

Gene Expression Analysis in Human Fetal Retinal Explants Treated with Docosahexaenoic Acid

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PURPOSE. To explore the effect of docosahexaenoic acid (DHA) on gene expression during human fetal retinal maturation.

METHODS. Human fetal retinal explants were cultured in serum-free Waymouth's medium supplemented with DHA or oleic acid (OA), using bovine serum albumin (BSA) as the vehicle. After 14 days in culture, fatty acid composition was assessed, and the abundance of 2400 cDNAs was examined with a human cDNA microarray system.

RESULTS. Transcript abundance remained unchanged for 82% and 90% of genes in the explants with added DHA or OA, respectively. Decreased expression was detected in 4% and 9% of genes, in explants supplemented with DHA or OA, respectively, whereas, 14% of genes in explants exposed to DHA and only 0.4% of genes in explants treated with OA showed increased expression. Transcripts displaying changes in abundance in explants supplemented with DHA encode for proteins involved in diverse biological functions, including neurogenesis, neurotransmission, and refinement of connectivity. These gene expression changes were not observed in explants supplemented with OA.

CONCLUSIONS. The effect of DHA deficiency on retinal function during human development can be partly explained by modifications in retinal gene expression by direct or indirect mechanisms. (*Invest Ophthalmol Vis Sci.* 2003;44:3170-3177) DOI:10.1167/iovs.02-1138

Multiple investigations support the role of DHA (C22:6 ω 3) in the development and function of the nervous system. Vision, audition, and learning abilities have been shown to depend on the adequate availability of ω 3 fatty acids during development.¹ High DHA levels are found in particular brain regions and in specific cell types such as retinal photoreceptors.^{2,3} Disc membranes in photoreceptor outer segments are highly enriched with DHA, which represents roughly 50% of total acyl chains in the phospholipids.⁵ Restricted availability of DHA may alter retinal development and maturation with effects on visual function, as suggested by evaluation of retinal function in DHA-deficient humans and nonhuman primates.⁴⁻⁶ In accordance, retinopathy is a common feature in diseases of oxidative phosphorylation in which DHA synthesis is reduced by impaired mitochondrial β -oxidation.⁷

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The purpose of this work was to investigate the effect of DHA on gene expression in human fetal retinal explants and thus to gain insights into the processes that may be altered as a consequence of ω 3 fatty acid deficiency. Explants of neural retina, devoid of RPE, from human fetuses of 13 to 18 weeks' gestational age, were used as the experimental model to investigate changes in the gene expression profile. Retinal explants remain viable for up to 30 days in culture (Hoffman DR, Hoffman LH, ARVO Abstract 283, 1995) and undergo a morphologic differentiation process that results in the formation of the inner and outer nuclear layers, inner and outer plexiform layers, and a ganglion cell layer. The defined culture medium was supplemented with DHA to assess the effect on gene expression; whereas OA (18:1 ω 9) was added to separate explants to determine the specificity of DHA's effects. Microarray technology was used to monitor simultaneously the expression of more than 2000 genes from the limited amount of RNA obtained from the fetal retinal explants. Differential expression of select transcripts was corroborated by RT-PCR. Addition of DHA resulted in modification of the relative abundance of transcripts encoding for several proteins involved in retinal neurogenesis and maturation, suggesting that these processes are affected by nutritional DHA deficiency.

MATERIALS AND METHODS

Tissue Specimens and Culture Conditions

Ocular tissue was obtained from aborted fetuses provided by the Cecilia H. and Ida Green Center for Reproductive Biology Sciences at the University of Texas Southwestern Medical Center (Dallas, Texas) or Advanced Bioscience Resources (Alameda, CA) and in accordance with the Donors Anatomic Gift Act of the State of Texas. The study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review board of University of Texas Southwestern.

Fetal human retinas (13-18 weeks) were divided into two sections, with the central fovea removed by a 2-mm trephine. The neural retina was transferred to sterile lens paper suspended over medium on a stainless-steel mesh pedestal. One half of each retina was placed in a 35-mm culture dish containing Waymouth's medium (MB 752/1; Gibco Life Technologies, Rockville, MD) supplemented with penicillin, streptomycin, 2 mM glutamine, and 27 μ M DHA or OA (Nu-Check Prep, Elysian, MN) complexed with 0.02% essential fatty-acid-free BSA (Sigma-Aldrich, St. Louis, MO). Sodium salts of fatty acids were sonicated into a warm aqueous solution of BSA under a flow of nitrogen. The aqueous mixtures were filter sterilized, and the fatty acid concentrations were determined by gas chromatography and stored under nitrogen at -70°C . Aliquots were thawed every 2 days for addition to the culture medium. Fresh solutions were prepared for each set of 14-day studies. The second retinal section, used as a control, was incubated in Waymouth's medium with 0.02% BSA. Explants were maintained at 37°C in a humidified incubator gassed with 5% CO_2 and 95% air, and the culture medium was replaced every 2 days. Explants were harvested at day 14 for RNA extraction and fatty acid analysis. For histologic examination, tissues were fixed in phosphate-buffered formaldehyde (4% for 24 hours) and stained with toluidine blue-acid fuchsin. Specimens were embedded in methacrylate and cut at 2 μm for light microscopy.

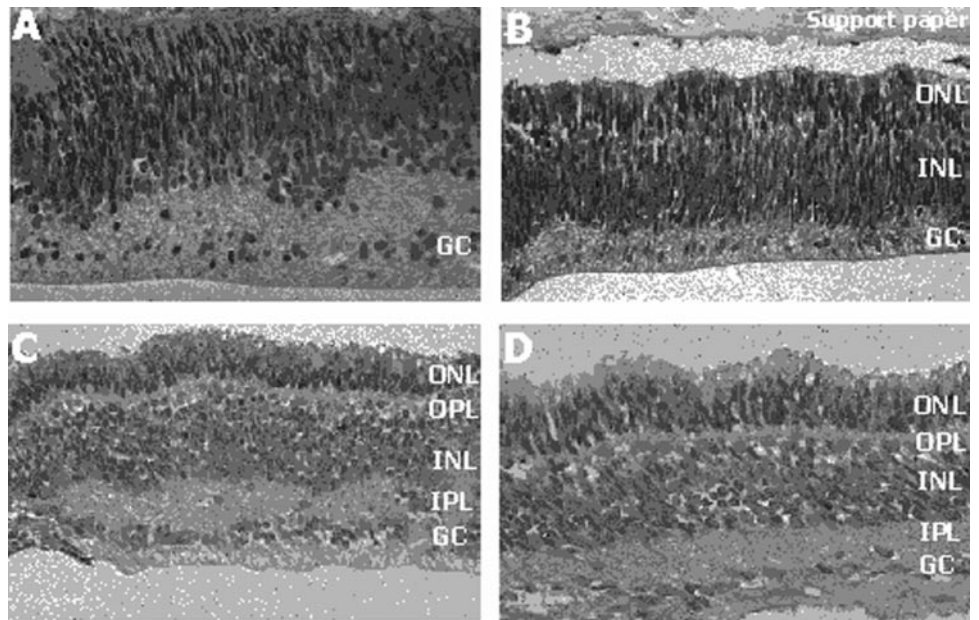


FIGURE 1. Neural retinal explant from a 13-week-old human fetus at 0 (A), 10 (B), 20 (C), and 30 days of culture (D). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GC, ganglion cell layer. Magnification: (A, D) $\times 133$; (B) $\times 150$; (C) $\times 100$.

Lipid Analysis

Retinal explants were washed three times with phosphate-buffered saline (PBS) and homogenized under nitrogen in PBS, and the lipids were extracted with chloroform, methanol, and butylated hydroxytoluene (1:2:0.02). Fatty acid methyl esters were prepared and quantified by capillary column gas chromatography, as previously described.⁸ The fatty acid composition of retinal explants is reported as the relative weight percentage of all identifiable fatty acids.

RNA Purification, cDNA Synthesis, and Hybridization

RNA was extracted from explants (TRIZOL; GibcoBRL), according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA, previously treated with DNase I, and labeled with biotinyl or dinitrophenyl nucleotide analogues using a human gene microarray system (Micromax Human cDNA Microarray System I; NEN Life Science Products, Inc., Albany, MA), after the manufacturer's instructions.

Hybridization was performed overnight at 65°C by simultaneous incubation of biotinyl- and dinitrophenyl-labeled cDNA to the gene array on the glass slide and followed by several washes at room temperature with 0.5 \times SSC (0.15 M sodium chloride, 0.015 M sodium citrate [pH 7.0]), 0.01% sodium dodecyl sulfate (SDS); 0.06 \times SSC, 0.01% SDS and finally, with 0.06 \times SSC. Biotinyl and dinitrophenyl cDNAs hybridized to any spot in the array were sequentially detected by a signal-amplification system (TSA; NEN Life Science Products, Inc.) using cyanine-3 and -5 as fluorescent reporters for experimental and control cDNA, respectively.

Signal Detection, Quantification, and Data Processing

Scanning was conducted by NEN. Cyanine-3 and -5 channels were scanned sequentially, at low/medium and high settings of laser power and photomultiplier voltage. Cyanine-3 and -5 scanning differences were balanced based on the arithmetic average of the cyanine-3/-5 ratios for control genes in the array.

Data processing was performed first by subtracting local background from signal for every spot and for every channel separately. Spots were considered for analysis when the difference between the signal ratio obtained from low- and high-power laser scanning was less than 33% (2264 and 2242 of 2400 spots for the DHA and OA incubations, respectively). Gene expression differences were considered for

analysis when the ratio of fatty acid to BSA transcript abundance (r) was 3.0 or more or 0.33 or less.

RT-PCR Analysis of Differential Gene Expression

Approximately 1 μg of total RNA from retinal explants was converted to cDNA with oligo dT₁₅ and reverse transcriptase (Reverse Transcription System; Promega Corp., Madison, WI). Serial dilutions of the cDNA were used for PCR amplification of portions of select genes with specific oligonucleotide primers and *Taq*DNA Polymerase (Invitrogen Corp., Carlsbad, CA). PCR products of the predicted size were obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α -crystallin, NeuroD, and ornithine decarboxylase-1 cDNAs. Caveolin 1, patched NMDA receptor 1, and synaptotagmin cDNAs could not be consistently amplified by RT-PCR.

RESULTS

Fatty Acid Supplementation

Explants of human neural retina devoid of RPE, obtained from fetuses of 13 to 18 weeks' gestational age, underwent morphologic differentiation in culture which resulted in formation of well-defined inner and outer nuclear layers, inner and outer plexiform layers, and a ganglion cell layer (Fig. 1). Supplementation of the culture medium with DHA induced a change in the fatty acid profile in the retinal explants (Table 1). An increase (1.7–3.6-fold) in C20–C22 ω 3 long-chain polyunsaturated fatty acids (LCPUFAs) after 14 days in culture was noted, whereas, total ω 6 LCPUFAs remained unchanged and C22:5 ω 6 decreased.

Differential Gene Expression Analysis

The cDNA chip used for differential gene expression analysis in the retinal explants included 2400 human genes. However, in accordance with the data analysis protocol, 5.7% and 6.5% of all genes were judged unreliable and excluded from the results of the DHA and OA experiments, respectively, because the fluorescence measurements with low- and high-intensity laser scanning differed more than 33% for these spots. Overall results, depicted in Table 2A, indicated changes in transcript relative abundance for 412 of 2264 genes analyzed in retinal

TABLE 1. Fatty Acid Composition of Retinal Explants after 14 Days in Culture

Fatty Acid	+DHA (%)	-DHA (%)	Ratio* +DHA/-DHA
Saturated	52.3 ± 1.3	51.0 ± 0.4	1.0
Monounsaturated	23.2 ± 1.6	27.7 ± 0.1	0.8
ω3-PUFA	11.0 ± 0.2†	6.5 ± 0.1	1.7
ω6-PUFA	13.6 ± 1.0	14.8 ± 0.4	0.9
Total	100.1	100.0	
ω3-LCPUFA	10.90 ± 0.20†	6.31 ± 0.04	1.7
C20:5 ω3	0.51 ± 0.00†	0.14 ± 0.01	3.6
C22:5 ω3	0.23 ± 0.00†	0.10 ± 0.02	2.3
C22:6 ω3	9.24 ± 0.20†	4.29 ± 0.18	2.2
ω6-LCPUFA	12.9 ± 1.00	14.1 ± 0.40	0.9
C20:4 ω6	8.20 ± 1.06	8.89 ± 0.23	0.9
C22:5 ω6	1.38 ± 0.07†	1.88 ± 0.02	0.7

Data are mean ± SD of triplicate determinations. PUFA, polyunsaturated fatty acids; LCPUFA, long chain PUFA (>18 carbons in length).

* Ratio refers to relative fatty acid abundance in explants exposed to DHA (+DHA) compared with that in explants without DHA (-DHA).

† Values are significantly different at $P < 0.0005$.

explants cultured in the presence of DHA compared with those maintained in BSA only. In explants incubated with DHA, an increased expression ratio ($r \geq 3.0$) was detected in 322 (14%) of genes, whereas 90 (4%) showed a reduced ratio ($r \leq 0.3$). When the culture medium was supplemented with OA, the expression ratio was increased in 9 (0.4%) and decreased in 210 (9.4%) genes. Ribosomal protein genes and other housekeeping genes exhibited a ratio of experimental (fatty acid) to control (BSA) expression within the range considered not significantly different ($0.3 \geq r \leq 3.0$) under DHA or OA supplementation (Table 2B). RT-PCR was used to verify that the expression level of the GAPDH gene in retinal explants

supplemented with DHA or OA was comparable to that in the corresponding control with BSA ($r = 1.0$).

Expression Analysis of Genes Related to Lipid Metabolism

Genes for the transcription factors RXR-γ, RXR-β, PPARβ, SREBP-1, and SREBP-2, whose activity and/or expression is regulated by fatty acid ligands, were included in the gene microarray. Supplementation of the retinal explant culture medium with DHA or OA did not modify their expression ratio (not shown).

Genes related to lipid catabolism that are transcriptionally regulated in response to fatty acid levels are included in Table 3. In explants supplemented with DHA, increased levels were detected for lipoprotein lipase mRNA ($r = 3.8$) and long-chain acyl-coenzyme A (CoA) synthetase mRNA, the enzyme that catalyzes the first step in β-oxidation ($r = 3.3$). Explants exposed to DHA also showed higher abundance of transcripts encoding for enzymes directly involved in the fatty acid β-oxidation cycle, such as: branched chain α-ketoacid dehydrogenase E1 β-subunit ($r = 3.6$), cytosolic acetoacetyl CoA thiolase ($r = 3.0$), and 3-oxoacyl-CoA thiolase ($r = 3.1$). In accordance with a predominant effect on the expression of genes for lipid catabolism, no significant changes were detected in the abundance of fatty acid synthase (FAS) transcripts in explants treated with DHA ($r = 0.4$). Supplementation with OA was associated with reduced expression of the mRNA for the peroxisomal enoyl-CoA hydratase-like protein ($r = 0.3$). Genes related to cholesterol biosynthesis were also affected by incubation with DHA (Table 3). The mRNA for 3-hydroxy-3-methylglutaryl CoA synthase, the enzyme that catalyzes the rate limiting step in cholesterol biosynthesis, was not significantly modified in explants supplemented with DHA ($r = 1.6$); however, its relative abundance was reduced in explants treated with OA ($r = 0.3$). Transcripts for farnesyl-protein transferase (β subunit) and 2,3-oxidosqualene-lanosterol cyclase displayed

TABLE 2. Gene Expression Analysis in Retinal Explants Cultured with DHA or OA
A. Overall Results

	FA Supplement			
	DHA	(%)	OA	(%)
Number of cDNA spots included in the array	2400		2400	
Number of cDNA spots considered for analysis	2264		2242	
Unchanged FA/BSA expression ratio*	1852	82.0	2023	90.2
Decreased FA/BSA expression ratio*	90	4.0	210	9.4
Increased FA/BSA expression ratio*	322	14.0	9	0.4

B. Expression Ratio for Housekeeping Genes

Gene	GenBank Acc. No.	Ratio	
		DHA/BSA	OA/BSA
Ribosomal protein L29	U10248	1.0	0.7
Ribosomal protein S19	M81757	1.0	0.6
Ribosomal protein L1	D23660	1.0	0.9
Ribosomal protein S5	U14970	1.1	0.7
Ribosomal protein S12	X53505	1.1	0.8
Nuclear ribonucleoprotein particle (hnRNP) C protein	M16342	1.1	0.9
Ribosomal protein L5	U14966	1.3	0.8
Ribosomal protein S10	U14972	0.7	1.1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	M33197	1.0	0.7

The ratio DHA/BSA or OA/BSA refers to transcript abundance in explants exposed to DHA or OA complexed to BSA compared to the level in their corresponding controls exposed only to BSA.

* FA/BSA expression ratio represents DHA/BSA and OA/BSA ratios.

TABLE 3. Expression Analysis of Genes Related to Lipid Metabolism

Gene	GenBank Acc. No.	Ratio	
		DHA/BSA	OA/BSA
Genes for proteins involved in lipid metabolism			
Type-2 phosphatidic acid phosphohydrolase	AF17116	4.8	0.6
Farnesyl-protein transferase β subunit	L00635	4.6	0.4
Lipoprotein lipase (EC 3.1.1.34)	X14390	3.8	1.1
2,3 oxidosqualene lanosterol cyclase	U22526	3.7	0.5
Branched chain α -ketoacid dehydrogenase E1 β subunit	U50708	3.6	0.5
Long-chain acyl-CoA synthetase	D10040	3.3	0.6
Stearoyl CoA desaturase	Y13647	3.3	NA
Liver 3-oxoacyl-CoA thiolase	S70154	3.1	0.4
Cytosolic acetoacetyl-CoA thiolase	X14813	3.0	0.4
Peroxisomal enoyl-CoA hydratase-like protein	U16660	1.6	0.3
3-hydroxy-3-methylglutaryl coenzyme A synthase	L25798	1.6	0.3
Genes for proteins involved in eicosanoid biosynthesis			
PAF acetylhydrolase IB, γ subunit	D63391	5.2	0.6
Phospholipid hydroperoxide glutathione peroxidase	X71973	5.6	0.7
Lipocortin	X05908	4.2	0.9
Leukotriene A4 hydrolase	J02959	0.2	1.1
Genes for proteins involved in lipid trafficking			
Synaptojanin (splice variant)	AF009040	5.2	0.6
Apolipoprotein J	J02908	4.4	0.8
AP-3 complex β 3B subunit	AF022152	4.3	0.3
Caveolin 1	Z18951	4.0	0.8
LDL-R related protein	X13916	3.4	0.4
LDL receptor related protein 105 (LRP3)	AB009462	0.3	1.2
hB FABP	AJ002962	0.1	0.6

Ratios are as described in Table 2. In bold: ratio of fatty acid to BSA transcript abundance ≥ 3.0 or ≤ 0.33 ; NA, not available.

DHA-dependent increase in the expression ratio (4.6 and 3.7, respectively). In contrast, the mRNA for mevalonate pyrophosphate decarboxylase was not significantly modified by DHA ($r = 2.1$).

Analysis of genes involved in eicosanoid biosynthesis (Table 3) revealed elevated levels of the mRNA for lipocortin, an endogenous inhibitor of phospholipase A2 ($r = 4.2$) and also of the mRNA for phospholipid hydroperoxide glutathione peroxidase ($r = 5.6$), a negative regulator of lipoxygenase activity, in the explants treated with DHA. Moreover, reduced expression of leukotriene A4 hydrolase mRNA ($r = 0.2$) and increased expression of the platelet-activating factor acetylhydrolase IB γ subunit mRNA ($r = 5.2$) are also consistent with the anti-inflammatory role proposed for $\omega 3$ LCPUFA. None of these changes was detected in the explants cultured with OA.

Changes in mRNA abundance for several proteins involved in lipid transport and intracellular trafficking are depicted in Table 3. The mRNA for apolipoprotein J, a HDL-associated protein that is abundant in retina and pigmented epithelium, displayed an increase in response to DHA supplementation in the culture medium ($r = 4.4$). Conversely, the mRNA abundance for LDL receptor-related protein 105 (LRP3), a receptor apparently involved in uptake of apolipoprotein E-containing proteins, was reduced ($r = 0.3$). Under the same experimental conditions, the mRNA for the hB FABP, a protein that determines the intracellular availability of free fatty acids and fatty acyl-CoA, displayed a substantial reduction ($r = 0.1$). The abundance of the mRNAs for neuron-specific protein components of coated vesicles specifically involved in LCPUFA trafficking was higher in retinal explants cultured with DHA than in those with BSA. The same was the case of the mRNA for the synaptojanin splice variant ($r = 5.2$) and the AP-3 complex β 3B subunit mRNA ($r = 4.3$). The latter was reduced in explants supplemented with OA ($r = 0.3$). An elevated ratio ($r = 4.0$) of mRNA for caveolin 1, an essential protein component of caveolae, was also found.

Differential Expression of Genes Related to Retinal Neurogenesis and Maturation

Table 4 depicts the expression ratios of genes encoding for proteins involved in neuronal differentiation, as determined by microarray analysis. RT-PCR validation of differential expression for select genes is also included. In the retinal explants supplemented with DHA, the transcription factors of the basic helix-loop-helix (bHLH) family, b-PAS protein JAP3, and NeuroD had expression ratios of 8.1 and 3.1, respectively. RT-PCR confirmed augmented expression of the NeuroD gene. Similarly, the mRNA for Patched, a receptor that acts as a negative regulator of Hedgehog signaling, displayed increased levels in DHA-treated explants ($r = 11.6$). The abundance of the h-neuralized mRNA was lower in the explants exposed to DHA than in the control condition ($r = 0.3$), and the expression ratio for the neurotrophic factor neuroleukin was 4.0 in retinal explants with added DHA and 0.3 in those with OA.

Table 4 also includes genes for protein components of the neuronal intracellular cytoskeleton and extracellular matrix that displayed differential expression in retinal explants. Supplementation with DHA augmented mRNA levels for laminin B2 chain (an extracellular membrane protein that is able to stimulate neurite outgrowth in astrocytes; $r = 3.6$), human microtubule-associated protein 1A (MAP1A, a protein that is specifically expressed in brain; $r = 3.9$), and myelin/oligodendrocyte glycoprotein (an integral membrane protein expressed in the oligodendrocyte cell surface and the outermost surface of myelin sheaths; $r = 3.5$). There was decreased expression of SCG10 mRNA (a stathmin homologue and neuronal growth-associated protein; $r = 0.3$) and neurofilament-66 (a cytoskeleton protein expressed in human fetal brain; $r = 0.2$). In addition, transcripts for N Wasp (a protein that interconnects actin polymerization with signal transduction pathways; $r = 0.3$) and N-cadherin mRNA (a receptor of the neuronal adherens junction system involved in cell adhesion and neurite

TABLE 4. Neuronal Genes that Display a Differential Expression Ratio in Retinal Explants

Gene	GenBank Acc. No.	Array Ratio		PCR Ratio	
		DHA/ BSA	OA/ BSA	DHA/ BSA	OA/ BSA
Patched homologue	U43148	11.6	0.7		
bHLH-PAS protein JAP3	U60415	8.1	0.6		
NMDA receptor, key subunit of	D13515	7.1	0.6		
α B-crystallin, Rosenthal fiber component	S45630	6.0	0.8	3.3	1.1
Brain angiogenesis inhibitor 2	AB005298	5.6	0.4		
Serotonin 1D receptor (5-HT1D)	M81590	4.8	0.4		
K ⁺ channel β 2 subunit	U33429	4.6	0.4		
Excitatory amino acid transporter 4	U18244	4.5	0.5		
Glutamate receptor flop isoform (GluR3-flop)	U10302	4.5	NA		
h-Neuro-d4 protein	U43843	4.3	NA		
Ceramide glucosyltransferase	D50840	4.2	0.6		
Neuroleukin	K03515	4.0	0.3		
EHK-1 receptor tyrosine kinase	X95425	4.0	NA		
MAP1A	U14577	3.9	NA		
Hkf-1	D76444	3.9	0.5		
Brain ryanodine receptor	AB001025	3.6	0.6		
Laminin B2 chain	J03202	3.6	0.5		
Myelin/oligodendrocyte glycoprotein-25.1 kDa	U64564	3.5	0.6		
Glutamate/kainate receptor subunit (EAA5)	U16127	3.3	0.6		
Ornithine decarboxylase, odc1 mRNA	X55362	3.2	0.4	1.0	1.0
NeuroD	D82347	3.1	0.4	4.6	1.0
K ⁺ channel β subunit (clone hKv β 3)	L39833	3.0	0.9		
Neuropeptide Y-like receptor	X71635	0.3	0.5		
SCG10, stathmin homologue	S82024	0.3	0.5		
Related adhesion focal tyrosine kinase	S80542	0.3	0.4		
h-Neuralized	U87864	0.3	0.4		
Syntaxin 7	U77942	0.3	0.7		
N-cadherin	S42303	0.3	1.9		
Brain ankyrin (brank-2)	X56958	0.3	0.5		
Ankyrin G (ANK-3)	U13616	0.2	0.3		
N Wasp	D88460	0.3	NA		
Neurofilament-66	S78296	0.2	0.9		
Synaptotagmin	M55047	0.2	NA		
Opioid-binding cell adhesion molecule	L34774	0.2	1.5		

Ratios are as described in Table 2. In bold: ratio of fatty acid to BSA transcript abundance ≥ 3.0 or ≤ 0.33 ; NA, not available.

growth that is also transiently expressed during blood-retina barrier maturation⁹; $r = 0.3$) were reduced in DHA-treated tissues, whereas, transcripts for other cadherins did not display significant changes (not shown).

Transcript for the synaptic vesicle proteins brain ankyrin (brank-2) and synaptotagmin, showed reduced levels in explants with added DHA ($r = 0.3$ and 0.2 , respectively). The expression ratio for ankyrin G (ANK-3) was low in explants supplemented with either DHA or OA ($r = 0.2$ and 0.3 , respectively). The mRNA for the secretory vesicle receptor, syntaxin 7, was also diminished in the retinal explants treated with DHA ($r = 0.3$).

Gene expression for proteins related to cell excitability and neurotransmission, such as receptors, transporters, and channels, is also reported in Table 4. Transcripts for various proteins involved in glutamate neurotransmission displayed significant changes in explants supplemented with DHA: the excitatory amino acid transporter 4 ($r = 4.5$), the glutamate/kainate receptor subunit (EAA5, $r = 3.3$), the AMPA glutamate receptor flop isoform (GluR3-flop, $r = 4.5$), and the key subunit of the NMDA receptor ($r = 7.1$). In explants treated with DHA, increased levels of the mRNA for ornithine decarboxylase odc1 (the enzyme that catalyzes the biosynthesis of putrescine, a precursor for gamma amino butyric acid [GABA] during development; $r = 3.2$) was detected. Whereas, the level of the mRNA for glutamate decarboxylase (the enzyme that catalyzes GABA biosynthesis in adults) was not modified ($r =$

1.8). RT-PCR analysis failed to detect differences in the expression ratio for the ornithine decarboxylase gene in explants exposed to DHA or OA (Table 4).

Differential Expression of Genes Related to Apoptosis

Table 5 shows differential expression of genes for several proteins that protect cells from apoptosis (Ref-1 redox factor, TRAF5, NF κ B p65 subunit, MEK5) and for gene products that are hallmarks of the apoptotic process or that have a proapoptotic role (requiem, TRAIL receptor 2, MAP kinase-activating death domain protein [MADD], apoptosis-specific protein, amyloid β -peptide binding protein). The mRNA for antiapoptotic NF κ B p65 subunit displayed increased abundance in explants supplemented with DHA ($r = 3.6$) and reduced levels in the explants with OA ($r = 0.3$). Proapoptotic TRAIL receptor 2 (DR5) mRNA showed a similar pattern of expression (DHA, $r = 3.4$; OA, $r = 0.3$).

DISCUSSION

Effect of DHA Supplementation on Gene Expression

The advent of microarray technologies allows the simultaneous monitoring of the expression of thousands of genes. This

TABLE 5. Differential Expression of Genes Related to Apoptosis

Gene	GenBank Acc. No.	Ratio	
		DHA/BSA	OA/BSA
Cytochrome c1	X06994	4.5	0.4
Requiem homologue (hsReq)	U94585	4.3	NA
Ref-1, redox factor	S43127	4.1	0.6
TRAF5	AB000509	4.0	NA
ZIP-kinase	AB007144	3.9	0.8
NF- κ B transcription factor p65 subunit	L19067	3.6	0.3
ERK1 mRNA for protein serine/threonine kinase	X60188	3.6	0.7
TRAIL receptor 2 (DR5)	AF016266	3.4	0.3
Apoptosis specific protein	Y11588	3.4	0.5
Amyloid β -peptide binding protein	U96132	3.3	0.4
MEK5	U25265	3.3	0.4
MAP kinase-activating death domain protein (MADD)	U77352	3.0	0.5
DNA-dependent protein kinase catalytic subunit	U34994	2.1	0.3
TRAF family member-associated NF- κ B activator TANK	U63830	0.3	1.1
Cdk-inhibitor p57KIP2 (KIP2)	U22398	0.1	0.8

Ratios are as described in Table 2. In bold: ratio of fatty acid to BSA transcript abundance ≥ 3.0 or ≤ 0.33 ; NA, not available.

powerful tool allowed us to investigate the effect of DHA on transcript abundance for more than 2200 genes in human fetal retina explants. Despite the fact that transcripts are not the ultimate gene product, regulation of gene expression is frequently achieved at the transcriptional level. Although there is not a direct relationship between mRNA and protein abundance, very low levels of mRNA most likely imply limited protein synthesis and conversely, augmented mRNA suggests an increase in translation.¹⁰ Therefore, screening for changes in the expression of many genes may unravel cellular and biochemical pathways underlying the functional consequences of altered DHA levels during retinal development.

Gene Expression Changes in Cellular Pathways Regulated by Fatty Acids

The microarray allowed an investigation of the effect of DHA supplementation on the expression level of genes that are known targets of transcriptional regulation by fatty acids, such as those involved in the control of lipid metabolism. The mRNA for transcription factors that control the expression of genes involved in lipid metabolism, such as PPAR and SREBP,^{11,12} were not significantly affected by supplementation of the explant culture medium with either DHA or OA. DHA supplementation did not alter gene expression for proteins related to fatty acid anabolism; however, increased expression of genes encoding enzymes involved in fatty acid oxidation was detected in retinal explants treated with DHA but not those treated with OA. Consequently, the results are consistent with a predominance of fatty acid catabolism that would lead to intracellular accumulation of two-carbon end products in explants cultured with DHA.

Given that cholesterol biosynthesis is sensitive to fatty acid regulation, the expression level of genes coding for enzymes involved in this pathway was also investigated. Explants treated with DHA showed increased expression of farnesyl protein transferase (β subunit) and 2,3-oxidosqualene-lanosterol cyclase; however, transcripts for mevalonate pyrophosphate decarboxylase and the rate-limiting enzyme 3-hydroxy-3-methylglutaryl CoA synthase remained unchanged. Therefore, it is not possible to speculate on the consequences of DHA supplementation in cholesterol synthesis in retinal explants.

The precise role of different fatty acid-binding proteins is not completely understood. However, members of this family have been reported to participate in early and late phases of

cellular fatty acid uptake, in intracellular trafficking, and in phospholipid acyl chain exchange.¹³ Under the conditions of DHA supplementation, retinal explants showed a remarkable decrease of mRNA for hB FABP, a fatty acid-binding protein that influences the intracellular availability of free fatty acids and fatty acyl-CoA. Conversely, the retinal explants exposed to DHA showed increased abundance of transcripts encoding for neuron-specific components of coated vesicles involved in LCPUFA intracellular trafficking in nerve terminals, such as synaptotagmin and for AP-3 complex $\beta 3B$ subunit. The increase in the mRNA for caveolin 1, an essential component of caveolae, is of special interest, given the role of these membrane structures in modulating signal transduction. Emerging information suggests that caveolae are involved in cholesterol transport and in sorting membrane proteins (receptors and other components of signal transduction pathways) to specific domains in the plasma membrane.¹⁴ Evidence indicates that caveolin-1 directly interacts with proteins that bear lipid-binding motifs (Hedgehog, eNOS, and kinases involved in cell proliferation) and controls their activity by targeting them to distinct membrane phospholipid domains. This mechanism appears to determine where and when these proteins function and may facilitate the spatial integration of the signal transduction machinery. Hence, increased expression of the caveolin-1 gene may have an impact in the development and functional maturation of the retina.

Expression Changes in Genes Related to Retinal Development

Development of the retina involves differentiation of a pseudostratified epithelium that gives rise to different retinal cell types, arranged in layers and functionally connected. The whole process comprises cell proliferation, differentiation, and apoptosis and relies on extracellular matrix interactions, intracellular signal transduction, and specific regulation of gene expression. As determined by microarray analysis, DHA supplementation in retinal explants modified the expression of genes for various protein components of the neuronal intracellular cytoskeleton and the extracellular matrix.

At late stages of retinal development, different transcription factors of the bHLH appear to promote the generation of distinct subclasses of retinal neurons from a common pool of progenitors. Vertebrate bHLH/Per-ARNT-Sim (bHLH/PAS) proteins, forming heterodimers with other family members, par-

ticipate in development of the central nervous system in *Drosophila*. In the retinal explants supplemented with DHA, transcripts for the bHLH b-PAS protein JAP3 (whose specific function is not known) displayed a large increase. Likewise, transcripts for the bHLH protein neuroD, which functions as a differentiation factor for neurogenesis,^{15,16} increased in explants exposed to DHA, as did the mRNA for the neurotrophic factor neuroleukin. On the contrary, the abundance of the mRNA for neuralized homologue, which is also implicated in cell fate determination,¹⁷ displayed lower levels in explants exposed to DHA than in the corresponding control. The consequences of the substantial increase of the mRNA for Patched homologue on neural growth and differentiation deserves further investigation, given the role of Patched receptor as a repressor of Hedgehog signaling.^{18,19}

Transcripts for several proteins related to electrical excitability and neurotransmission appeared highly expressed in the retinal explants supplemented with DHA—particularly, those involved in glutamate neurotransmission, such as subtypes of glutamate receptors that are involved in neural development, neural plasticity, and neurodegeneration. *N*-methyl-D-aspartate (NMDA) and GABA-activated Ca⁺² channels appear to participate in synaptogenesis during retinal development.^{20,21} GABA is present in certain retinal neurons before synapses are formed and temporarily increases intracellular Ca⁺² levels in selected retinal cells.²² It is also suggested that GABA participate in synaptogenesis of cone photoreceptors during retinal maturation.²³ In humans, GABA synthesis is detected as early as 12 weeks of gestation in undifferentiated cells of the inner neuroblast zone. Moreover, synthesis increases between 16 and 25 weeks, both in amacrine and ganglion cells.²⁴ It is apparent that the system that utilizes putrescine instead of glutamate as a precursor for GABA synthesis predominates in early developmental stages of the mammalian retina.²⁵ Putrescine seems to be necessary to sustain both rod outer segment and cone outer segment differentiation in chick embryo retinas.²⁶ In accordance, the expression of ornithine decarboxylase is developmentally regulated in rat embryos, and its activity parallels putrescine content in the retina. An increased level of the ornithine decarboxylase mRNA was found in human fetal retina explants supplemented with DHA; whereas, glutamate decarboxylase mRNA did not exhibit significant changes. Genes encoding different subunits of GABA receptors showed no significant differences in expression in retinal explants treated with DHA. The mRNA for the transactivation domain of NFκB increased in explants exposed to DHA and showed reduced levels in explants supplemented with OA. NFκB is known as an antiapoptotic transcription factor; however, in neurons it appears to participate in transmission of transient glutamatergic signals to the nucleus.²⁷

DHA is suggested to exert a protective effect from apoptosis in neuronal cell lines,²⁸ to promote the survival of photoreceptors during early stages of development, and to mimic glial-derived neurotrophic factor, which promotes apical differentiation.²⁹ In the absence of DHA, photoreceptors start progressive apoptosis, which leads to degeneration.³⁰ Taking into account that development of the vertebrate retina is accompanied by apoptosis in neurons and glia, particularly at stages when synaptic connections are established, the expression level of some genes related to the apoptotic process was investigated in the retinal explants cultured with DHA. The microarray analysis revealed a significant increase in the mRNA for ref-1; a bifunctional redox factor/AP endonuclease that functions as negative modulator of retinal apoptosis and is normally downregulated during cell death in the retina.³¹ However, cell death/survival decisions appear to depend mainly on the ability of proapoptotic and antiapoptotic factors to interact and to trigger/prevent mitochondrial membrane permeabiliza-

tion.³² Therefore, the consequences of increased levels of transcripts for ref-1 and other protein components of the apoptosis signaling cascade (cytochrome c, TRAF5, ZIP kinase, NFκB subunit p65, ERK1 kinase, TRAIL receptor2, apoptosis-specific protein, MEK5, MADD) in retinal explants supplemented with DHA remain to be elucidated.

The broad range of functional categories of genes included in the microarray used in this study provide an overview of the effect of DHA supplementation on retinal explant gene expression. Changes in transcript abundance were detected for genes encoding for proteins that participate in biochemical pathways that are well-known targets for PUFAs, such as lipid β-oxidation and cholesterol biosynthesis. The addition of DHA also modified the expression of genes related to neurogenesis and neuronal function. Although the experimental approach undertaken in the current study does not allow a distinction between direct or indirect consequences of DHA supplementation, the results open a venue to study novel cellular and biochemical responses under the transcriptional influence of DHA or DHA metabolites in developing human retina. Further studies are needed to determine the precise mechanism for the reported effects of DHA on gene expression.

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