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Dietary long-chain polyunsaturated fatty acids upregulate expression of *FADS*3 transcripts ☆

Holly T. Reardon [a], Andrea T. Hsieh [a], Woo Jung Park [a], Kumar S.D. Kothapalli [a], Joshua C. Anthony [b,1], Peter W. Nathanielsz [c], J. Thomas Brenna [a,*]

[a] Division of Nutritional Sciences, Cornell University, Ithaca, New York, USA
[b] Mead Johnson Nutrition, 2400 West, Lloyd Expressway, Evansville, Indiana, USA
[c] Center for Pregnancy and Newborn Research, University of Texas Health Science Center, San Antonio, Texas, USA

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ABSTRACT

The fatty acid desaturase (FADS) gene family at 11q12-13.1 includes *FADS*1 and *FADS*2, both known to mediate biosynthesis of omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA). *FADS*3 is a putative desaturase due to its sequence similarity with *FADS*1 and *FADS*2, but its function is unknown. We have previously described 7 *FADS*3 alternative transcripts (*AT*) and 1 *FADS*2 *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 *FADS*1 and both *FADS*2 transcripts were downregulated by at least 50% in the L3 group compared to controls. In contrast, *FADS*3 *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 *FADS*3 expression. Significant upregulation of *FADS*3 *AT* was also observed in human liver-derived HepG2 cells after DHA or ARA treatment. The PPARγ antagonist GW9662 prevented *FADS*3 upregulation, while downregulation of *FADS*1 and *FADS*2 was unaffected. Thus, *FADS*3 *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 *FADS*3 *AT* compared to other FADS gene products.

1. Introduction

Biosynthesis of long-chain polyunsaturated fatty acids (LCPUFA) requires introduction of *cis* double bonds by the Δ5 and Δ6 desaturases, encoded by the *FADS*1 and *FADS*2 genes, respectively. *FADS*1 and *FADS*2 span a 100 kb cluster on the long arm of chromosome 11 (11q12-13.1), together with a third member of the gene family, designated *FADS*3. *FADS*3 is a putative fatty acid desaturase gene due to its high degree of sequence homology with *FADS*2 (62%) and *FADS*1 (52%), but no function for *FADS*3 has been demonstrated experimentally [1].

Although its exact function is unknown, genetic evidence suggests *FADS*3 plays an important role in lipid metabolism and diseases. For example, single nucleotide polymorphisms in *FADS*3 have been associated with plasma sphingolipids and triglyceride levels, and with risk of myocardial infarction [2,3]. Expression of *FADS*3 is altered in familial combined hyperlipidemia [3], and *FADS*3 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 *FADS*3 expression resulted in the discovery of seven alternative transcripts (*AT*) of *FADS*3 with distinctive patterns of expression in primate tissues [5]. In addition, we recently reported an alternative splice variant for *FADS*2 [6]. *FADS*2 *AT*1 and at least five of the *FADS*3 *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 *FADS*3 *AT* encoded functional fatty acid

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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* Corresponding author. Tel.: +607 255 9182; fax: +607 255 1033.
E-mail address: jtb4@cornell.edu (J. Thomas Brenna).
[1] Present address: Campbell Soup Company, One Campbell Place, Box 48k, Camden, NJ 08103, USA.

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desaturases, they were likely to be regulated similarly to the classical desaturase genes, *FADS*1 and *FADS*2. 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 *FADS*1 and *FADS*2 [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 *FADS*1 and *FADS*2 gene expression has been shown to occur via PPARα and the sterol response element binding protein, SREBP-1c [11]. Nutrients, hormones, and drugs regulating *FADS*1 and *FADS*2 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 *FADS*3 *AT* and *FADS*2 *AT*1 similarly to classical *FADS1* and *FADS*2, 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.

2. Experimental Procedures

2.1. 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.

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.

2.2. 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).

2.3. 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.

2.4. 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 (US Biologicals) in a 3:1 ratio of fatty acid to albumin, and incubated for 5 h 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 h in media containing 0.5% FBS.

2.5. 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).

3. Results

3.1. FADS expression in baboon liver

The splicing and expression patterns of the seven alternative transcripts (*AT*) of *FADS*3 and the one splice variant for *FADS*2