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Modulation of blood cell gene expression by DHA supplementation in hypertriglyceridemic men☆,☆☆,★

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Abstract

Our previous study with docosahexaenoic acid (DHA) supplementation to hypertriglyceridemic men showed that DHA reduced several risk factors for cardiovascular disease, including the plasma concentration of inflammatory markers. To determine the effect of DHA supplementation on the global gene expression pattern, we performed Affymetrix GeneChip microarray analysis of blood cells [treated with lipopolysaccharide (LPS) or vehicle] drawn before and after the supplementation of DHA from the hypertriglyceridemic men who participated in that study. Genes that were significantly differentially regulated by the LPS treatment and DHA supplementation were identified. Differential regulation of 18 genes was then verified by quantitative real-time polymerase chain reaction (qRT-PCR). Both microarray and qRT-PCR data showed that DHA supplementation significantly suppressed the expression of low-density lipoprotein (LDL) receptor and cathepsin L1, both of which were also up-regulated by LPS. DHA supplementation also suppressed oxidized LDL (lectin-like) receptor 1 (OLR1). However, LPS did not induce OLR1 mRNA expression. Enrichment with Gene Ontology categories demonstrated that the genes related to transcription factor activity, immunity, host defense and inflammatory responses were inversely regulated by LPS and DHA. These results provide supporting evidence for the anti-inflammatory effects of DHA supplementation, and reveal previously unrecognized genes that are regulated by DHA and are associated with risk factors of cardiovascular diseases.
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Keywords: Docosahexaenoic acid; Gene expression; Inflammation; LDL receptor; Oxidized LDL receptor; Cathepsin L1; Cardiovascular disease

1. Introduction

Chronic inflammation is one of the key pathological conditions promoting the development and progression of a number of inflammatory diseases including cardiovascular disease (CVD), diabetes and cancer [1–3]. Inflammation is induced in response to infection or tissue injury for host defense or wound healing, respectively. Inflammation can also be induced in the absence of

Abbreviations: CVD, Cardiovascular disease; CTSL1, cathepsin L1; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acids; LDLR, low-density lipoprotein receptor; LPS, lipopolysaccharide; OLR1, oxidized LDL (lectin-like) receptor 1, PAMP, pathogen associated molecular pattern; PPAR delta, peroxisome proliferator-activator receptor delta, PTGES, prostaglandin E synthase, PUFA, polyunsaturated fatty acids; qRT-PCR, quantitative real-time polymerase chain reaction, SFA, saturated fatty acid; TLR, Toll-like receptor.

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★ The protocol is listed by the identifier NCT00728338 at http://clinicaltrials.gov.

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infection or tissue injury. It has been suggested that hypoxia in adipose tissues, ER stress and oxidative stress can induce inflammation [4]. Toll-like receptors (TLRs) are a family of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) present in microbes [5,6]. The activation of TLR by PAMPs initiates signaling pathways that lead to the expression of broad arrays of proinflammatory gene products necessary for the elimination of invading microbes. However, recent studies revealed that certain TLRs can be activated by saturated fatty acids and other endogenous molecules in the absence of infection, and that polyunsaturated fatty acids (PUFA), particularly (n-3) PUFA, inhibit agonist-induced activation of TLR [7–9]. These results suggest that sterile inflammation (inflammation with noninfectious origin) and consequent risk of development and progression of chronic diseases can be modulated by dietary fatty acids.

It is generally accepted that diets rich in (n-3) fatty acids are cardioprotective [10,11]. However, the underlying mechanisms are not fully understood. Diets high in saturated, trans and (n-6) PUFA were shown to promote inflammation, while those rich in (n-3) PUFA reduced it [12,13]. Results from epidemiological studies reported an inverse association between concentrations of plasma (n-3) PUFA and markers of inflammation [14-17]. Intervention studies with fish oils or individual (n-3) PUFA demonstrated decreases in the symptoms of inflammatory diseases and in the plasma levels and *ex vivo* production of inflammatory markers [18–20]. While numerous studies demonstrated anti-inflammatory effects of fish oil, the mechanism by which fish oil exert anti-inflammatory effects is not well understood. Moreover, most previous human feeding studies that investigated the effects of (n-3) fatty acids on inflammation were carried out with fish oil and did not differentiate between the effects of eicosapentaenoic acid [EPA, 20:5 (n-3)] and docosahexaenoic acid [DHA; 22:6 (n-3)], the two major components in fish oil. However, there is a growing body of recent evidence that suggests specific effects of these fatty acids on markers of inflammation [21,22]. This may account for some of the reported inconsistencies in the fish oil supplementation studies on markers of inflammation, as concentrations of these two fatty acids can vary widely in marine oils [23]. Though both EPA and DHA share many anti-inflammatory characteristics, there may be qualitative and quantitative differences in their biological actions.

We have previously reported that DHA supplementation (in the absence of EPA) to healthy and hypertriglyceridemic men reduced the plasma and *ex vivo* produced concentrations of inflammatory cytokines, eicosanoids and other markers of inflammation [24,25]. DHA supplementation also reduced a number of other risk factors for CVD, including fasting and postprandial triglycerides, the number of total and small dense low-density lipoprotein (LDL) particles and the number of chylomicron remnant particles, blood pressure and heart rate in hypertriglyceridemic men [26,27]. Here, we determined the effects of DHA on the pattern of global gene expression in blood cells [untreated or treated with lipopolysaccharide (LPS), a TLR4 ligand] from hypertriglyceridemic men using microarray gene chip analysis.

2. Methods and materials

2.1. Study design and participants

The study protocol was approved by the institutional review boards of the University of California Davis and the Veterans Administration Medical Center, Mather, CA. Details regarding the study participants and design have previously been described elsewhere [26,27]. Briefly, moderately hypertriglyceridemic but otherwise healthy men were enrolled and completed a double-blind, placebo-controlled, parallel study with two metabolic periods: baseline (first 8 days) and intervention (last 90 days). Participants were randomized into one of two groups, where one received 7.5 g/day DHA oil capsules containing 3.0 g/day DHA, produced in *Crypthecodinium cohinii* (Martek Biosciences Corp., Columbia, MD, USA), and no EPA and the other group received 7.5 g/day extra virgin olive oil capsules. During the baseline period, participants did not receive supplements. Clinical chemistry and hematology panels for all qualified participants were within normal ranges with the exception of blood lipids. All selected participants had serum C-reactive protein concentrations of 1–10 mg/L; fasting serum triglycerides concentrations of 150–400 mg/dl (1.70–4.53 mmol/L), total cholesterol <300 mg/dl (7.78 mmol/L), LDL cholesterol <220 mg/dl (5.69 mmol/L) and a body mass index between 22 and 35 kg/m^2. Blood samples were collected for *in vitro* treatment. The effects of DHA supplementation on profiles of lipids and lipoproteins and biochemical markers of inflammation have been previously reported [25–27].

2.2. Blood sample collection, LPS treatment and RNA isolation

Four subjects in the DHA-supplemented group were randomly selected for the current microarray study. The changes in plasma triglyceride levels of these four subjects are presented in Supplemental Table 2. Blood samples were treated with either LPS (*E. coli* O111:B4; List Biological Laboratories, Inc., Campbell, CA, USA) or vehicle. The treatment protocol was as described [28]. Briefly, two 10-ml heparinized venous blood samples were collected on day 0 and day 91 from each subject. Whole blood was then diluted 1:2 with sterile RPMI 1640 culture medium containing 25 mmol/L HEPES and L-glutamine (Invitrogen, Carlsbad, CA, USA) and then split into two 30-ml aliquots and stimulated with either 10 μg/ml ultra pure LPS or sterile endotoxin-free water alone for 4 h at 37°C, 5% CO_2. To simplify RNA isolation, erythrocytes were lysed by a two-step process, and the resulting leukocyte pellets were lysed with Buffer RLT (Qiagen), homogenized with QIAshredder spin columns (Qiagen) and stored at −80°C until all the samples were collected. Total cellular RNA from leukocytes was isolated using RNeasy Midi Kit (Qiagen) according to the manufacturer's instructions. RNA quality and integrity were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

2.3. DNA microarray analysis

Purified total RNA (5 μg) was used for cDNA synthesis (SuperScript III First Strand Synthesis System; Invitrogen, Carlsbad, CA, USA) followed by *in vitro* transcription to incorporate biotin labels and subsequent hybridization to Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) microarrays, which represent 38,500 well-characterized genes, per manufacturer's protocol. The arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GeneChip Scanner 3000. Affymetrix CEL files were imported into the Bioconductor affy R package and subjected to baseline correction, normalization and calculation of gene expression values using Robust Multichip Average procedure. Genes that were detected in at least 50% of the microarrays (P<.05 of the signed rank test) were considered for further analyses. Gene expression values were exported into cluster for hierarchical clustering and self-organizing map procedure and visualized as heat maps in Java TreeView. DHA and LPS-specific changes were determined using paired t test. False discovery rate was controlled using Bonferroni. Lists of probe sets that were significantly differentially regulated by DHA or LPS or any combinations thereof were exported into the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov). Gene Ontology categories and BioCarta and KEGG pathway maps were evaluated against the lists of probe sets. Data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) (accession numbers: GSE20114, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20114).

2.4. Quantitative real-time polymerase chain reaction

Eighteen significantly differentially regulated genes, as determined by GeneChip microarray data analysis, were verified using SYBR green quantitative real-time polymerase chain reaction (qRT-PCR). Forward and reverse primers were designed by Oligo Perfect Designer (Invitrogen) based on published full-length cDNA sequences (Supplemental Table 1). Purified total RNA (2 μg) from each subject per time point per treatment was used for cDNA synthesis as described above. The qRT-PCRs were conducted on a 384-well plate format in triplicates (12 μl/well) that included 25 ng cDNA, 170 nmol/L primers, and *Power* SYBR Green PCR Master Mix buffer (Applied Biosystems) and performed using an ABI 7900HT Fast Real-Time PCR System and documented by SDS software v.2.2.2 (Applied Biosystems, Foster City, CA, USA). To confirm specific PCR amplification, a dissociation curve for each gene was programmed after the final cycle. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [29], which normalizes against beta actin endogenous control gene and the day 0 vehicle control group computed average ΔCt. The data represent the percent changes relative to day 0 vehicle control as mean±S.E.M. (n=4). Differences between d 0 LPS and day 0 vehicle-treated samples (LPS effects) and between day 0 vehicle- and day 91 vehicle-treated samples (DHA effects) were tested by using a paired Student's t test using Excel 2003. Statistical significance was set at P<.05.

3. Results

Comparisons were made between LPS and vehicle-treated control samples to detect the LPS effects and between day 0 and day 91

Table 1
Selected genes regulated by DHA supplementation[a]

Gene name	Gene symbol	Fold of	*P* values
Up-regulated by DHA			
Protein serine/threonine kinases			
Mitogen-activated protein kinase kinase kinase 3	MAP3K3	1.79	.0089
Protein kinase N2	PKN2	1.78	.022
Mitogen-activated protein kinase 14	MAPK14	1.76	.022
Serine/threonine kinase 38	STK38	1.73	.014
Mitogen-activated protein kinase 1	MAPK1	1.56	.03
Transcription regulator or activator/nucleotide binding			
CUGBP, Elav-like family member 2	CELF2	2.02	.0064
Cell division cycle and apoptosis regulator 1	CCAR1	1.84	.0015
Transcription factor CP2	TFCP2	1.78	.0035
CUGBP, Elav-like family member 1	CELF1	1.72	.001
Enzymes			
Phosphorylase, glycogen, liver	PYGL	1.75	.038
Protein phosphatase 1, regulatory (inhibitor) subunit 12 B	PPP1R12B	1.69	.039
Other			
Interferon (alpha, beta and omega) receptor 2	IFNAR2	1.74	.006
Enoyl coenzyme A hydratase domain containing 1	ECHDC1	1.69	.0022
Transforming growth factor, beta receptor	TGFBR1	1.64	.0069
Cathepsin S	CTSS	1.63	.0014
Down-regulated by DHA			
Lipoprotein receptor			
Oxidized low-density lipoprotein (lectin-like) receptor 1	OLR1	−2.84	.0046
Low-density lipoprotein receptor	LDLR	−1.64	.041
Transcription factors/regulator of transcription			
Peroxisome proliferator-activated receptor gamma	PPARG	−2.3	.035
SKI-like oncogene	SKIL	−1.91	.0028
Peroxisome proliferator-activated receptor delta	PPARD	−1.8	.013
Activating transcription factor 5	ATF5	−1.57	.048
Aryl hydrocarbon receptor	AHR	−1.53	.024
Other			
Prostaglandin E synthase	PTGES	−2.1	.014
Cytochrome p450, family 51, subfamily A, polypeptide 1	CYP51A1	−1.8	.05
Nuclear receptor interacting protein 3	NRIP3	−1.64	.024
Fatty acid desaturase 1	FADS1	−1.63	.0268
Syndecan 2	SDC2	−1.63	.028
Cathepsin L	CTSL1	−1.59	.0014
Heparin-binding EGF-like growth factor	HBEGF	−1.54	.0165

[a] Numbers represent folds of the control value. Positive numbers represent increase; negative numbers represent decrease.

samples to detect the DHA-specific effects. Interindividual differences were controlled by making pairwise comparisons within subjects. Hierarchical clustering and self-organizing maps were carried out, and the results were then visualized in Java TreeView (Supplemental Figure 1). Clustering of all samples showed that the effect of LPS stimulation was greater than that of the DHA supplementation. Therefore, the effect of DHA supplementation was evaluated in two separate analyses, one for the LPS-treated samples and the other for the vehicle-treated samples.

3.1. Effects of in vitro LPS stimulation on gene expression

To better understand the anti-inflammatory effects of DHA, whole blood samples before and after the 91-day DHA supplementation were stimulated with LPS, a TLR4 ligand, for 4 h. Over 7500 genes were differentially regulated; 2592 genes were induced and 4955 genes were suppressed by LPS. A number of proinflammatory genes, including chemokines, interleukins, TNFα and prostaglandin E synthase (PTGES), were up-regulated by LPS. On the other hand, chondroitin sulfate, proteoglycan 2, chemokine receptors, CD1d, CD31, CD36 and IL-8 receptor beta were suppressed by LPS, consistent with a previous report [30].

3.2. Effects of DHA supplementation on gene expression

DHA-specific effects on gene expression were evaluated by comparing samples before and after the 91-day supplementation. One hundred fifty-eight genes (158) were up-regulated by DHA supplementation, including a number of regulatory kinases, such as MAPK1, MAPK14, MAP3K3 and STK38; glycogen phosphorylase (liver) and protein phosphatase 1; transcription regulators, such as CUGBP, Elav-like family member 1 and 2 (CELF1 and CELF2), interferon (α, β, and ω) receptor 2 (IFNAR2); TGF beta receptor 1 (TGFBR1) and cathepsin S (CTSS) (Table 1). Gene Ontology analysis revealed that DHA up-regulated genes with protein serine/threonine kinase activity, transcription activator/regulator activity and macromolecule metabolic process (Table 1). Thirty genes (30) were down-regulated by DHA supplementation, including LDL receptor (LDLR), oxidized LDL (lectin-like) receptor (OLR1), PPAR gamma and delta (PPARG and PPARD), fatty acid desaturase 1 (FADS1), cathepsin L (CTSL1), activating transcription factor 5 (ATF5), PTGES and heparin-binding EGF-like growth factor (HBEGF) (Table 1). Gene Ontology analysis revealed that DHA down-regulated genes with LDL receptor activity and transcription factor activity (Table 1), and biological processes of immune response, inflammatory response, lipid transport and steroid metabolism (data not shown).

3.3. The interactions between LPS and DHA

To further understand the mechanism(s) underlying DHA's anti-inflammatory effects, we compared the gene profiles that showed opposite effects between LPS and DHA. Eighteen genes (18) were induced by LPS but suppressed by DHA supplementation. These include chemokine CCL-23, PTGES, PPARD and CTSL1 (Table 2). Gene Ontology

Table 2
Selected genes reciprocally modulated by LPS and DHA[a]

Gene name	Gene symbol	Fold of (LPS;DHA)	*P* value (LPS; DHA)
Up-regulated by LPS, but down-regulated by DHA			
Chemokines			
Chemokine (C-C motif) ligand 23	CCL23	12.78; −1.76	.00029; .0055
Transcription factor/regulator of transcription			
SKI-like oncogene	SKIL	1.55; −1.91	.0036; .0028
Peroxisome proliferator-activated receptor delta	PPARD	2.23; −1.80	.006; .013
Nuclear receptor interacting protein 3	NRIP3	2.83; −1.64	.0019; .024
Other			
Prostaglandin E synthase	PTGES	5.08; −2.10	.00053; .014
Cathepsin L1	CTSL1	1.56; −1.59	.002; .0014
Formin-like 3	FMNL3	1.97; −1.55	.0046; .045
Complement component 3	C3	4.75; −1.55	.000007; .033
Down-regulated by LPS, but up-regulated by DHA			
Protein serine/threonine kinase			
Mitogen-activated protein kinase kinase kinase 3	MAP3K3	−2.68; 1.79	.000065; .0089
Mitogen-activated protein kinase 14	MAPK14	−1.78; 1.76	.038; .022
Serine/threonine kinase 38	STK38	−1.53; 1.73	.023; .014
Other			
CUGBP, Elav-like family member 2	CELF2	−1.51; 2.02	.046; .0064
Reticulon 3	RTN3	−2.1; 1.8	.011; .0043
Advillin	AVIL	−2.7; 1.78	.0024; .015
Dedicator of cytokinesis 11	DOCK11	−2.12; 1.76	.0018; .00036
Phosphorylase, glycogen, liver	PYGL	−1.75; 1.75	.010; .038
Erythrocyte membrane protein band 4.1	EPB41	−2.23; 1.74	.0048; .024
Interferon (alpha, beta and omega) receptor 2	IFNAR2	−1.86; 1.74	.010; .006
Asialoglycoprotein receptor 2	ASGR2	−2.18; 1.69	.0039; .022

[a] Numbers represent folds of the control value. Positive numbers represent increase; negative numbers represent decrease.

Table 3
Selected genes similarly modulated by LPS and DHA[a]

Gene name	Gene symbol	Fold of (LPS;DHA)	*P* value (LPS; DHA)
Up-regulated by both LPS and DHA			
Transcription factor, regulator/nucleotide binding			
AT rich interactive domain 5B (MRF1-like)	ARID5B	3.65; 1.90	.0010; .0085
Forkhead box O1A	FOXO1	1.70; 1.75	.013; .0059
CUGBP, Elav-like family member 1	CELF1	2.36; 1.72	.0065; .0010
E74-like factor 2 (ets domain transcription factor)	ELF2	2.07; 1.69	.0018; .0029
Mediator complex subunit 13	MED13	1.95; 1.66	.0069; .0043
RAR-related orphan receptor A	RORA	2.13; 1.65	.0016; .0023
Other			
CDC42 small effector 2	CDC42SE2	1.87; 1.67	.0459; .0097
C-type lectin domain family 2, member D	CLEC2D	1.91; 1.65	.0214; .0213
Down-regulated by LPS and DHA			
Transcription factor/regulator of transcription			
Peroxisome proliferator-activated receptor gamma	PPARG	−2.6; −2.3	.0121; .0348
Hypermethylated in cancer 1	HIC1	−1.48; −1.64	.0097; .0011
Other			
c-mer proto-oncogene tyrosine kinase	MERTK	−6.52; −2.22	.0005; .0340
Rho GTPase activating protein 18	ARHGAP18	−3.91; −1.92	.0004; .0043
Fibronectin 1	FN1	−1.76; −1.67	.0103; .0152
Fatty acid desaturase 1	FADS1	−3.03; −1.63	.001; .0268

[a] Numbers represent folds of the control value. Positive numbers represent increase; negative numbers represent decrease.

analysis revealed that the molecular functions of these genes include transcription factor and chemokine activity (Table 2). The biological processes of these genes include immune, defense and inflammatory response (data not shown). Forty-five (45) genes were down-regulated by LPS but up-regulated by DHA supplementation. These include MAP3K3, MAPK14, STK38, CELF2 and INFAR2 (Table 2). Many of these gene products have serine/threonine kinase activity (Table 2).

Interestingly, we also found genes that showed the similar trend by LPS and DHA. Sixteen (16) genes were up-regulated by both DHA supplementation and LPS treatment. These include the AT rich interactive domain 5B (ARID5B) and CELF1 (Table 3). Six (6) genes were down-regulated by both treatments. These genes include PPARG and FADS1 (Table 3).

3.4. Conformation of microarray results with qRT-PCR

We selected 18 genes that represented the major and significant changes that are relevant to risk of cardiovascular disease (Figs. 1 and 2). The qRT-PCR results confirmed the microarray results by showing up-regulation of CELF1, CCL2, CCL7, CTSL1, HBEGF, IL1A, PPARD, PTGES and LDLR and down-regulation of CELF2, MAP3K3, STK38 and IFNAR2 genes by LPS (Fig. 1A–B). Moreover, DHA supplementation significantly down-regulated basal mRNA levels of LDLR and CTSL1 ($P<.05$), both of which were up-regulated by LPS ($P<.09$ and $P<.01$, respectively), consistent with the microarray results. DHA supplementation also appeared to down-regulate OLR1 expression ($P<.06$) (Fig. 2). However, LPS did not up-regulate OLR1 expression (Fig. 1A).

4. Discussion

We reported here the effects of DHA on global gene expression in blood cells (untreated or treated with LPS) in hypertriglyceridemic men in a 91-day double-blinded and placebo-controlled intervention trial. Supplementation of DHA (3.0 g/day) for 91 days resulted in changes of basal mRNA expression as well as mRNA expression in response to LPS of a number of genes in hypertriglyceridemic men, as determined by microarray analysis. To our knowledge, this is the first time to report the effects of DHA supplementation on global basal gene expression as well as gene expression in response to LPS in human blood cells. Although the fold-expressions regulated by DHA supplementation were mild (generally less than twofold of vehicle controls), consistent with a fish oil supplementation study in humans [31], qRT-PCR analysis has confirmed some significant changes in gene expression of genes that are involved in the risk of CVD.

Both lipoprotein receptor LDLR and OLR1 were down-regulated by DHA supplementation (Table 1). The most dramatically down-regulated gene by DHA supplementation was OLR1. OLR1 is a type II membrane glycoprotein and a member of scavenger receptors [32]. OLR1 is expressed in macrophages, endothelial cells and smooth muscle cells and is known to be involved in the uptake of proatherogenic modified form of LDL leading to foam cell formation [33]. OLR1 is prominently expressed in advanced atherosclerotic plaques of humans [34]. Therefore, the down-regulation of OLR1 may be a part of cardioprotective effects of DHA. On the contrary, it was reported that the saturated fatty acid palmitic acid enhances OLR1 expression and promotes uptake of oxidized LDL into macrophage cells [35].

Low-density lipoprotein receptor is responsible for the uptake of plasma LDL particles by receptor-mediated endocytosis [36]. Familial hypercholesterolemia is caused by the lack of or dysfunctional LDLR expression [37,38]. LDLR knockout mice develop hypercholesterolemia and atherosclerosis when fed a high-fat/cholesterol/cholate-

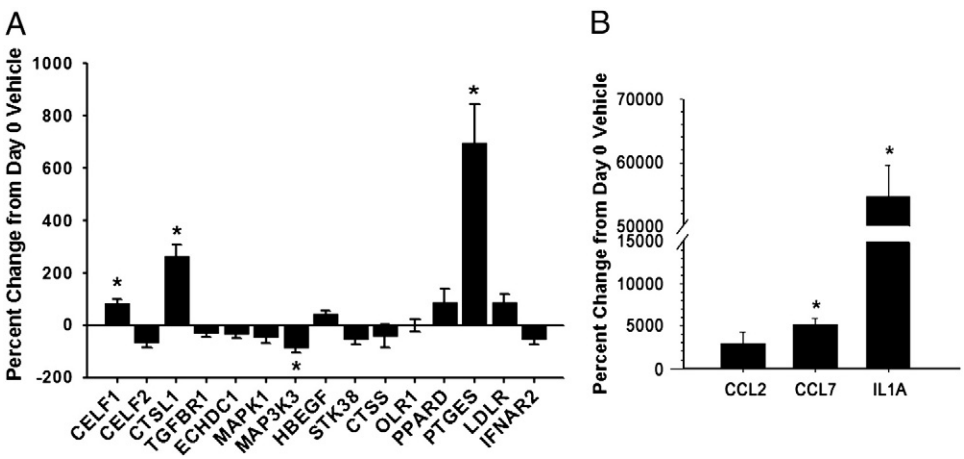

Fig. 1. Quantitative RT-PCR confirms changes in mRNA expression of selected genes in blood cells in response to LPS. (A) Percent change of mRNA expression of selected genes in response to LPS. The selected genes are alphabetically listed. Positive changes indicate induction by LPS, and negative changes indicate suppression by LPS. (B) Percent change of CCL2, CCL7 and IL1A mRNA expression induced by LPS. Data are presented as mean±S.E.M. ($n=4$). *Significantly different from those in the day 0 vehicle group, $P<.05$.

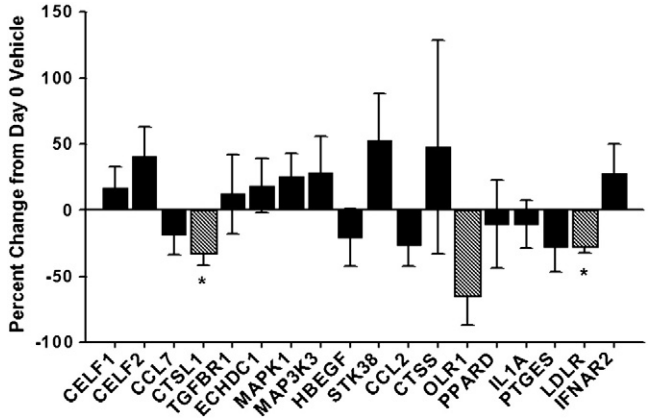

Fig. 2. Quantitative RT-PCR confirms changes in mRNA expression of the selected genes in blood cells in response to DHA supplementation. Percent change of mRNA expression of the selected genes in response to DHA. The selected genes are alphabetically listed. Positive changes indicate induction by DHA, and negative changes indicate suppression by DHA. Data are presented as mean±S.E.M. (n=4). *Significantly different from those in the day 0 vehicle group, P<.05.

containing diet [39]. These results suggest that down-regulation of LDLR may enhance atherosclerosis risk. Our gene expression analyses were performed with mRNA samples derived from whole blood. Thus, down-regulation of LDL receptors by DHA reflects the effects of DHA on nucleated blood cells. It is not known whether DHA also down-regulates LDLR expression in liver, the major organ clearing LDL cholesterol in blood. It has been reported that DHA supplementation elevated, although not significantly, LDL concentration, but significantly decreased the small dense LDL particles in hypertriglyceridemic men [27]. In addition, DHA supplementation decreased concentrations of fasting triacylglycerol, large very low-density lipoprotein (VLDL) and intermediate-density lipoproteins but elevated small VLDL particles, large LDL particles and the mean diameter of LDL particles (0.6 nm) in fasting plasma [27]. Moreover, DHA supplementation decreased remnant-like chylomicron particles and several markers of inflammation [25,26]. Therefore, although the increase in LDL cholesterol and suppression of LDLR mRNA expression may be considered undesirable, the benefits provided by DHA supplementation could more than offset any adverse effects.

Another down-regulated gene by DHA supplementation was cysteine protease CTSL1. It has been shown that CTSL1 is overexpressed in lesions of the inflammatory arterial diseases abdominal aortic aneurysm and atherosclerosis, in which extensive vascular remodeling occurs [40]. Moreover, overexpressed CTSL1 in atherosclerotic lesions has been linked to the death of macrophages, necrotic core formation and development of atherosclerotic plaque instability [41]. Interestingly, CTSL1 was shown to be induced by proinflammatory cytokines [40] as well as by LPS (Fig. 1A), suggesting the role of inflammation in the development and progression of atherosclerosis. However, a supplementation study with fish oil (1.8 g EPA+DHA/day) for 26 weeks in an elderly healthy population [31] shows that basal LDLR, not OLR1 and CTSL1, was significantly down-regulated by fish oil supplementation. The discrepancies may be due to the differences between fish oil and DHA supplementation, lower EPA+DHA intakes and/or lower basal levels of OLR1 and CTSL1 in the healthy population.

We have reported previously that DHA supplementation caused an 11% decrease in the number of circulating neutrophils, but did not change the number of lymphocytes, monocytes, basophils and eosinophils [25,26]. It increased DHA concentration in the red blood cells from 2.91 to 8.12 wt% of total fatty acids (3.53-fold) [25,26]. We did not analyze the fatty acid composition of white blood cells, but anticipate large increases in their DHA concentration as well. We therefore attribute the changes in gene expression to the large changes in DHA concentration rather than the small changes in cell compositions in the whole blood.

In summary, our study reveals a group of genes that are associated with cardiovascular disease risk, but previously unrecognized for being regulated by DHA. Further studies on how DHA regulates the expression of these genes will unveil the mechanisms by which DHA provides cardioprotective effects. We hope that our microarray results can be an important source of information that can help identify additional genes whose expression is regulated by DHA supplementation.

4.1. Statement of authors' contribution to manuscript

K.D. analyzed data, performed statistical analyses, wrote paper and had primary responsibility for final content. L.Z. conducted research, wrote paper and had primary responsibility for final content. Y.A. conducted research and wrote paper. M.V. conducted research. R.L.R. provided oversight. J.P.G. provided oversight on the microarray analysis. D.S.K. designed research, wrote paper and had primary responsibility for final content. D.H.H. designed research, wrote paper and had primary responsibility for final content as a corresponding author. All the authors have read and approved the final version of the manuscript.

Appendix A. Supplementary data

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