Compensatory induction of Fads1 gene expression in heterozygous Fads2-null mice and by diet with a high n-6/n-3 PUFA ratio[s]

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Abstract In mammals, because they share a single synthetic pathway, n-6/n-3 ratios of dietary PUFAs impact tissue arachidonic acid (ARA) and DHA content. Likewise, SNPs in the human fatty acid desaturase (FADS) gene cluster impact tissue ARA and DHA. Here we tested the feasibility of using heterozygous Fads2-null-mice (HET) as an animal model of human FADS polymorphisms. WT and HET mice were fed diets with linoleate/α-linolenate ratios of 1:1, 7:1, and 44:1 at 7% of diet. In WT liver, ARA and DHA in phospholipids varied >2× among dietary groups, reflecting precursor ratios. Unexpectedly, ARA content was only <10% lower in HET than in WT livers, when fed the 44:1 diet, likely due to increased Fads1 mRNA in response to reduced Fads2 mRNA in HET. Consistent with the RNA data, C20:3n-6, which is elevated in minor FADS haplotypes in humans, was lower in HET than WT. Diet and genotype had little effect on brain PUFAs even though brain Fads2 mRNA was low in HET. No differences in cytokine mRNA were found among groups under unstimulated conditions.[Jlr] In conclusion, differential PUFA profiles between HET mice and human FADS SNPs suggest low expression of both FADS1 and 2 genes in human minor haplotypes.—Su, H., D. Zhou, Y-X. Pan, X. Wang, and M. T. Nakamura. **Compensatory induction of Fads1 gene expression in heterozygous Fads2-null mice and by diet with a high n-6/n-3 PUFA ratio.** *J. Lipid Res.* 2016. **57:** 1995–2004.

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Linoleic acid (C18:2 n-6; LA) and α-linolenic acid (C18:3 n-3; ALA) are essential FAs because they cannot be synthesized in mammals and must be obtained from diet (1, 2). They are precursors of the physiologically essential highly unsaturated FAs (HUFAs) arachidonic acid (C20:4 n-6; ARA) and DHA (C22:6 n-3), respectively. The n-6 and n-3 FA families share the same synthetic pathway that involves a series of desaturation and elongation (2, 3). Therefore, competition exists between n-6 and n-3 FAs for utilizing the enzymes, and overconsumption of one results in a significant decrease in products of the other (4, 5). In general, the n-6 and n-3 families have an opposite effect in the progress of inflammation through their derivatives (6). Upon activation of phospholipase A_2, ARA and its n-3 counterpart EPA are released from cell membrane phospholipids (PLs) and compete for synthesis of eicosanoids such as prostaglandins and leukotrienes (7). Generally, 2-series prostaglandins and 4-series leukotrienes derived from ARA promote inflammation, whereas EPA-originated 3-series prostaglandins and 5-series leukotrienes are less inflammatory or anti-inflammatory (6, 8, 9). Also, docosanoids such as resolvins and neuroprotectins derived from DHA were reported to have anti-inflammatory effects (10). Chronic inflammation contributes to the development of several diseases including atherosclerosis, cancer, and neurological disorders (11–13).

Recently, large-scale, genome-wide association studies identified strong associations of SNPs in the region of the fatty acid desaturase (FADS) gene cluster with disease or its risk factors such as blood lipids (14–16), blood glucose (16), and asthma (17). Minor alleles (designated by the

Abbreviations: ALA, alpha-linolenic acid; ARA, arachidonic acid; CE, cholesteryl ester; CO, corn oil; DGLA, dihomo-γ-linolenic acid; Elovl, elongation of very long chain fatty acids; FADS/Fads, fatty acid desaturase; FCO, flaxseed plus canola oil; HUFA, highly unsaturated fatty acid; HET, heterozygous Fads2-null mouse; Il1b, interleukin 1 beta; Il6, interleukin 6; LA, linoleic acid; LSD, least significant difference; PL, phospholipid; RBC, red blood cell, erythrocyte; Rpl7a, ribosomal protein L7alpha; SO, soybean oil.

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NCBI dbSNP database) in the FADS gene cluster, where two desaturase genes, FADS1 and FADS2, for the ARA and DHA synthesis are located, were generally associated with a lower product/substrate ratio of n-6 and n-3 PUFAs in serum or red blood cells (RBCs), indicating lower capacity of ARA, DHA synthesis (18–22). Furthermore, the allele frequency of the FADS cluster differs among populations; some minor alleles in most populations occur predominantly in certain populations (23, 24), suggesting environmental effects on the allele frequency. Taken together, the genetic capacity of endogenous synthesis of ARA and DHA likely interacts with environmental factors and affects disease susceptibility and survival. Although the environmental factors that affect the allele frequency in the FADS gene cluster are yet to be identified, a dietary n-6/n-3 ratio is likely one of them. For example, a reduced capacity in synthesis when combined with a diet with a high LA/ALA ratio may attenuate elevation of tissue ARA and subsequent overreaction of inflammatory response, whereas it may compromise adequate supply of DHA that can be compensated if a diet contains sufficient preformed DHA.

Our laboratory created Fads2-null mice, in which synthesis of ARA and DHA was disabled. Because heterozygous Fads2-null mice (HET) have reduced delta-6-desaturase expression, we conducted this study to address if the HET mouse is suitable as a model of human polymorphism in the FADS gene cluster. To achieve this objective, we investigated the effects of Fads2 genotypes on tissue ARA and DHA abundance in liver, brain, gastrocnemius muscle, and RBCs and on the gene expression of synthetic pathway of these HUFAs under diets with a varying ratio of dietary precursors, LA and ALA.

MATERIALS AND METHODS

Animals and diets

All the animal experiments were approved by the University of Illinois Institutional Animal Care and Use Committee and were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Production of the Fads2-null mouse was described previously (3). The mouse was subsequently backcrossed to C57BL/6J background. Heterozygous and WT mice from our breeding colony were mated to produce WT and HET animals for the study. Until 2 months of age, the animals were fed a standard purified diet, AIN93G diet (Dyets Inc., Bethlehem, PA). At 2 months, 18 WT and 18 HET male mice were evenly divided into three dietary groups and were singly housed in a 12 h light/dark cycle. They had free access to food and water. Body weight was recorded every week. Three experimental diets were prepared by adding 7% (w/w) soybean oil (SO), corn oil (CO), and a mixture of flaxseed and canola oil (FCO) to a fat-free AIN93G diet (Dyets Inc.) to achieve the ratio of LA/ALA 7:1, 44:1, and 1:1, respectively, as shown in **Table 1**. After being fed the respective diets for 14 weeks, mice were fasted overnight before euthanization. Final body weight was recorded. Blood was drawn by cardiac puncture after mice were anesthetized under isoflurane gas. Plasma and erythrocytes (RBCs) were separated by centrifuging at 800 g for 10 min at 4°C. Liver, brain, and gastrocnemius were removed and snap frozen in liquid N_2. All samples were stored in a −80°C freezer until further analysis.

TABLE 1. FA composition of experimental diets

	FCO	SO	CO
16:0	4.4	10.2	10.6
18:0	2.5	4.5	1.8
18:1	53.2	22.7	27.3
18:2 n-6	18.4	54.8	53.2
18:3 n-3	18.4	7.8	1.2
n-6:n-3	1:1	7:1	44:1

Values are wt. % of total FAs. Each diet contains 7% fat. FCO was prepared by mixing flaxseed oil and canola oil in a ratio of 1:4.

Plasma lipid analysis

Plasma total cholesterol and TGs were measured by enzymatic kits (Cholesterol E and L-Type TG M; Wako Life Sciences Inc., Mountain View, CA) according to the manufacturer's instructions.

Total RNA extraction and quantitative PCR

Total RNA was isolated from liver, brain, and gastrocnemius muscle with a Trizol reagent and a purification kit Direct-Zol™ RNA Miniprep (Zymo Research Corp., Irvine, CA). About 25 mg of liver and 60 mg of brain and gastrocnemius muscle were used. After adjusting the RNA concentration to 100 ng/µl, 10 µl from each sample was prepared for the synthesis of cDNA by a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Then, 25ng cDNA template was amplified by a 7300 real-time PCR system (Applied Biosystems) under the following conditions: 95°C for 10 min, then 15 s at 95°C and 1 min at 60°C for 35 cycles. The primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences were previously published for the primers of Fads2 (25), elongase of very long chain fatty acid (Elovl) 5 (26), interleukin 1 β (Il1b) (27), interleukin 6 (Il6) (27), and tumor necrosis factor (Tnf) (28). These three cytokines are chosen as markers of proinflammatory condition (29). Additional genes were analyzed using the following primer pairs: Fads1, 5′ primer, 5′-CCAGCTTTGAACCCAC-CAA-3′ and 3′ primer, 5′-CATGAGGCCCATTCGCTCTA-3′; Elovl2, 5′ primer, 5′-GCTAATGGCATGACGGACA-3′ and 3′ primer, 5′-GTTCCCCGGCACTTCATTT-3′; ribosomal protein L7α (Rpl7a as a housekeeping gene) 5′ primer, 5′-AACTTCG-GCATTGGACAGG ACA-3′, and 3′ primer, 5′-TTTGAGCCGCTT-GTAGAGGATAGC-3′. A standard curve was constructed in every run to account for the efficiency of each primer set. A pooled cDNA was sequentially diluted to half each time from 100 to 3.1 ng and was used for the standard curve construction. All gene expression data were normalized with Rpl7a. In addition, to compare gene expression between different genes and organs, a normalizing factor $2^{-\Delta\Delta Ct}$ was back-calculated from the standard curves using the following formula:

$$\Delta\Delta Ct = [(Ct\ of\ Gene\ X\ in\ Tissue\ Y\ at\ 25\ ng - Ct\ of\ Rpl7a\ in\ Tissue\ Y\ at\ 25\ ng) - (Ct\ of\ Fads2\ in\ liver\ at\ 25\ ng - Ct\ of\ Rp17a\ in\ liver\ at\ 25\ ng)]$$

Thus, all RNA expression data were expressed relative to the mean of liver Fads2, which was set as 100.

Total lipid extraction, thin-layer chromatography, and methylation

A one-step extraction-methylation method was used for brain, gastrocnemius, and RBC samples (30). Briefly, ~100 mg tissue was added to a glass tube containing 2 ml of 4:1 methanol and hexane and 100 µg butylated hydroxytoluene. After homogenization on ice and flushing a tube with nitrogen for 20 s, 200 µl of acetyl

chloride was slowly added. The tube was then flushed with nitrogen, capped tightly, and incubated for 1 h in a water bath at 100°C. Five milliliters of 6% K_2CO_3 was added to the tube after cooling on ice. After centrifugation at 3,200 g for 3 min, the upper layer containing FA methyl esters was transferred to a 2.0 ml vial. For liver samples, total lipid was first extracted from 150 mg of frozen samples by the method of Folch (31). The lipid extracts were separated with thin-layer chromatography to cholesteryl ester (CE), TG, and PL fractions, which were then methylated individually (3).

FA analysis

Methylated FAs in hexane were analyzed using a GC-2010 Plus gas chromatograph (Shimadzu, Columbia, MD) equipped with a flame ionization detector and a DB-FFAP capillary column (15 m × 0.1 mm inner diameter, 0.1 μm film thickness; Agilent Technologies, Santa Clara, CA). A program was based on the method of Masood (32). FA data were analyzed by GC Solution (Shimadzu) and presented as weight percent of total FAs.

Statistical analyses

Data are presented as mean ± SD (n = 6 for each group). Six animals per group was sufficient to reach a power of 0.8 with a type I error rate of 0.05. All data were analyzed using two-way ANOVA (2 genotypes × 3 diets), and when significance was found, data sets were further analyzed using one-way ANOVA followed by a post hoc test using the least significant difference (LSD) multiple comparison. Analysis by another post hoc test, Duncan's multiple range test, yielded essentially identical results to LSD. Difference was considered significant at $P < 0.05$.

RESULTS

Both diet and genotype affected mRNA expression of desaturases and elongases in liver

No significant dietary or genotype effect was observed in body weight, tissue weight, plasma lipids, or liver TG, although brain was heavier in SO-fed HET than in CO-fed WT, and plasma cholesterol was lower in SO-fed HET than in CO-fed HET by LSD test (supplemental Table S1). In the liver of WT, the CO group with a high ratio of dietary n-6/n-3 precursors increased the mRNA of all 4 synthetic enzymes, Fads2, Fads1, Elovl5, and Elovl2, compared with SO and FCO groups, whereas none of four genes was different between SO and FCO (**Fig. 1A**). As expected, the expression of Fads2 gene in HET was about half of that in WT. Fads1 gene expression in HET liver was higher than that in WT in the FCO and SO groups, suggesting compensatory increase of Fads1 expression when Fads2 expression is limited. However, in the CO group, Fads1 in HET did not increase over WT, suggesting the Fads1 expression may have reached maximum in the CO group. On the other hand, the genotype had no effect on Elovl2 or Elovl5.

In the brain of WT, no dietary effect was observed in the expression of Fads2, Fads1, Elovl5, or Elovl2 (Fig. 1B). Fads2 expression in HET was about half of WT, but no compensatory change was observed in other genes, indicating constitutive expression in brain. Surprisingly, Elovl2 expression was an order of magnitude lower than other three genes. This expression pattern suggests that the role of the pathway in brain may be primarily supplying ARA, and that brain may acquire DHA mostly from circulation.

In the gastrocnemius muscle, mRNA of Fads2 and Fads1 was much lower than in liver and brain, suggesting a limited synthetic capacity. In the muscle of WT, unlike liver, the FCO group showed small but significantly higher Fads2 and Fads1 mRNA compared with the SO group, whereas the expression of these genes in the CO group fell in between (Fig. 1C). Like brain, no genotype effect was observed except Fads2 expression in HET was about half of WT.

Diet showed stronger effects than genotype on PUFA composition in liver

As shown in **Table 2**, two-way ANOVA revealed that the n-6/n-3 ratio of precursor FAs in diet had strong effects on the abundance of product HUFAs in liver PLs in both genotypes. ARA in the SO and CO groups was nearly 2× and 3×, respectively, higher than that of FCO, whereas DHA in FCO and SO was more than twice as that in the CO group as dietary ALA was increased. C22:5n-6 significantly increased only in the CO group, whereas EPA decreased from 4% in FCO to <1% in SO and CO groups. In liver TGs, as the dietary LA to ALA ratio increased, all products of LA were significantly increased, whereas all ALA products were decreased significantly although the abundance of each HUFA is 2% or less in TGs (supplemental Table S2). PUFAs in the liver CE fraction also showed dietary effects similar to FAs in TGs (supplemental Table S3). In WT animals, LA was significantly lower, and ARA was higher in CO than in SO in all PL, TG, and CE fractions, although ARA in TGs did not reach statistical significance (Table 2; supplemental Tables S2 and S3), reflecting induction of genes of the synthetic pathway (Fig. 1A).

Contrary to our expectation, the magnitude of the genotype effect was weaker than the dietary effect. The HET animals exhibited significantly lower ARA than WT in liver PLs only in the CO group in which dietary LA/ALA was high, but the difference was small, a decrease of only 9% (24% and 22% in WT and HET, respectively) (Table 2). A decline of ARA and DHA with a larger magnitude was found in liver CE (supplemental Table S3), confirming a previous observation (3). The most consistently found genotype effects across dietary treatment were a decrease in dihomo-γ-linolenic acid (C20:3 n-6; DGLA) and an increase in C20:2n-6 in liver PLs from HET compared with WT (Table 2). Also, DGLA in HET was significantly lower in TG and CE fractions of liver (supplemental Tables S2 and S3). Even though decreased expression of Fads2 in HET mice, neither DHA nor C22:5 n-6, the products of the second desaturation by Δ6 desaturase, decreased in any lipid fractions of HET except a small decline of DHA in the CE fraction (Table 2; supplemental Tables S2 and S3). This lack of genotype effects suggests that the second desaturation by Δ6 desaturase is not rate limiting.

Brain PUFA composition was less affected by diet and genotype than in liver

As presented in **Table 3**, the diet had a significant effect on all PUFAs except the LA in brain. However, as expected,
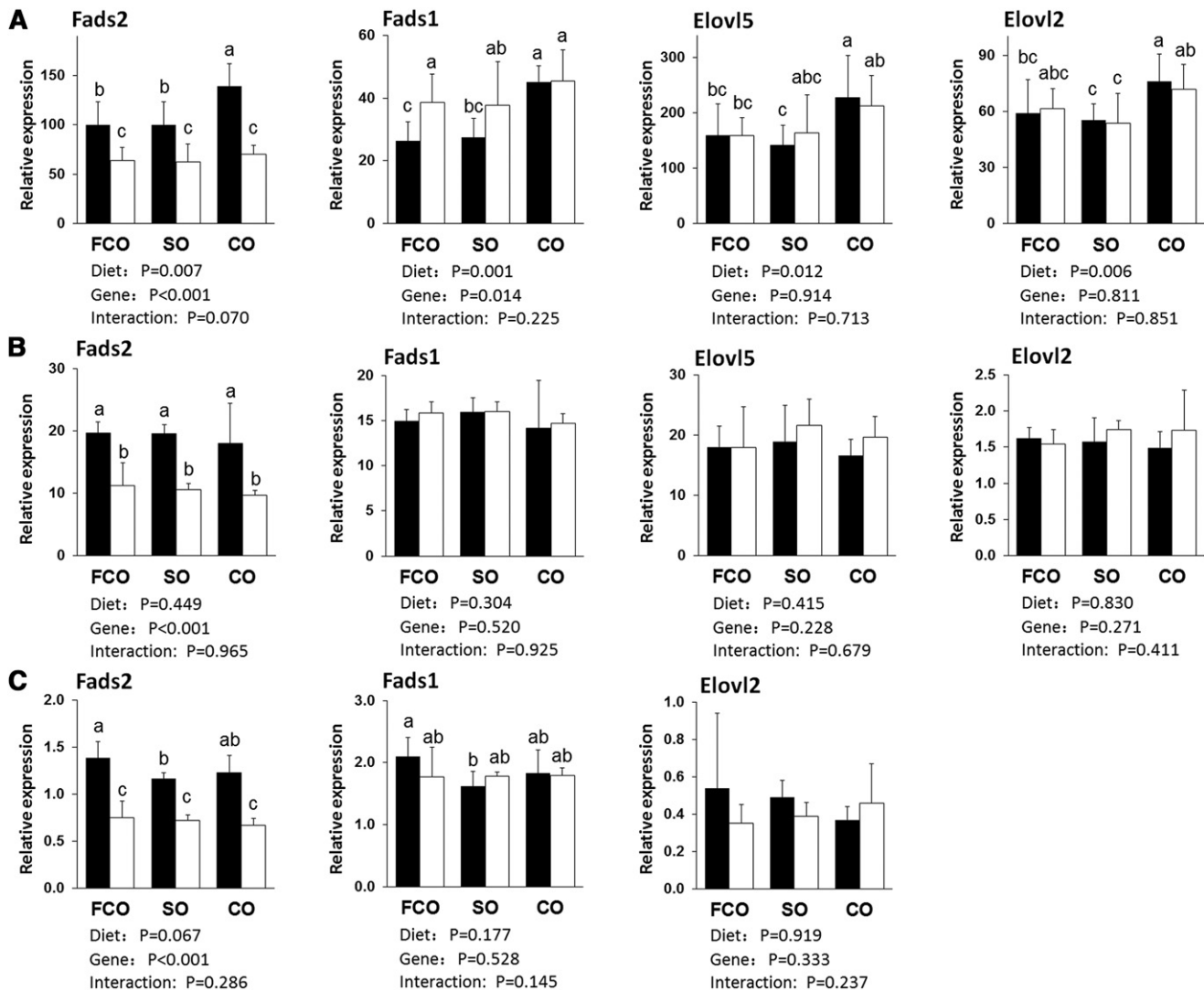
Fig. 1. Expression of genes for HUFA synthesis in liver (A), brain (B), and gastrocnemius muscle (C). A: In liver, CO diet with high LA/ALA ratio increased Fads2, Fads1, Elovl2, and Elovl5 mRNA in WT mice liver. HET showed elevated Fads-1 expression compared with WT. In HET mice, Fads2 mRNA was about half of that in WT. B: Except for decreased Fads2 expression in HET, no other dietary or genotype effect was found in brain. The expression of Elovl2 was an order of magnitude lower than other three genes. C: In gastrocnemius muscle, Fads2 in HET was about half in WT. Expression of Fads genes were much lower than in liver and brain. Black bars, WT; white bars, HET. Values were normalized with the housekeeping gene (Rpl7a) and expressed as relative to mean liver Fads2, which was set as 100. Mean ± SD, n = 6. Bars without same letter were significantly different, $P < 0.05$ by LSD multiple comparison. P values for the effect of diet, gene, and their interaction were calculated by two-way ANOVA.

the changes in ARA and DHA, the two major HUFAs in brain, were much smaller compared with the changes in liver PLs. In HET, the level of DGLA was significantly lower compared with WT. In general, the brain PUFA composition was maintained in a narrow range, indicating the presence of a mechanism to resist dietary and genetic influence.

PUFA composition in gastrocnemius muscle was strongly affected by diet but much less by genotype

As shown in **Table 4**, high dietary LA/ALA ratio significantly elevated proportions of all n-6 PUFAs. The LA level in the SO and CO groups was more than twice of that in FCO. The ARA in the SO and CO groups doubled and tripled, respectively, compared with the FCO group. The n-3 family exhibited an opposite trend. Particularly,

the ALA in the FCO groups was 20-fold higher than that in the CO group, and the DHA level in FCO was twice as that in CO (Table 4). The genotype effect was less pronounced; the DGLA level was lower in HET compared with WT, whereas C20:2 n-6 was increased in HET (Table 4).

PUFA composition in RBCs was similar to that in liver PLs

As shown in **Table 5**, the diet had a very strong effect on all n-6 PUFAs except on DGLA. The CO and SO groups showed similar ARA level, which was twice as much as that in the FCO group. The n-3 PUFAs showed an opposite trend of n-6 PUFAs. As seen in the other tissues, DGLA showed lower percentage in HET than in WT, whereas the 20:2 n-6 increased in HET compared with WT. No genotype effect was found in n-3 PUFA composition.

TABLE 2. PUFA composition of liver PLs (wt. %)

| Fatty Acid | FCO | | SO | | CO | | P | | |
	WT	HET	WT	HET	WT	HET	Diet	Gene	Gene × Diet
18:2 n-6	13.65 ± 0.81^b	14.11 ± 1.2^b	16.16 ± 1.86^a	17.49 ± 1.96^a	13.24 ± 1.68^b	16.75 ± 0.86^a	***	**	*
18:3 n-6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:2 n-6	0.34 ± 0.03^{bc}	0.41 ± 0.08^{ab}	0.29 ± 0.07^c	0.46 ± 0.07^a	0.31 ± 0.04^c	0.44 ± 0.04^a		***	
20:3 n-6	2 ± 0.28^a	1.45 ± 0.2^b	2.07 ± 0.34^a	1.33 ± 0.08^{bc}	1.84 ± 0.28^a	1.15 ± 0.15^c	*	***	
20:4 n-6	8.77 ± 1.01^d	8.5 ± 1.3^d	17.76 ± 1.1^c	16.49 ± 0.97^c	24.15 ± 1.21^a	22.16 ± 0.9^b	***	**	
22:4 n-6	0.05 ± 0.01^c	0.05 ± 0.04^c	0.21 ± 0.11^b	0.27 ± 0.13^b	0.39 ± 0.02^a	0.37 ± 0.02^a	***		
22:5 n-6	0.04 ± 0.01^b	0.05 ± 0.02^b	0.19 ± 0.15^b	0.25 ± 0.17^b	2.37 ± 0.29^a	2.25 ± 0.29^a	***		
18:3 n-3	0.64 ± 0.13^a	0.67 ± 0.1^a	0.19 ± 0.03^b	0.22 ± 0.05^b	0.04 ± 0.04^c	0.03 ± 0.02^c	***		
20:3 n-3	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:5 n-3	4.36 ± 0.29^a	3.87 ± 0.87^b	0.66 ± 0.13^c	0.55 ± 0.21^{cd}	0.11 ± 0.03^d	0.09 ± 0.04^d	***		
22:5 n-3	0.81 ± 0.02^a	0.79 ± 0.1^a	0.41 ± 0.03^b	0.35 ± 0.07^c	0.12 ± 0.01^d	0.1 ± 0.02^d	***		
22:6 n-3	13.62 ± 0.72^a	13.36 ± 0.54^a	10.82 ± 0.49^b	10.54 ± 0.59^b	5.55 ± 1.36^c	4.98 ± 0.18^c	***		
Total n-6	24.85 ± 0.77^c	24.56 ± 1.28^c	36.68 ± 1.24^b	36.29 ± 1.57^b	42.3 ± 1.33^a	43.12 ± 0.85^a	***		
Total n-3	19.43 ± 0.65^a	18.68 ± 1.06^a	12.09 ± 0.62^b	11.66 ± 0.87^b	5.81 ± 1.37^c	5.2 ± 0.17^c	***		
n-6/n-3	1.28 ± 0.08^c	1.32 ± 0.13^c	3.04 ± 0.17^b	3.13 ± 0.23^b	7.64 ± 1.83^a	8.3 ± 0.38^a	***		
AA/DHA	0.65 ± 0.10^c	0.64 ± 0.09^c	1.65 ± 0.16^b	1.57 ± 0.14^b	4.53 ± 0.89^a	4.46 ± 0.19^a	***		

n. d., not detected. Values are means ± SD (n = 6); data that do not share same superscript letter(s) within a row are significantly different, $P < 0.05$ by LSD multiple comparison. Effects of diet, gene, and their interaction were calculated by two-way ANOVA; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Proinflammatory cytokine gene expression

As low-grade inflammation is implicated to be involved with the development of chronic diseases in tissues including liver and brain, we investigated if abundance of tissue ARA affected cytokine expression. Shown in **Table 6** is the mRNA of proinflammatory cytokines in liver and brain. No significant difference was found in any cytokines tested, indicating neither the diet nor the genotype had an influence on these cytokines gene expression in an unstimulated condition.

DISCUSSION

Impact of dietary LA/ALA ratio on HUFA synthesis

The enzyme activity (33) and mRNA (34) of Δ6 desaturase increase when animals are fed a diet free of n-6 and n-3 essential FAs. The activity of Δ6 desaturase, the first step of ARA and DHA synthesis is primarily regulated at the transcriptional level by transcription factors such as sterol regulatory element-binding protein (SREBP) 1c (35), PPARα (36), and Elk (37). Elk is likely to mediate the effect of growth hormone on Fads2 (38, 39). Strong associations of SNPs in the FADS gene cluster with HUFA profiles and chronic diseases further suggest physiological importance of transcriptional regulation of the pathway. In this study, we found that gene expression of Fads2 as well as Fads1, Elovl 5, and Elovl 2 were also regulated by a ratio of dietary LA and ALA. The CO diet with LA/ALA ratio of 44:1 increased the expression of all four genes required for synthesis of DHA and ARA in the liver of WT compared with the SO diet with the ratio of 7:1. This upregulation of synthetic pathway in the CO group is likely a mechanism to compensate the decreased conversion of dietary ALA to DHA, but the upregulation also increased the conversion of LA to ARA, resulting in a large increase in ARA in liver PL and CE fractions and a decrease in LA in liver PL, TG, and CE fractions. In contrast, changing the LA/ALA ratio to 1:1 from 7:1 did not alter the expression of the desaturase and elongase genes in liver even though ARA in the

TABLE 3. PUFA composition of brain (wt. %)

| Fatty Acid | FCO | | SO | | CO | | P | | |
	WT	HET	WT	HET	WT	HET	Diet	Gene	Gene × Diet
18:2 n-6	0.41 ± 0.06^b	0.45 ± 0.04^{ab}	0.65 ± 0.24^{ab}	0.65 ± 0.05^{ab}	0.42 ± 0.04^b	1.03 ± 1.22^a			
18:3 n-6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:2 n-6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:3 n-6	0.44 ± 0.01^a	0.39 ± 0.04^b	0.35 ± 0.06^b	0.27 ± 0.06^c	0.25 ± 0.02^c	0.20 ± 0.02^d	***	***	
20:4 n-6	7.54 ± 0.38^b	7.51 ± 0.58^b	8.63 ± 0.41^a	8.29 ± 0.39^a	8.80 ± 0.52^a	8.68 ± 0.37^a	***		
22:4 n-6	1.71 ± 0.06^c	1.70 ± 0.10^c	2.06 ± 0.15^b	1.97 ± 0.04^b	2.33 ± 0.15^a	2.31 ± 0.08^a	***		
22:5 n-6	0.08 ± 0.02^b	0.09 ± 0.02^b	0.15 ± 0.03^b	0.15 ± 0.04^b	0.84 ± 0.15^a	0.81 ± 0.08^a	***		
18:3 n-3	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:3 n-3	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:5 n-3	0.12 ± 0.01^a	0.14 ± 0.05^a	0.05 ± 0.03^b	0.06 ± 0.02^b	0.04 ± 0.01^b	0.05 ± 0.03^b	***		
22:5 n-3	0.29 ± 0.02^a	0.31 ± 0.04^a	0.13 ± 0.02^b	0.13 ± 0.02^b	0.07 ± 0.02^c	0.08 ± 0.01^c	***	*	
22:6 n-3	13.12 ± 1.56^a	13.03 ± 1.20^a	12.76 ± 1.12^a	12.05 ± 0.93^{ab}	11.28 ± 1.25^b	11.25 ± 0.89^b	**		
Total N-6	10.18 ± 0.40^c	10.13 ± 0.66^c	11.85 ± 0.52^b	11.32 ± 0.43^b	12.64 ± 0.77^a	13.03 ± 0.91^a	***		
Total N-3	13.53 ± 1.58^a	13.48 ± 1.24^a	12.94 ± 1.14^a	12.25 ± 0.95^{ab}	11.38 ± 1.27^b	11.38 ± 0.89^b	***		
N-6/N-3	0.76 ± 0.06^c	0.75 ± 0.03^c	0.92 ± 0.06^b	0.93 ± 0.06^b	1.12 ± 0.06^a	1.15 ± 0.14^a	***		
AA/DHA	0.58 ± 0.04^c	0.58 ± 0.02^c	0.68 ± 0.05^b	0.69 ± 0.04^b	0.78 ± 0.05^a	0.77 ± 0.04^a	***		

n. d., not detected. Values are means ± SD (n = 6); data that do not share same superscript letter(s) within a row are significantly different, $P < 0.05$ by LSD multiple comparison. Effects of diet, gene, and their interaction were calculated by two-way ANOVA; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

TABLE 4. PUFA composition of gastrocnemius muscle (wt. %)

	FCO		SO		CO		P		
Fatty Acid	WT	HET	WT	HET	WT	HET	Diet	Gene	Gene × Diet
18:2 n-6	8.69 ± 1.34^b	8.47 ± 1.24^b	19.59 ± 2.73^a	19.10 ± 4.61^a	20.11 ± 2.81^a	19.16 ± 4.59^a	***		
18:3 n-6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:2 n-6	0.07 ± 0.03^c	0.09 ± 0.03^c	0.17 ± 0.04^b	0.22 ± 0.05^{ab}	0.20 ± 0.03^b	0.26 ± 0.07^a	***	*	
20:3 n-6	0.33 ± 0.12^{bc}	0.26 ± 0.11^c	0.41 ± 0.09^{ab}	0.28 ± 0.12^{bc}	0.50 ± 0.14^a	0.39 ± 0.16^{abc}	*	*	
20:4 n-6	1.53 ± 0.74^c	1.68 ± 0.61^c	3.30 ± 0.64^{bc}	3.68 ± 1.61^{ab}	5.22 ± 1.91^a	5.36 ± 2.58^a	***		
22:4 n-6	0.06 ± 0.03^c	0.07 ± 0.02^c	0.33 ± 0.05^{bc}	0.36 ± 0.16^b	0.94 ± 0.33^a	0.89 ± 0.44^a	***		
22:5 n-6	0.06 ± 0.03^c	0.06 ± 0.03^b	0.43 ± 0.15^b	0.61 ± 0.44^b	3.04 ± 0.99^a	3.16 ± 1.86^a	***		
18:3 n-3	3.43 ± 1.20^a	3.22 ± 0.85^a	1.60 ± 0.34^b	1.52 ± 0.46^b	0.17 ± 0.09^c	0.16 ± 0.07^c	***		
20:3 n-3	0.03 ± 0.01^a	0.03 ± 0.01^a	0.01 ± 0.00^b	0.02 ± 0.02^{ab}	0.02 ± 0.01^{ab}	0.03 ± 0.01^a			
20:5 n-3	0.46 ± 0.39^a	0.23 ± 0.07^b	0.09 ± 0.02^b	0.07 ± 0.02^b	0.06 ± 0.06^b	0.05 ± 0.03^b	***		
22:5 n-3	1.64 ± 0.70^{ab}	1.78 ± 0.75^a	1.12 ± 0.35^b	1.07 ± 0.63^b	0.35 ± 0.14^c	0.43 ± 0.31^c	***		
22:6 n-3	8.68 ± 4.93^a	9.12 ± 3.98^a	7.18 ± 1.70^{ab}	8.63 ± 4.00^a	3.50 ± 1.17^b	4.05 ± 2.68^b	**		
Total n-6	10.74 ± 0.48^c	10.62 ± 0.63^c	24.24 ± 1.94^b	24.25 ± 2.74^b	30.01 ± 1.24^a	29.22 ± 1.36^a	***		
Total n-3	14.23 ± 4.68^a	14.38 ± 3.89^a	10.00 ± 1.71^b	11.31 ± 4.04^{ab}	4.10 ± 1.23^c	4.72 ± 2.95^c	***		
n-6:n-3	0.84 ± 0.33^b	0.79 ± 0.23^b	2.49 ± 0.49^b	2.54 ± 1.40^b	7.74 ± 1.76^a	7.80 ± 3.53^a	***		
AA/DHA	0.18 ± 0.77^c	0.18 ± 0.52^c	0.46 ± 0.16^b	0.43 ± 0.38^b	1.49 ± 0.06^a	1.32 ± 0.12^a	***		

n. d., not detected. Values are means ± SD (n = 6); data that do not share same superscript letter(s) within a row are significantly different, $P < 0.05$ by LSD multiple comparison. Effects of diet, gene, and their interaction were calculated by two-way ANOVA; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

liver PLs decreased to half. This lack of upregulation in response to decreased ARA suggests that the pathway of DHA and ARA synthesis is primarily regulated to meet DHA requirement rather than ARA requirement in liver. The mechanism underlying this differential regulation by DHA and ARA is yet to be elucidated.

In contrast, the brain gene expression was unaffected by diet. Our results are consistent with studies by Igarashi et al. (40–42) that reported upregulation of synthetic genes in liver but not in brain when rats were fed diets devoid of n-3 FAs for 15 weeks even though brain DHA decreased by nearly 40%. Skeletal and heart muscle in mice contains high DHA (43, 44) compared with other species such as rats (45), pigs (46), and humans (47, 48). Our study showed that unlike brain, the DHA content in gastrocnemius muscle markedly decreased and partly replaced with 22:5 n-6 when the dietary LA/ALA ratio was increased, although it is currently unknown if this change affects physiological function of skeletal muscle. The mechanism underlying the higher expression of both Fads2 and Fads1 mRNA in the WT FCO than in WT SO in muscle is unknown, but it is possible that the pathway may be under feedback regulation by ARA in the muscle.

Differences in FA profiles between HET mice and human FADS SNPs

Our study revealed critical differences in FA profiles between HET mouse model and human SNPs in the FADS gene cluster. **Figure 2** summarizes the changes in the gene expression and FA compositions in HET animals compared with WT (shown in red). Also, changes in FA profile in minor alleles of human SNPs compared with major alleles are overlaid in Fig. 2 (shown in blue). Although HET mice and human minor SNPs share similar changes in FA profiles, human SNPs showed more extensive changes (Fig. 2). There is a notable difference in the abundance of DGLA between HET

TABLE 5. PUFA composition of RBCs (wt. %)

	FCO		SO		CO		P		
Fatty Acid	WT	HET	WT	HET	WT	HET	Diet	Gene	Gene × Diet
18:2 n-6	8.1 ± 0.38^c	8.82 ± 0.79^{bc}	11.25 ± 1.38^a	12.17 ± 1.19^a	10.44 ± 0.94^{ab}	11.55 ± 3.51^a	***		
18:3 n-6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:2 n-6	0.14 ± 0.05^c	0.19 ± 0.09^{bc}	0.24 ± 0.05^b	0.35 ± 0.1^a	0.24 ± 0.03^b	0.26 ± 0.05^b	***	*	
20:3 n-6	1.38 ± 0.21^a	1.02 ± 0.2^b	1.61 ± 0.33^a	0.98 ± 0.23^b	1.42 ± 0.24^a	1.03 ± 0.29^b		***	
20:4 n-6	9.84 ± 1.49^b	9.89 ± 1.18^b	17.68 ± 2.23^a	16.87 ± 1.65^a	18.67 ± 2.46^a	18.21 ± 5.23^a	***		
22:4 n-6	0.26 ± 0.12^c	0.25 ± 0.14^c	1.42 ± 0.31^b	1.42 ± 0.25^b	2.14 ± 0.39^a	2.14 ± 0.64^a	***		
22:5 n-6	0.15 ± 0.17^b	0.15 ± 0.16^b	0.45 ± 0.15^b	0.47 ± 0.17^b	2.46 ± 0.59^a	2.39 ± 0.8^a	***		
18:3 n-3	0.52 ± 0.34^a	0.62 ± 0.26^a	0.18 ± 0.13^b	0.18 ± 0.12^b	0.03 ± 0.01^b	0.02 ± 0.03^b	***		
20:3 n-3	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.			
20:5 n-3	2.91 ± 0.48^a	2.82 ± 0.57^a	0.45 ± 0.15^b	0.32 ± 0.17^{bc}	0.03 ± 0.02^c	0.03 ± 0.01^c	***		
22:5 n-3	1.85 ± 0.31^a	1.84 ± 0.29^a	0.86 ± 0.16^b	0.81 ± 0.26^b	0.18 ± 0.04^c	0.17 ± 0.05^c	***		
22:6 n-3	7.9 ± 1.01^a	7.7 ± 0.87^a	6.08 ± 0.7^b	5.92 ± 1.13^b	2.65 ± 0.45^c	2.54 ± 0.74^c	***		
Total n-6	19.73 ± 1.54^c	20.13 ± 0.81^c	32.41 ± 1.83^b	31.92 ± 1.61^b	35.13 ± 2.63^a	35.33 ± 3.4^a	***		
Total n-3	13.18 ± 1.44^a	12.98 ± 1.49^a	7.58 ± 0.81^b	7.24 ± 1.45^b	2.88 ± 0.48^c	2.77 ± 0.76^c	***		
n-6:n-3	1.5 ± 0.06^c	1.57 ± 0.24^c	4.32 ± 0.55^b	4.58 ± 1.05^b	12.41 ± 1.64^a	13.86 ± 4.72^a	***		
AA/DHA	1.24 ± 0.05^c	1.3 ± 0.24^c	2.92 ± 0.3^b	2.93 ± 0.57^b	7.12 ± 0.59^a	7.18 ± 0.15^a	***		

n. d., not detected. Values are means ± SD (n = 6); data that do not share same superscript letter(s) within a row are significantly different, $P < 0.05$ by LSD multiple comparison. Effects of diet, gene, and their interaction were calculated by two-way ANOVA; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

TABLE 6. Cytokine gene expression in liver and brain

	FCO		SO		CO	
	WT	HET	WT	HET	WT	HET
Liver						
Il1b	0.66 ± 0.28	0.62 ± 0.51	0.75 ± 0.42	0.89 ± 0.68	0.54 ± 0.10	0.66 ± 0.24
Il6	0.21 ± 0.16	0.15 ± 0.11	0.15 ± 0.08	0.20 ± 0.13	0.14 ± 0.06	0.11 ± 0.05
Tnf	0.21 ± 0.13	0.29 ± 0.30	0.23 ± 0.23	0.42 ± 0.35.	0.14 ± 0.05	0.16 ± 0.08
Brain						
Il1b	0.16 ± 0.02	0.15 ± 0.03	0.16 ± 0.05	0.13 ± 0.03	0.12 ± 0.05	0.14 ± 0.06
Il6	0.12 ± 0.07	0.17 ± 0.11	0.21 ± 0.12	0.15 ± 0.08	0.14 ± 0.06	0.16 ± 0.13

Means ± SD (n = 6); values were normalized with the housekeeping gene (Rpl7a) and expressed as relative to mean liver Fads2, which was set as 100.

mice and human FADS SNPs. Decreased DGLA was the most consistent change of FA profile in HET mice, which can be explained by decreased Fads2 mRNA and increased Fads1 mRNA in liver (Fig. 2). In contrast, DGLA is increased in the human FADS minor alleles, suggesting the expression of both FADS2 and FADS1 is decreased (Fig. 2, shown in orange). Our study and human studies suggest that control of FADS2, the first step of ARA and DHA synthesis, alone is insufficient for regulating the synthesis rate, and requires coordinated regulation of both FADS2 and FADS1.

Because the SNPs occurring in FADS2 and FADS1 genes exhibit a strong linkage disequilibrium (18, 22), it is likely that the expression of FADS2 and FADS1 is regulated together in most haplotypes, and that expression of both
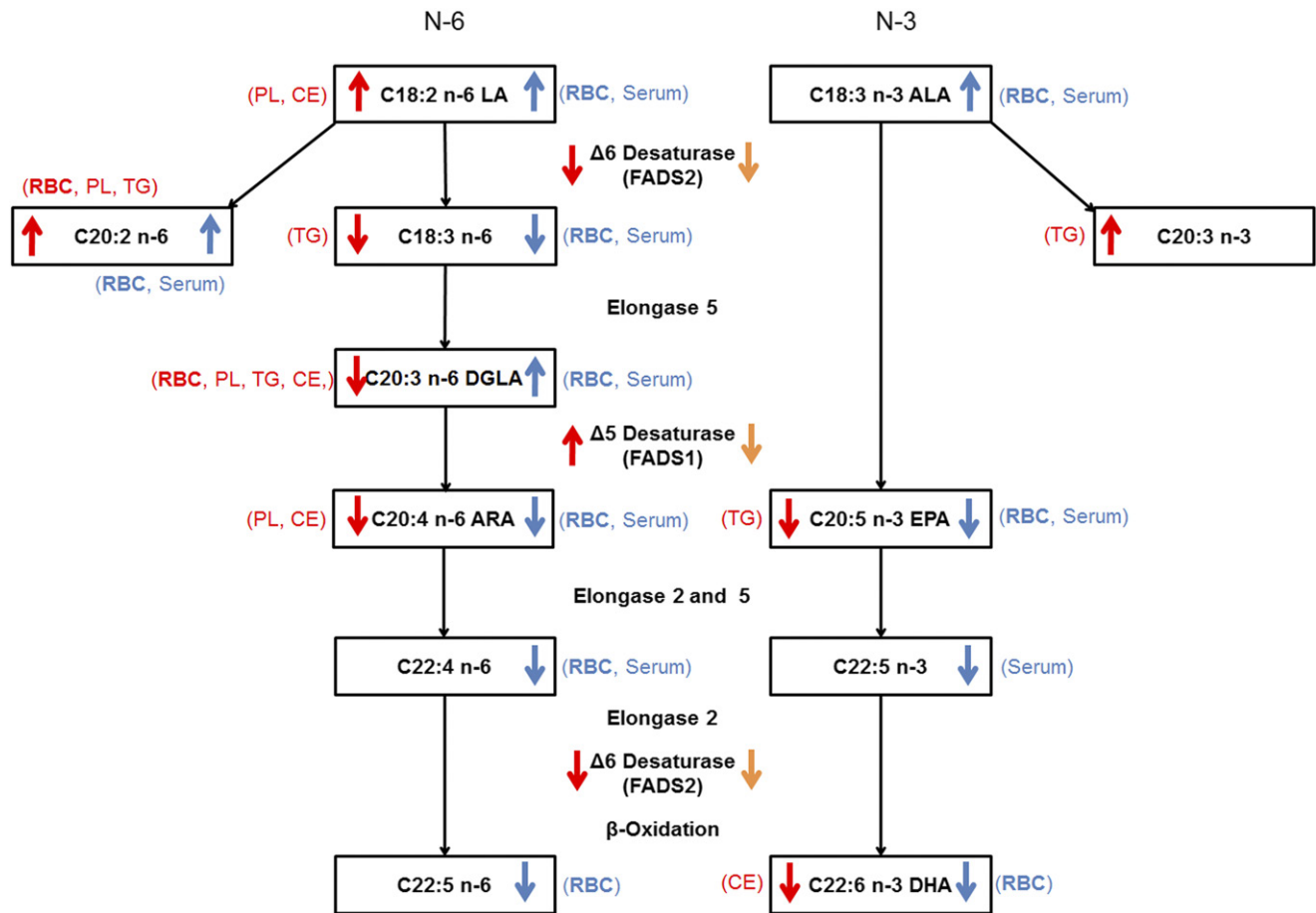

Fig. 2. Summary of genotype effects of HET mice and comparison with minor alleles in human FADS gene cluster. Red arrows: changes in gene expression in liver and FA abundance in RBC and liver in HET mice compared with WT; lipid fractions in liver and RBC total lipid that showed a significant change are shown in parentheses in red. Blue arrows: changes in FA abundance in RBC (21) and serum (22) in minor alleles of SNPs compared with major alleles in the human FADS cluster; specimens that showed a significant change are shown in parentheses in blue. Orange arrows: likely changes in human FADS2 and FADS1 expression based on the comparison with the HET mouse gene expression and FA profiles; increased LA, ALA, and C20:2 n-6, as well as decreased C18:3 n-6, indicate decreased expression of FADS2, whereas increased DGLA in contrast to HET mouse and a more extensive decrease in downstream products suggest a decrease in FADS1 expression in human minor alleles. RBC, the common data from both human and mice, is shown in bold. Although no data of gene expression or FA profiles in human liver are available, the serum FA profile likely reflects the hepatic synthesis because the FAs in fasting serum are largely originated from liver.

FADS1 and FADS2 are lower in the minor alleles than the major alleles in the FADS gene cluster. In addition, because the transcription start sites of FADS2 and FADS1 genes are located only 20 kb apart in diverging orientation in human genome (2), these two genes may share the same regulatory elements, resulting in functional alleles affecting expression of both genes. Indeed, studies with rodents showed that both Fads2 and Fads1 genes were induced, albeit Fads1 in lesser extent, in response to fat-free diet, triolein diet and administration of a PPARα agonist (36, 49). Taken together, as seen in other pathways, coordination between the upstream and downstream of a pathway seems to be important for the regulation of the DHA and ARA synthesis in haplotypes as well as in the physiological responses.

A study reported that the major haplotype in the FADS cluster emerged in humans after divergence from Neanderthals and before exodus from Africa during the human evolution, suggesting upregulation of the synthetic capacity to supply DHA for larger brain as an important event in human evolution (50). In addition to strong associations of haplotypes in the FADS gene cluster with tissue ARA and disease risks, allele frequency differs among populations. As mentioned in the introduction, a "minor allele" becomes major in some populations, indicating that both haplotypes have a survival advantage depending on the environment they live in (23, 24). The conservation of haplotypes and differing occurrences of the FADS cluster underscore the physiological importance of the endogenous synthesis of ARA and DHA. Further study is warranted to investigate the interactive effects of environment and SNPs in the FADS gene cluster on health.

Lack of effects of tissue HUFAs on baseline cytokine expression

The n-3 FAs have been shown to decrease inflammatory cytokines both in vivo (51, 52) and in vitro (53, 54). However, we observed no difference in cytokine expression between groups under an unstimulated condition despite a large difference in the tissue ARA/(EPA + DHA) ratio among the dietary treatments. Consistent with our results, Moranis et al. (55) reported that mice with an n-3-deficient diet showed no difference in Il6 and Il10 concentrations in plasma and brain cortex as compared with the ones with normal diet. Also, Huang et al. (56) found that compared with an SO-based diet, fish oil significantly decreased dextran sulfate sodium-induced Tnf, Il1b, and Il6 secretion in colon but not in untreated groups. Because regulatory enzymes for eicosanoids synthesis such as phospholipases A2 and cyclooxygenase-2 are activated and induced, respectively, in response to inflammation (57, 58), a high tissue ARA/(EPA + DHA) is likely to have a minimal effect on cytokine production in basal condition, while it could exaggerate a response against an inflammatory stimulus by increasing proinflammatory eicosanoids such as prostaglandin E2. The overproduction of prostaglandins may further promote the inflammation by amplifying the cytokine signaling and may form a positive feedback on its own production (8).

CONCLUSIONS

In conclusion, a diet with a high ratio of LA/ALA (44:1) induced all four genes for ARA and DHA synthesis in WT liver compared with a diet with LA/ALA of 7:1 in response to decreased tissue DHA, whereas a diet with LA/ALA of 1:1 did not change expression of synthetic genes from the 7:1 diet despite marked decrease in ARA, suggesting that DHA exerts stronger regulation to the synthetic pathway than ARA. ARA in liver PL was small but significantly lower in HET than WT when fed a high LA/ALA diet, whereas DHA was unchanged in HET in all tissues examined. Fads1 was induced in response to decreased Fads2 expression in HET liver, resulting in decreased DGLA in all tissues examined. This Fads1 induction in HET is likely to have attenuated the decline of HUFA supply. In contrast to the HET mouse, minor alleles of SNPs in the human FADS cluster show increased DGLA in serum and RBC, as well as a strong linkage disequilibrium, suggesting reduced expression of both FADS2 and FADS1 genes.

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