. Non-alcoholic fatty liver disease (NAFLD) is now the most common liver disease in Western countries and about 20-30% of the population is affected by this disease³⁴⁾. NAFLD is strongly associated with features of metabolic syndrome such as insulin resistance³⁵⁾. In accordance with this, the ability of THA to strongly suppress the accumulation of fat in the liver of mice could efficiently prevent the development of metabolic syndrome. The activities of hepatic TG metabolism-related enzymes are shown in Table 4. These results mean that the suppression of fat accumulation in the liver is mainly controlled by the suppression of fatty acid synthesis because FAS and ME activities decreased significantly compared to that in the control. The average values of FAS and ME activity gradually decreased according to an increase in the number of double bonds and carbon atoms in n-3HUFA. Specifically, the suppression ability was THA > DHA > EPA. mRNA data also supports these results and the levels of ACC1 and AAC2 mRNAs, relating to fatty acid synthesis, also decreased according to an increase in the number of double bonds and carbon atoms in n-3HUFA (Table 5). This tendency was also observed in the mRNA for steroyl-CoA desaturase. Monocytes and macrophages play an important

Fig. 2 Comparison of the distribution of the main fatty acids in (A) liver, (B) muscle, (C) heart, (D) kidney, (E) testis, (F) brain, (G) epididymal fat, (H) adrenal gland, and (I) spleen. Values represent mean $\pm$ SE ($n = 5$). 14:0; myristic acid, 16:0; palmitic acid, 16:1n-7; palmitoleic acid, 18:0; stearic acid, 18:1n-9; oleic acid, 18:3n-3; α -linolenic acid, 20:4n-6; arachidonic acid, 20:5n-3; eicosapentaenoic acid (EPA), 22:5n-3; docosapentanoic acid (DPA), 22:6n-3; docosahexaenoic acid (DHA), 24:6n-3; tetracosahexaenoic acid (THA).

role in the development of atherosclerosis in vascular endothelial cells; therefore, MCP1 is thought to be one of the key factors for the prevention of cardiovascular disease³⁶⁾. As previously predicted, all n-3HUFAs could suppress the MCP1 mRNA levels significantly compared to that in the control. This result means that n-3HUFAs can suppress metabolic syndrome. It is reported that the gene expression of SREBP1c was suppressed by n-3PUFA^{37–39)}. However, our results did not suppress it. Probably, the detection method we employed in this study might be one cause because the method cannot distinguish SREBP1a and 1c. The other reason is the mouse species. The used mouse was C57BL/KsJ-*db/db* mouse. This mouse expresses much SREBP1 compared to normal C57BL6 mouse and this original high SREBP1 level might affect the expression of the mRNA by n-3PUFA administration. However, the suppression would have worked in transcriptive activity level and the suppression of gene expression concerning fatty acid synthesis would occur as the result. In contrast, the suppression ability of TG levels in the serum was in the order EPA>DHA>THA (Table 6). EPA had the strongest ability to suppress the TG level; the same was observed in our previous report. The EPA-TG group had increased insulin levels and this result was the same as in our previous report¹⁷⁾. Significant increases in adiponectin levels were observed in all n-3 HUFAs-TG groups compared to those in the control group. The adiponectin levels in DHA-TG and THA-TG groups were almost the same. These results indicate that the number of double bonds and carbon atoms of n-3HUFAs affect the serum parameters. The distribution of fatty acids in the respective group are summarized in Fig. 2. Interestingly, the EPA-TG group characteristically accumulated a high content of 22:5n-3, an intermediate product that is formed from 20:5n-3 to 22:6n-3 in the liver, muscle, heart, kidney, testis, adrenal gland, and spleen of animals. 24:6n-3 is characteristically found in the muscle, heart, testis, and spleen of the THA-TG group; however, the contents were not high even though the mice were administrated 24:6n-3. In contrast, the THA-TG group expressed a relatively high content of 22:6n-3 in the liver, muscle, heart, kidney, testis, adrenal gland, and spleen, and the level was almost the same as that in the DHA-TG group. This fact would mean that most of the administrated 24:6n-3 was beta-oxidized to form 22:6n-3. However, the accumulation of TG in the liver was suppressed in the order of THA (24:6n-3), DHA (22:6n-3), and EPA (20:5n-3). There might be a possibility that 24:6n-3 acts as the reserve compound of 22:6n-3 and be converted to 22:6n-3 when needed because only THA-TG group was detected the accumulation of 24:6n-3 (about 0.3%) in liver. Therefore, the continuous conversion of 22:6n-3 from 24:6n-3 might succeed in suppressing the accumulation of TG in the liver, lower the hepatic FAS and ME enzymatic activities, and lower hepatic ACC2 mRNA

levels, among others. The relative contents of 20:5n-3 and 22:5n-3 in the muscle, heart, kidney, adrenal gland, and spleen of the EPA-TG group were higher than those in the DHA-TG and THA-TG groups. However, the relative content of 22:6n-3 in the EPA-TG group in the organs and tissues was almost the same as that in the control group. These results suggest that the 20:5n-3 administered was converted to 22:5n-3 quickly; however, the formed 22:5n-3 was not converted to 22:6n-3 smoothly. The biosynthesis of 22:6n-3 from 20:5n-3 might be not effective. This idea was supported by previous report⁴⁰⁾. High relative contents of n-3HUFAs and 20:4n-6 were not detected in epididymal fat. Probably, these functional fatty acids are purposely transferred to organs and tissues where they are needed and not accumulated in the epididymal fat. The fatty acid composition of the brain was almost the same among the 5 groups, even when the mice species were different. It is known that the transportation of fatty acids into the brain is controlled by the blood brain barrier (BBB)⁴¹⁾. It is likely that the required fatty acid composition of the brain is not different between mice species and would be strictly controlled by the BBB.

5 CONCLUSION

THA (24:6n-3) is one of the n-3HUFAs found in marine organisms²¹⁾. THA is thought to be synthesized positively in starfish. The high accumulation of THA in starfishes could be caused by the decrease in the activation of enzymes related to beta-oxidation to form DHA from THA in the peroxisome of starfish. The accumulation of THA in Baltic herring and the flathead flounder could be related to the food chain because starfish are fish food²¹⁾. Therefore, THA is not a special n-3HUFAs and we can obtain it from fish. The order of activities relating to the suppression of hepatic TG and fatty acid synthesis among n-3HUFAs was found to be THA>DHA>EPA in this study. In our previous study, the order was DHA>DPA>EPA. Therefore, a combination of the two results suggests that the order of their activities among n-3HUFAs is THA>DHA>DPA>EPA. This was the same as that observed in the cell experiment and the order clearly translates to the rule that “the number of double bonds and carbon atoms in n-3HUFAs structure relates to their health functions”. There are many ideas to develop drugs for the treatment or suppression of metabolic syndrome. In this study, the level of cholesterol in the blood was not improved by n-3HUFAs; however, other factors such as TG level in the serum and liver, adiponectin levels in the blood, etc., were improved. New drugs for the treatment of metabolic syndrome could be developed using the rule confirmed in this study. The effect of THA on the formation of resolvins and protectins⁴²⁾ was not confirmed in this study and will be our next

topic of study.

Conflict of interest

The authors declare no conflicts of interest.

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