Published in final edited form as:
Prostaglandins Leukot Essent Fatty Acids. 2013 January ; 88(1): 15–19. doi:10.1016/j.plefa.2012.02.003.

Dietary long-chain polyunsaturated fatty acids upregulate expression of *FADS3* transcripts

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Abstract

The fatty acid desaturase (FADS) gene family at 11q12-13.1 includes *FADS1* and *FADS2*, both known to mediate biosynthesis of omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA). *FADS3* is a putative desaturase due to its sequence similarity with *FADS1* and *FADS2*, but its function is unknown. We have previously described 7 *FADS3* alternative transcripts (AT) and 1 *FADS2* AT conserved across multiple species. This study examined the effect of dietary LCPUFA levels on liver FADS gene expression *in vivo* and *in vitro*, evaluated by qRT-PCR. Fourteen baboon neonates were randomized to three diet groups for their first 12 weeks of life: C: Control, no LCPUFA; L: 0.33% docosahexaenoic acid (DHA)/ 0.67% arachidonic acid (ARA) (w/w); and L3: 1.00% DHA/ 0.67% ARA (w/w). Liver *FADS1* and both *FADS2* transcripts were downregulated by at least 50% in the L3 group compared to controls. In contrast, *FADS3* AT were upregulated (L3>C), with four transcripts significantly upregulated by 40% or more. However, there was no evidence for a shift in liver fatty acids to coincide with increased *FADS3* expression. Significant upregulation of *FADS3* AT was also observed in human liver-derived HepG2 cells after DHA or ARA treatment. The PPARγ antagonist GW9662 prevented *FADS3* upregulation, while downregulation of *FADS1* and *FADS2* was unaffected. Thus, *FADS3* AT were directly upregulated by LCPUFA by a PPARγ-dependent mechanism unrelated to regulation of other desaturases. This opposing pattern and mechanism of regulation suggests a dissimilar function for *FADS3* AT compared to other FADS gene products.

Keywords

docosahexaenoic acid; arachidonic acid; polyunsaturated fatty acids; fatty acid desaturase; FADS3; alternative splicing

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All authors report no conflicts of interest.

Introduction

Biosynthesis of long-chain polyunsaturated fatty acids (LCPUFA) requires introduction of *cis* double bonds by the Δ5 and Δ6 desaturases, encoded by the *FADS1* and *FADS2* genes, respectively. *FADS1* and *FADS2* span a 100kb cluster on the long arm of chromosome 11 (11q12-13.1), together with a third member of the gene family, designated *FADS3*. *FADS3* is a putative fatty acid desaturase gene due to its high degree of sequence homology with *FADS2* (62%) and *FADS1* (52%), but no function for *FADS3* has been demonstrated experimentally [1].

Although its exact function is unknown, genetic evidence suggests *FADS3* plays an important role in lipid metabolism and diseases. For example, single nucleotide polymorphisms in *FADS3* have been associated with plasma sphingolipids and triglyceride levels, and with risk of myocardial infarction [2, 3]. Expression of *FADS3* is altered in familial combined hyperlipidemia [3], and *FADS3* is one of the six most highly expressed genes at the implantation site in mice at the initiation of pregnancy [4].

Early attempts in our laboratory to characterize *FADS3* expression resulted in the discovery of seven alternative transcripts (AT) of *FADS3* with distinctive patterns of expression in primate tissues [5]. In addition, we recently reported an alternative splice variant for *FADS2* [6]. *FADS2 AT1* and at least five of the *FADS3* AT were conserved from chickens to humans [7]. Despite this evidence of crucial roles in essential processes, functions of the splice variants remain unclear.

Patterns of regulation can often provide clues to function; we reasoned that if the *FADS3* AT encoded functional fatty acid desaturases, they were likely to be regulated similarly to the classical desaturase genes, *FADS1* and *FADS2*. These two genes encode desaturases required for biosynthesis of the omega-3 and omega-6 LCPUFA docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6). These two products of the biosynthetic pathway have been shown to decrease the classical transcripts of *FADS1* and *FADS2* [8]. DHA and ARA are both known to bind members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (especially PPARα and PPARγ), which form heterodimers with the retinoid X receptor (RXR) and influence gene expression [9, 10]. The effect of dietary LCPUFA on *FADS1* and *FADS2* gene expression has been shown to occur via PPARα and the sterol response element binding protein, SREBP-1c [11]. Nutrients, hormones, and drugs regulating *FADS1* and *FADS2* are known to regulate both in concert, with the same directionality of change [12], as would be expected for genes functioning in the same biosynthetic pathway.

Here we asked whether dietary LCPUFA affect expression of *FADS3* AT and *FADS2 AT1* similarly to classical *FADS1* and *FADS2*, both *in vivo* and *in vitro*. Neonatal baboons were fed infant formula with varying levels of DHA and ARA for 12 weeks, and liver fatty acids and FADS gene expression examined. In vitro, human liver-derived HepG2 cells were studied to determine whether the observed effects were reproducible in human cells, and if it was a direct response to a fatty acid or an endocrine response.

Experimental Procedures

Animals and diets

All baboon work was carried out at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX. Animal protocols were approved by the SFBR and Cornell University Institutional Animal Care and Use Committee (IACUC, protocol # 02–105.) Diets and feeding protocols were described in detail previously [13]. Briefly, fourteen

baboon neonates were randomized to one of three diet groups with varying concentrations of ARA and DHA, as described in Table 1. The infant formulas used for C and L groups correspond to the human infant formulas Enfamil and Enfamil LIPIL, respectively, and the L3 group was targeted to have three-fold higher DHA concentration, corresponding with the upper end of DHA levels found in human breast milk worldwide [14]. These diets were identical to a subset of those used in recently reported human studies [15, 16]. As shown in Table 1, analysis of the prepared diets showed that the actual concentrations used were slightly higher than target values, since the diets were prepared within tolerances designed to account for losses and variation during manufacturing and storage. Infant baboons consumed the experimental diets until 12 weeks of life, when tissues were harvested for lipid and RNA extraction.

Quantitative real-time PCR

Baboon liver RNA was extracted as described previously [5]. For HepG2 cells, RNA was extracted using the RNeasy kit (Qiagen), and RNA quality was checked by agarose gel electrophoresis to verify RNA integrity and by 260/280 nm ratios on a NanoDrop 2000 (Thermo Scientific). cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was carried out using SYBR Green Master Mix (Roche) on a LightCycler 480 instrument (Roche). Human and baboon PCR primers were obtained from Integrated DNA Technologies (sequences available upon request), except for 18S, which was obtained from Qiagen as a QuantiTect Primer Assay. PCR primers designed for FADS splice variants were validated by cloning and sequencing PCR products. PCR reaction efficiency was calculated from standard curves, and reactions were assessed by both melting curves and by running on agarose gels to verify reaction products and the absence of primer-dimers. Quantitative cycle (Cq) values were determined using LightCycler 480 SW1.5.0SP3 software, version 1.5.0.39 (Roche). Relative quantification was carried out using the method of Pfaffl [17], taking into account reaction efficiency and using multiple reference genes for greater accuracy (β-actin and GAPDH for baboon experiments; β-actin, GAPDH, and 18S for HepG2 cell experiments).

Fatty acid analysis

Lipids from baboon liver samples were extracted and fatty acids analyzed by covalent adduct chemical ionization tandem mass spectrometry as described in detail previously [13]. Percent conversion of substrates (S) to products (P) was calculated as: [(P) / (S + P)] * 100, and normalized to the control group.

Cell treatments

For all experiments, human HepG2 hepatocellular carcinoma cells were maintained within ten passages of the original passage received from the ATCC. HepG2 cells were grown in MEM with 10% FBS (media and serum obtained from HyClone) in a humidified environment at 37°C with 5% CO_2. For fatty acid treatment, free fatty acids were first non-covalently bound to fatty-acid free bovine serum albumin (BSA). Fatty acid sodium salts were suspended in PBS, then mixed with fatty-acid free bovine serum albumin (U.S. Biologicals) in a 3:1 ratio of fatty acid to albumin, and incubated for 5 hours at 37°C. Fatty acids conjugated to albumin were sterilized by passage through a syringe filter before cell treatments. Cells were treated with 100 μM of DHA-BSA, ARA-BSA, palmitic-BSA, and/or 2 μM GW9662 (Sigma) for 78 hours in media containing 0.5% FBS.

Statistical methods

Data are presented as mean ± standard deviation. Bootstrapping and randomization techniques were used in REST 2009 software (Qiagen) to calculate significance of fold changes in expression for qRT-PCR experiments. Statistical analysis of changes in fatty acid conversion was conducted using Student's t-test to compare LCPUFA supplementation with control. Linear regression analysis of fatty acid data was carried out in SAS v.9.2 (SAS Institute, Cary NC).

Results

FADS expression in baboon liver

The splicing and expression patterns of the seven alternative transcripts (AT) of *FADS3* and the one splice variant for *FADS2* have been described in detail previously [5, 6]. Because of shared sequences across transcripts, it was not possible to design PCR primers unique to the full-length *FADS3* classical transcript. However, the splice variants could each be assessed by quantitative real-time PCR (qRT-PCR), with the exception of *FADS3 AT2*, which was present at levels too low to quantify accurately. Thus, to assess the effect of dietary LCPUFA on *FADS3* expression, six of the alternative transcripts were evaluated by qRT-PCR in samples obtained from baboon livers after 12 weeks on diets L3, L, or C (described in Materials and Methods). As shown in Figure 1, *FADS3 AT1, AT3, AT4*, and *AT7* were about 40% upregulated in the highest LCPUFA group, L3, relative to control. *FADS3 AT5* and *AT6* also had apparently elevated mean expression in L3, but it was not statistically significant. However, *FADS3 AT5* was significantly upregulated in the intermediate group L compared to control. Interestingly, *AT1* and *AT7* had a significant U-shaped expression response, with lower expression in L compared to control, but higher expression in L3.

As shown in Figure 2, the other two members of the FADS gene cluster had an entirely different pattern of regulation. *FADS1, FADS2*, and the alternative transcript *FADS2 AT1* were all downregulated in both the L and the L3 groups relative to control. The magnitude of expression change was similar for both genes; *FADS1* and total *FADS2* transcripts were reduced by at least 50% for both L and L3. These data are consistent with previous studies showing lower appearance of labeled 22:6n-3 from labeled 18:3n-3 in liver and blood pools of animals fed with 22:6n-3 in a diet with DHA-ARA similar to the present L diet compared to the C diet [18].

Liver PUFA substrate-product ratios

To investigate whether *FADS3* upregulation affected desaturase reactions, levels of fatty acid substrates and products in baboon liver were evaluated as indicators of apparent desaturase activity. DHA and ARA were not included in ratios because their concentrations reflected both biosynthesis and incorporation pre-formed from the experimental diets. Instead, several other substrate/product pairings were used to infer activity, as shown in Figure 3. *FADS2*-encoded Δ6-desaturase activity catalyzes the desaturation of 18:2n-6 to 18:3n-6. The percent conversion to the 18:3n-6 product was significantly decreased in L compared to C, but L3 was not significantly different from control, though the L and L3 means were similar. Diet 20:4n-6 is zero in the C treatment and 0.64 % (w/w) in both the L and L3 treatments, consistent with the concept that ARA downregulated both L and L3.

Conversion of 22:4n-6 to 22:5n-6 is presumed to proceed via *FADS2*-mediated Δ6-desaturation. A linear regression against diet DHA yielded a significant slope ($p<0.05$) and thus a significant downward trend for decreasing percent conversion with increasing LCPUFA. The pairing of 18:3n-3 with the downstream product 20:5n-3 was used as an aggregate measure of total desaturase activity, since both *FADS1* and *FADS2* gene products

are required for 20:5n-3 synthesis. Substrate conversion resulting in 20:5n-3 was significantly decreased in L3 compared to C. Finally, a reaction catalyzed by a desaturase outside the FADS cluster, the conversion of 18:0 to 18:1 by stearoyl-coA desaturase, was also examined since this conversion is sensitive to overall dietary unsaturation. The downward trend in mean conversion with increasing LCPUFA was not significant. Thus, significant differences in pairwise comparisons and trends associated with more unsaturation implied steadily decreasing desaturation activity with increasing dietary LCPUFA. No substrate-product pairs were significantly increased by dietary LCPUFA, leaving no obvious candidates for FADS3 substrates.

FADS expression in response to LCPUFA supplementation in HepG2 cells

To investigate the mechanism for the effect of LCPUFA on *FADS3* transcripts, human liver-derived HepG2 cells were grown in low-serum media supplemented with albumin-bound fatty acids. Cells were treated either with docosahexaenoic acid (DHA, 22:6n-3) alone, arachidonic acid (ARA, 20:4n-6) alone, or palmitic acid (16:0) as a control. As summarized in Table 2, DHA alone significantly upregulated all *FADS3* transcripts except *AT7*. ARA alone significantly upregulated *FADS3 AT1* and *AT3*, while *FADS3 AT4, AT6*, and *AT7* increases were comparable to those observed with DHA treatment, but were not statistically significant. As in baboon livers, both fatty acids also significantly downregulated *FADS1* and both *FADS2* transcripts.

To understand the role of transcription factors in mediating these expression changes, the effect of co-incubation of fatty acids with the PPARγ antagonist GW9662 was evaluated. GW9662 treatment completely blocked the upregulation of *FADS3* transcripts observed with both DHA and ARA treatment, but had no effect on the downregulation of *FADS1* or *FADS2* transcripts.

Discussion

We have evaluated transcriptional changes in FADS genes in infant baboon liver in response to diets corresponding to the physiological range of LCPUFA found in human breastmilk worldwide. *FADS3* transcripts followed a pattern of regulation opposite to the other members of the FADS gene cluster, *FADS1* and *FADS2*. *FADS3* transcripts were upregulated by approximately 40% in livers of animals fed the highest LCPUFA diet, L3, compared to control. In contrast, the same diet downregulated *FADS1, FADS2*, and the alternative transcript *FADS2 AT1*, by at least 50%. The similarity of the downregulation of *FADS2* and *FADS2 AT1* suggests transcription-level control rather than any change in splicing regulation.

The expression changes observed in baboon liver were reproduced in human liver-derived HepG2 cells treated with either DHA or ARA. DHA produced statistically significant fold changes in *FADS3* isoforms, while ARA treatment produced similar magnitude fold-changes, though most were not statistically significant. Thus, there was no obvious difference in sensitivity of the *FADS3* response to DHA or ARA, but the DHA response was of greater precision in this experiment. Moreover, because the cell treatments did not differ in caloric or fat content from the control (palmitic acid treatment), the response was a specific, direct response to DHA or ARA rather than an endocrine mechanism or a general response to energy density of the diet/media. Both DHA and ARA have previously been shown to bind and activate the transcription factor PPARγ [19]. Co-incubation with the PPARγ antagonist GW9662 prevented *FADS3* upregulation by DHA or ARA, suggesting that the fatty acids acted by a PPARγ-dependent mechanism. In contrast, GW9662 did not prevent the downregulation of *FADS1* and both *FADS2* isoforms by DHA or ARA. Thus, *FADS3* expression is regulated in opposite sense to the other FADS genes, and occurs by a

different mechanism. Among the FADS3 AT, we note that the expression pattern for FADS3AT5 is unique because it is responsive to the L diet but not to the higher DHA level in L3. FADS3AT5 is the only AT that we have detected to retain an intron (between exons 8 and 9), though we cannot yet speculate on putative functions.

Extensive substrate screening in our laboratory has so far failed to uncover a substrate for any *FADS3* isoforms (unpublished data). Investigation of baboon liver fatty acids as evidence of desaturase enzyme activity produced evidence for downregulation of *FADS1* and *FADS2*, but no evidence for increased desaturation of any substrate/product pair to correspond with the upregulation of *FADS3* isoforms. This observation could be explained if *FADS3* isoforms function as desaturases on non-LCPUFA substrates. Alternatively, they may act as non-functional dominant negative inhibitors by binding non-productively to LCPUFA substrates, and removing them from availability for desaturase reactions. In favor of the latter theory, Δ5-desaturase, Δ6-desaturase, and stearoyl-coA desaturases are all downregulated by dietary unsaturated fatty acids [20, 21], probably as a mechanism to maintain the unsaturation index of cell membranes within certain limits. It is difficult to reconcile the opposite pattern of regulation of *FADS3* with a putative function as a desaturase. Moreover, dominant negative inhibition is a common mode of action for splice variants of other enzymes [22], and binding specificity for different fatty acids could explain the large number of splice variants for FADS3. Further studies are required to test this hypothesis.

We have demonstrated that *FADS3* is regulated by a different mechanism from other members of the FADS gene cluster, and expression is upregulated when other desaturases are downregulated. These results suggest that, despite a high degree of sequence similarity, *FADS3* isoforms have a function quite distinct from *FADS1* and *FADS2*, and may not encode functional desaturases at all. *FADS3* has been implicated in cardiovascular conditions of enormous public health import, so determining the true function of *FADS3* and its alternative transcripts should be a high priority. Further work characterizing regulation of *FADS3* transcription and alternative mRNA splicing may yield more clues to functional roles.

Acknowledgments

H.R. was supported by a Ruth L. Kirchstein-NRSA predoctoral training fellowship in reproductive sciences and genomics (Grant Number T32HD052471) from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NICHD or the NIH.

Funding

This work was funded by Mead Johnson Nutrition, Evansville, Indiana.

Abbreviations

ARA	arachidonic acid 20:4n-6
AT	alternative transcripts
DHA	docosahexaenoic acid 22:6n-3
LCPUFA	long-chain polyunsaturated fatty acids
PPAR	peroxisome proliferator-activated receptor
qRT-PCR	quantitative real-time polymerase chain reaction

RXR	retinoid X receptor
SREBP-1c	sterol response element binding protein 1c

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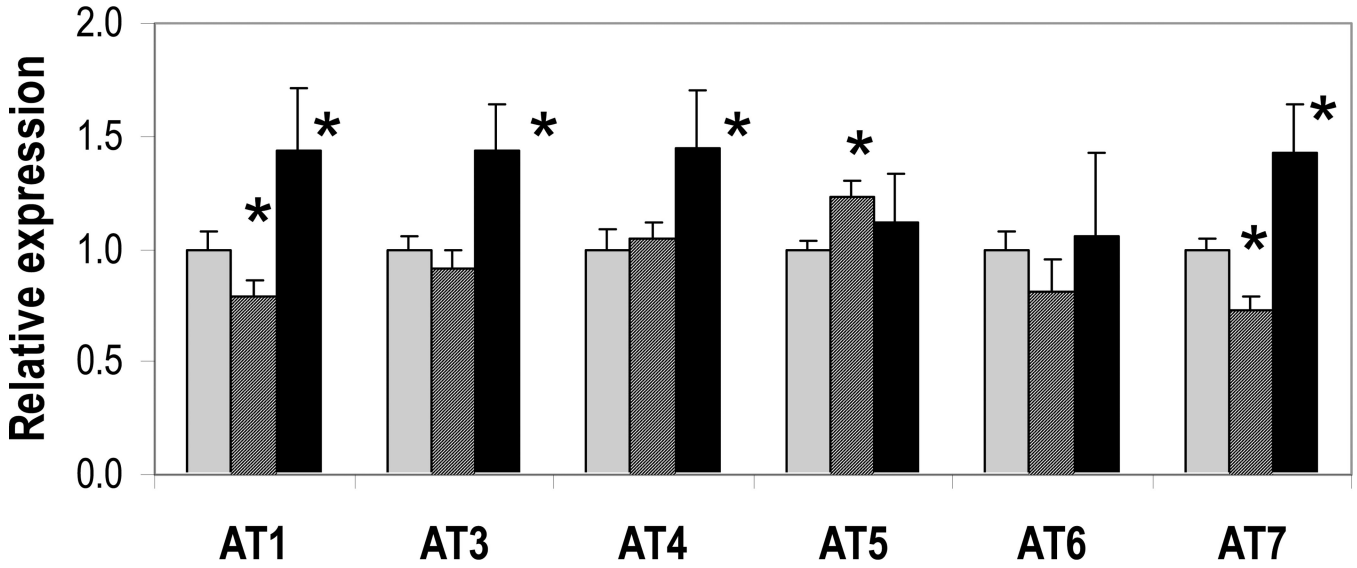

Figure 1. *FADS3* isoform expression is altered by dietary LCPUFA

FADS3 splice variant expression in livers from baboons fed diet C (gray bars), L (striped bars), or L3 (black bars) was measured by qRT-PCR, with GAPDH and beta-actin as reference genes. Most isoforms were significantly upregulated in the L3 diet relative to C (* $p < 0.05$ compared to C). Downregulated AT1 and AT7, and upregulated AT5, were observed for the intermediate diet, L.

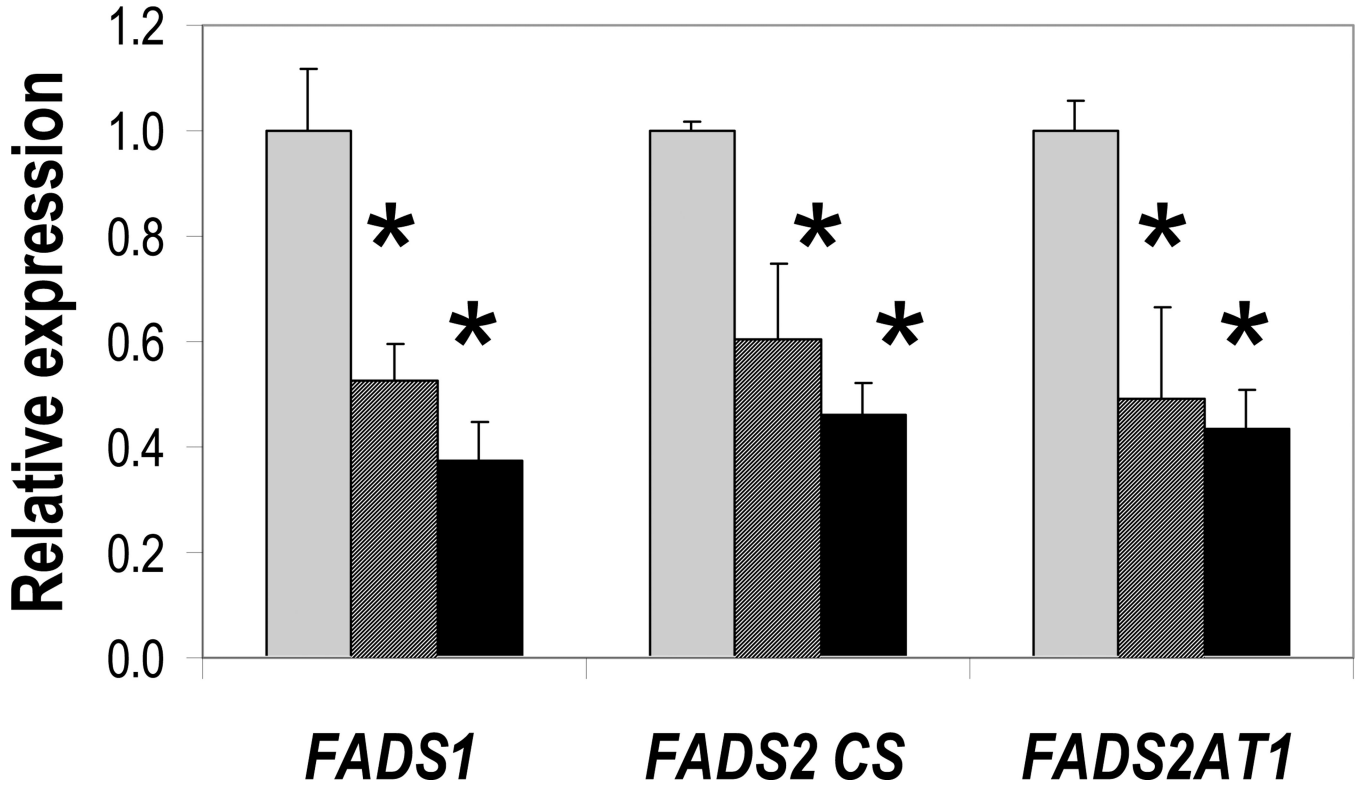

Figure 2. ***FADS1*** **and both** ***FADS2*** **isoforms are downregulated by dietary LCPUFA**

Expression in baboon liver from animals fed diet C (gray bars), L (striped bars), or L3 (black bars) was measured by qRT-PCR, with GAPDH and beta-actin as reference genes. * $p < 0.05$ compared to C group.

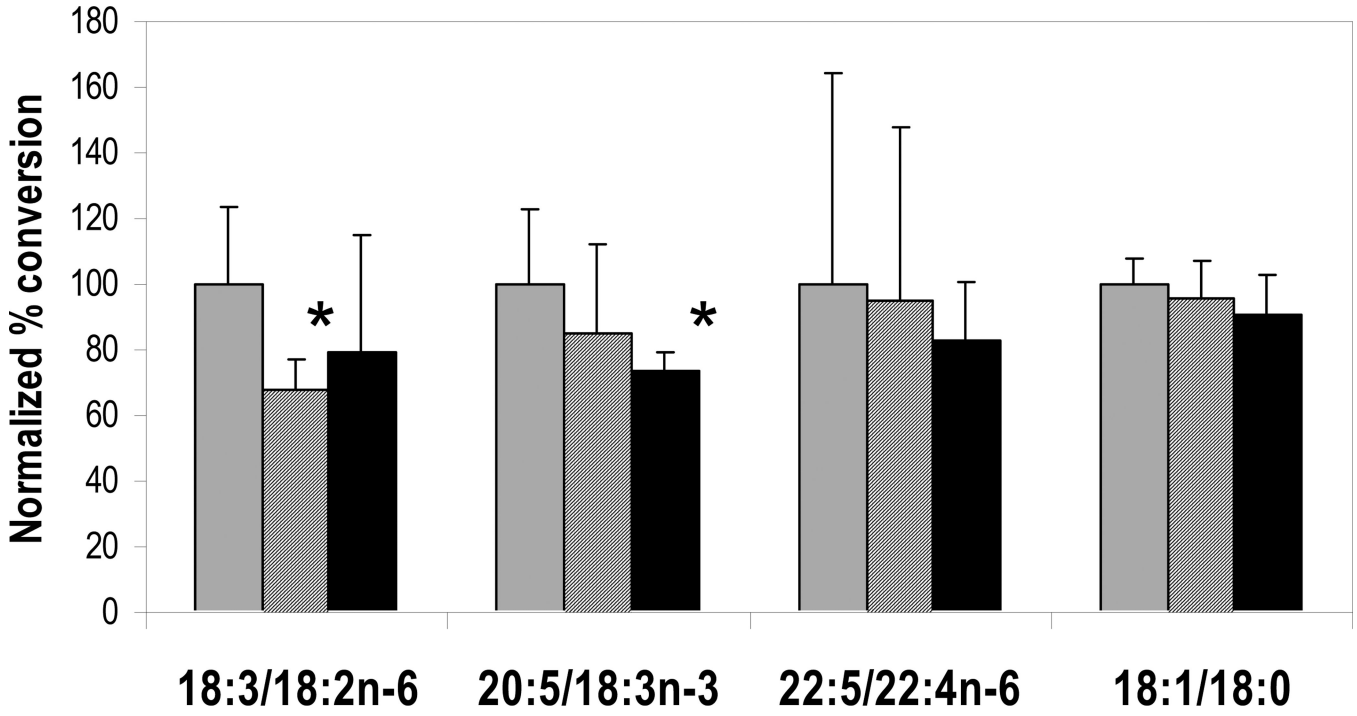

Figure 3. Apparent desaturase activity is consistent with downregulation

Percent conversion of fatty acid substrates into desaturated products (ratios shown, calculated as product/[substrate + product]) was measured in baboon liver and normalized to C group (gray bars). Group L is shown as striped bars, and L3 as black bars. * $p < 0.05$ compared to C group.

Table 1

Characteristics of experimental groups and diets

	C	L	L3
Number of animals (n)	5	4	5
Female	4	3	3
Male	1	1	2
DHA (% w/w)	0	0.42 ± 0.02 [0.33]	1.13 ± 0.04 [1.00]
ARA (% w/w)	0	0.77 ± 0.02 [0.67]	0.71 ± 0.01 [0.67]

Analyzed concentrations are shown as mean ± SD; target values are shown in brackets.

Table 2

Fold changes in FADS gene expression in HepG2 cells for LCPUFA treatments with or without the PPARγ antagonist GW9662, shown relative to control (palmitic acid).

Gene	DHA	DHA + GW9662	ARA	ARA + GW9662
FADS3 AT1	1.2**	1.1	1.3**	1.1
FADS3 AT3	1.2**	1.0	1.2**	1.1
FADS3 AT4	1.2*	0.93	1.2	1.1
FADS3 AT5	1.4*	0.92	0.91	1.1
FADS3 AT6	1.6**	0.85	1.4	0.92
FADS3 AT7	1.2	0.90	1.2	0.93
FADS2	0.59**	0.37**	0.47**	0.22**
FADS2 AT1	0.47**	0.34**	0.32**	0.31**
FADS1	0.81**	0.50**	0.65**	0.49**

* $p < 0.05$,

** $p < 0.01$ for being different from 1 (control = palmitic acid)