Docosahexaenoic Acid Improves the Nitroso-Redox Balance and Reduces VEGF-Mediated Angiogenic Signaling in Microvascular Endothelial Cells

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PURPOSE. Disturbances to the cellular production of nitric oxide (NO) and superoxide (O_2^-) can have deleterious effects on retinal vascular integrity and angiogenic signaling. Dietary agents that could modulate the production of these signaling molecules from their likely enzymatic sources, endothelial nitric oxide synthase (eNOS) and NADPH oxidase, would therefore have a major beneficial effect on retinal vascular disease. The effect of ω -3 polyunsaturated fatty acids (PUFAs) on angiogenic signaling and NO/superoxide production in retinal microvascular endothelial cells (RMECs) was investigated.

METHODS. Primary RMECs were treated with docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) for 48 hours. RMEC migration was determined by scratch-wound assay, proliferation by the incorporation of BrdU, and angiogenic sprouting using a three-dimensional model of in vitro angiogenesis. NO production was quantified by Griess assay, and phospho-eNOS accumulation and superoxide were measured using the fluorescent probe dihydroethidine. eNOS localization to caveolin-rich microdomains was determined by Western blot analysis after subfractionation on a linear sucrose gradient.

RESULTS. DHA treatment increased nitrite and decreased superoxide production, which correlated with the displacement of eNOS from caveolar subdomains and colocalization with the negative regulator caveolin-1. In addition, both ω -3 PUFAs demonstrated reduced responsiveness to VEGF-stimulated superoxide and nitrite release and significantly impaired endothelial wound healing, proliferation, and angiogenic sprout formation.

CONCLUSIONS. DHA improves NO bioavailability, decreases O_2^- production, and blunts VEGF-mediated angiogenic signaling. These findings suggest a role for ω -3 PUFAs, particularly DHA, in maintaining vascular integrity while reducing pathologic retinal neovascularization. (*Invest Ophthalmol Vis Sci.* 2010; 51:6815–6825) DOI:10.1167/iovs.10-5339

Retinal neovascularization (NV) is a serious complication of many ocular disease states, including diabetic retinopathy and retinopathy of prematurity (ROP). In these conditions,

uncontrolled blood vessel growth results in severe vision loss; therefore, dietary interventions that could inhibit the progress of these diseases are clearly advantageous. Indeed, such a role for ω -3 polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (EPA; C20:5 ω -3) and docosahexaenoic acid (DHA; C22:6 ω -3), in modulating angiogenesis is beginning to emerge.^{1–3} Dietary supplementation with ω -3 PUFAs has been shown to decrease pathologic retinal neovascularization in an animal model of oxygen-induced retinopathy (OIR).⁴ Although the health benefits of these fatty acids are now recognized for the prevention of cardiovascular disease, their mechanism of action and role in the modulation of vasoproliferative disorders are still poorly defined.

Proliferative retinopathies proceed in two steps: the primary initiating insult is endothelial cell (EC) death, which results in capillary closure, ischemic hypoxia of the inner retina, and angiogenic growth factor production, leading to the second proliferative stage, which is characterized by sight-threatening NV. There is now a considerable amount of evidence to suggest that in the initial phase, vascular closure is caused by an oxidative-nitrosative insult.^{5–7} In the healthy vasculature, nitric oxide (NO) produced from eNOS has important antiapoptotic and survival functions, resulting in vasoprotection.⁵ In disease, however, there is a decrease in NO bioavailability and an increase in the generation of oxygen free radicals or O_2^- ,⁶ the combined product of which is the highly reactive free radical peroxynitrite ($ONOO^-$). Overall, this causes a shift in the nitroso-redox balance toward one which is pro-apoptotic resulting in adverse consequences on vessel integrity and culminating in vascular occlusion.^{5,8}

The second proliferative NV phase is driven by vascular endothelial growth factor (VEGF) produced in response to tissue ischemia. The activity of VEGF is also dependent on free radical production, namely the production of eNOS and NADPH oxidase-derived NO and O_2^- , which act as second messengers to stimulate migration, proliferation, and angiogenesis.^{9–13} The role of ω -3 PUFAs in modifying these VEGF-mediated signaling cascades has not previously been described.

NO and O_2^- are highly reactive short-lived free radicals that often must be produced in close proximity to their site of action to activate downstream signaling events. eNOS, the predominant NO-producing enzyme in the vasculature, facilitates localized signaling events by means an N-terminal acylation moiety that allows its subcellular localization to the plasma membrane and, in particular, to caveolae or lipid raft subdomains.¹⁴ These cholesterol-rich microdomains act as signal transduction scaffolds that facilitate the clustering of cell-surface receptors with downstream effector or adaptor molecules also localized to these domains, such as VEGFR-2 and the NADPH oxidase complex.^{15,16} In addition, they are enriched in the caveolae coat protein caveolin-1 (Cav-1), which is a potent

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negative regulator of eNOS.¹⁷ Indeed, the importance of this subcellular targeting for eNOS function is evident in studies showing that removal of the acyl group from eNOS misroutes the enzyme and reduces its activation by VEGF.¹⁸ Thus, it is clear that any disruption of the localization of eNOS to caveolae or lipid rafts will have significant effects both on NO production and on the efficiency of downstream signal transduction.

PUFAs are incorporated into cellular membranes, where they can mediate their effects in 1 of 3 possible ways. First, they increase membrane fluidity and alter the subcellular localization of proteins.^{19,20} Second, through the competitive inhibition of the eicosanoid-synthesizing enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P-450 epoxygenases, these enzymes are necessary for arachidonic acid (AA; C20:4 ω -6)-derived eicosanoid production that is known to be proinflammatory and proangiogenic; ω -3 PUFAs compete with AA for access to these enzymes, reducing the production of AA-derived metabolites.²¹⁻²³ Third, ω -3 PUFAs can be metabolized by COX and LOX to generate a different series of eicosanoids that are less inflammatory and have decreased growth-promoting properties.^{24,25} Here, because of the importance of subcellular localization for eNOS function, we were particularly interested in the effect that PUFA supplementation would have on microdomain composition. Indeed, some studies have shown that in macrovascular human umbilical vein endothelial cells (HUVECs), ω -3 PUFAs can disrupt eNOS caveolar localization.^{26,27} However, this finding was not correlated with angiogenic signaling pathways. In addition, there are significant differences between macrovascular and microvascular endothelial cells; importantly, cells of microvascular origin have significantly more Cav-1 resulting in the decreased sensitivity of microvascular ECs to atorvastatin compared with microvasculature ECs.²⁸ Thus, such differences could potentially alter the responsiveness of microvascular ECs to ω -3 PUFA treatment and growth factor stimulation. There-

fore, our aim was to investigate the effects of ω -3 PUFAs on NO and ROS production and to correlate these with angiogenic signaling and changes in subcellular localization of eNOS to caveolae in retinal microvascular endothelial cells (RMECs). In addition, because previous studies indicate that EPA and DHA may have different potencies, suggesting that one may have a therapeutic advantage, a major aim of this study was to perform a direct comparison between EPA and DHA.²⁹

MATERIALS AND METHODS

Cell Culture and ω -3 PUFA Treatment

RMECs were isolated from bovine eyes and routinely cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Dartmouth, MA) supplemented with 20% porcine serum (PS; Sigma, St. Louis, MO), antibiotics (Primocin 100 μ g/mL; Invitrogen, Carlsbad, CA), 0.38% heparin, and 1% insulin for up to five passages in gelatin-coated flasks.³⁰ The phenotype of these cells was confirmed by their typical cobblestone appearance, and the absence of pericyte or smooth muscle cells was verified by the absence of immunocytochemical staining for muscle actins. For PUFA treatment, fatty acid-free bovine serum albumin (BSA; Fraction V; Sigma) was used as carrier to yield a final molar ratio of FA/BSA of 5:1 and to control wells treated with an equivalent amount of BSA.³¹ To determine the effects of EPA and DHA, confluent RMECs were maintained in 5% PS (basal) medium and were treated with each PUFA (Nu-Chek Prep, Inc., Elysian, MN) in the same medium for 48 hours, replacing the medium after 24 hours. The ω -6 fatty acid arachidonic acid (AA, 20:4) and the saturated fatty acid palmitic acid (PA, 16:0) (Nu-Chek Prep, Inc.) were used as controls, depending on the assay. For example, AA was used for the biochemical assays and PA for the structural isolation of caveolae microdomains. After treatment, cells maintained their typical cobblestone morphology. Fatty acid incorporation into cellular membranes was verified by gas-liquid chromatography³² (Table 1), which con-

TABLE 1. Fatty Acid Content in RMECs after 48-Hour Treatment, Analyzed by Gas-Liquid Chromatography

	C	EPA	DHA	AA	PA
Saturates					
Myristate (14:0)	2.11 $\pm$ 0.64	2.05 $\pm$ 0.61	1.64 $\pm$ 0.45	1.06 $\pm$ 0.04	3.87 $\pm$ 1.49
Palmitate (16:0)	20.45 $\pm$ 1.32	20.97 $\pm$ 2.59	20.57 $\pm$ 3.05	13.25 $\pm$ 1.18	31.25 $\pm$ 3.86*
Stearate (18:0)	15.39 $\pm$ 1.10	17.01 $\pm$ 3.00	17.42 $\pm$ 3.31	12.70 $\pm$ 0.67	14.39 $\pm$ 2.13
Arachidate (20:0)	0.15 $\pm$ 0.09	0.15 $\pm$ 0.15	0.13 $\pm$ 0.13	nd	0.30 $\pm$ 0.30
Behenate (22:0)	0.64 $\pm$ 0.03	0.05 $\pm$ 0.05	0.45 $\pm$ 0.10	0.04 $\pm$ 0.03*	0.35 $\pm$ 0.35
Lignocerate (24:0)	0.06 $\pm$ 0.06	0.08 $\pm$ 0.08	nd	0.02 $\pm$ 0.02	nd
Total SFA	43.17 $\pm$ 6.71	40.54 $\pm$ 5.50	40.06 $\pm$ 6.97	27.18 $\pm$ 1.85	50.15 $\pm$ 5.15#
Monosaturates					
Palmitoleate (16:1 ω 6)	0.34 $\pm$ 0.14	0.20 $\pm$ 0.20	0.21 $\pm$ 0.21	0.45 $\pm$ 0.04	nd
Oleate (18:1 ω 9)	15.46 $\pm$ 0.33	13.46 $\pm$ 0.38	12.67 $\pm$ 0.29	10.55 $\pm$ 0.41	8.20 $\pm$ 7.17
Vaccenate (18:1 ω 7)	2.74 $\pm$ 0.22	2.41 $\pm$ 0.32	2.58 $\pm$ 0.08	2.07 $\pm$ 0.21	1.25 $\pm$ 1.13
11-eicosenate (20:1 ω 9)	0.08 $\pm$ 0.08	nd	nd	nd	nd
Nervonate (24:1 ω 9)	nd	nd	nd	nd	nd
Total MUFA	18.72 $\pm$ 0.39	11.67 $\pm$ 4.50	11.79 $\pm$ 3.76	13.07 $\pm$ 0.56*	9.45 $\pm$ 8.29
ω6-polysaturates					
Linoleate (18:2 ω 6)	11.25 $\pm$ 1.51	7.07 $\pm$ 0.93	7.34 $\pm$ 0.59	5.58 $\pm$ 0.63	4.03 $\pm$ 3.62
Arachidonate (20:4 ω 6)	7.32 $\pm$ 1.93	3.95 $\pm$ 1.74	5.11 $\pm$ 2.11	24.76 $\pm$ 2.24*	3.44 $\pm$ 3.26
Total ω 6-PUFA	20.40 $\pm$ 2.30	8.93 $\pm$ 3.87#	10.26 $\pm$ 4.31#	30.44 $\pm$ 2.17	7.47 $\pm$ 6.88#
ω3-polysaturates					
Eicosapentenate (20:5 ω 3)	nd	9.18 $\pm$ 1.17*	0.78 $\pm$ 0.58	0.04 $\pm$ 0.04	nd
Docosahexanoate (22:6 ω 3)	0.25 $\pm$ 0.16	nd	14.21 $\pm$ 3.36*	0.07 $\pm$ 0.07	nd
Total ω 3-PUFA	0.25 $\pm$ 0.15	9.18 $\pm$ 1.17*#	15.38 $\pm$ 4.12*#	0.11 $\pm$ 0.06	nd
ω3/ω6 ratio	0.01	1.03	1.50	>0.01	nd

Results are expressed as percentage of total fatty acid content. C, control; nd, nondetected.

* $P < 0.05$ vs. control.

$P < 0.05$ vs. AA.

firmed a significant increase in EPA or DHA incorporation into cellular membranes.

Vascular Tube Formation

Vascular tube formation was determined using a three-dimensional model of angiogenesis.³³ Briefly, cells at a concentration of 1×10^5 per well were resuspended in DMEM containing 40% PS, mixed with an equal volume of basement membrane matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ) at 4°C, and plated to form spots. Spots were allowed to settle and polymerize for 30 minutes at room temperature and subsequently were bathed in DMEM containing 20% PS. The polymerized basement membrane matrix allows the formation of a three-dimensional matrix and facilitates the formation of a tubular endothelial network over the after 24 hours. The bathing medium was then aspirated, and a second layer of basement membrane matrix containing the various treatments in the presence and absence of VEGF was layered over the primary culture to produce a duplex culture. The secondary layer of basement membrane matrix was allowed to polymerize, and the duplex cultures were incubated for another 24 hours in 20% PS medium at 37°C. During this period, endothelial sprouts invade the secondary gel layer in numbers proportional to the strength of the angiogenic stimulus elicited by the constituents of the secondary gel layer. The cultures were then fixed in 4% paraformaldehyde (PFA) solution, and the numbers of endothelial sprouts passing the interface from the first layer to the second layer were counted using a phase-contrast microscope (Nikon, Tokyo, Japan). Typically, between 5 and 10 spots per treatment were analyzed in each experiment, and all experiments were repeated in at least three separate cell isolates.

Scratch-Wound Assay

To assess the effect of ω -3 PUFA on endothelial cell migration and proliferation, a scratch-wound assay was performed.³⁴ PUFA-treated RMEC monolayers were scratched with a sterile pipette tip to create a cell-free zone. An image of the wounded area was captured with an inverted microscope (0 hours), and the cells were allowed to recover the scraped area after treatment with 5% PS control medium or the same medium containing VEGF (10 ng/mL; Invitrogen) for 12 hours. The extent of healing was determined by measuring the denuded area of five fields per well at time 0 hours and after 12 hours using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Data are expressed as percentage of recovered area after 12 hours.

TUNEL Apoptosis Assay

Equivalent numbers of RMECs were seeded onto gelatin-coated coverslips and treated for 48 hours, as described. The cells were fixed with 4% PFA for 10 minutes at room temperature and incubated with a 0.1% Triton X-100–0.1% citrate permeabilization solution for 2 minutes on ice. Cells were then labeled with a commercial TUNEL reaction mixture (Roche, Germany) in accordance with the manufacturer's instructions. After extensive washing, the slides were mounted with mounting medium and visualized using a confocal fluorescence microscope. TUNEL-positive cells were stained green and were expressed as a percentage of the total number of cells. Five fields per treatment were analyzed, and the results were averaged to yield one data point.

Proliferation

Proliferation was determined by the incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA. ω -3 PUFA-treated monolayers were scratched as described. After 12 hours, medium was replaced with 5% PS medium containing BrdU (30 μ M; Sigma) for 1 hour at 37°C. Cells were then rinsed with PBS, fixed in ice-cold 99% ethanol for 20 minutes at –20°C, and rehydrated with PBS. Monolayers were heated for 1 minute in a microwave oven to break open the cells and expose the cellular DNA; this was followed by 30 minutes incubation with a blocking solution of PBS–0.1% Tween with 5% goat serum. BrdU-

positive cells were detected using an anti-BrdU (1:100; Dako, Glostrup, Denmark) antibody and subsequently a goat anti-mouse AlexaFluor 488-conjugated secondary antibody (1:500; Molecular Probes, Invitrogen). BrdU positive cells were visualized by confocal fluorescence microscopy, quantified, and expressed as a percentage of the total number of propidium iodide-labeled cells. At least 15 fields were captured per slide.

Superoxide Anion Detection

Superoxide anion release was detected using the superoxide-sensitive fluorescent probe dihydroethidine (DHE; Molecular Probes).^{12,13} RMECs were plated in black, clear-bottomed, 96-well plates (Nunc, Rochester, NY), grown until confluence, and treated for 48 hours as described. Cells were then pretreated with polyethylene glycol-superoxide dismutase (SOD; 100 U/mL) and apocynin (1 μ M) for 1 hour at 37°C and were loaded with 20 μ M DHE in Krebs-HEPES buffer for 40 minutes at 37°C. After washing, phorbol 12-myristate 13-acetate (PMA, 10 μ M) or VEGF (20 ng/mL, to reflect the acute nature of this assay) was added to the cells, and fluorescence was quantified immediately in a microplate reader (Safire; Tecan, Männedorf, Germany) at 592 nm emission for 60 minutes.

Nitric Oxide Production

After treatment, cells were incubated in the presence or absence of VEGF (10 ng/mL) in basal growth media for 18 hours at 37°C. Nitric oxide production was quantified in the presence and absence of L-NAME (1 mM) as nitrite accumulation in supernatants and was quantified using a colorimetric Griess assay with absorbance detected at a wavelength of 540 nm. Cells were lysed with 0.2 N NaOH, and nitrite concentration was normalized to total cellular protein content.

Isolation of Caveolae-Rich Fractions

A detergent-free extraction method was used to isolate caveolae-rich fractions with minor modifications.³⁵ After treatment, BRECs were washed twice with PBS and were harvested by scraping. The resultant pellet was resuspended in Mes-buffered saline (MBS; 150 mM NaCl/ 25 mM Mes (2-[N-Morpholino]ethanesulfonic acid, pH 6.5) containing 500 mM sodium carbonate and protease inhibitors on ice and was homogenized. Lysates were then centrifuged for 5 minutes at 10,000 rpm at 4°C, and equivalent amounts of protein were adjusted to 40% sucrose in MBS, placed at the bottom of an ultracentrifuge tube, and overlaid with 30%, 20%, 10%, and 5% sucrose in MBS with protease inhibitors. Samples were centrifuged overnight at 39,000 rpm for 18 hours in a swing out rotor ultracentrifuge (Optima TLX; Beckman Coulter, Brea, CA) at 4°C. Gradient fractions were collected sequentially from the top of the gradient to yield a total of nine fractions and were used immediately for immunoblot analysis. The optical densities (OD readings) of every band were measured and expressed as a percentage of the total OD reading for all the bands. Cells were also treated with 50 μ M of the long-chain saturated fatty acid palmitic acid (PA; 16:0), as described for the ω -3PUFAs and incorporation of PA into the membrane verified by gas-chromatography.

Western Blot Analysis

Cells were lysed, and cellular protein was prepared as described previously.³⁶ Equivalent amounts of protein (40 μ g) were resolved by SDS/PAGE (9% gel), and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Pall Life Sciences, Port Washington, NY). Membranes were probed with monoclonal anti-eNOS (1:500) (Transduction; BD Biosciences), polyclonal anti-nNOS (1:500) (Transduction; BD Biosciences), polyclonal anti-iNOS (1:1000) (Transduction; BD Biosciences), polyclonal Flk-1 Q antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal anti- β -actin (1:5000; Sigma) antibodies followed by anti-mouse, anti-rabbit, or anti-goat horseradish peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology). Protein bands were visualized using enhanced

chemiluminescence detection (ECL SuperSignal; Pierce, Rockford, IL), images were acquired (AutoChemi System; UVP, Upland, CA), and band densitometry was performed on images (Labworks v4.08 software; UVP).

P-eNOS Detection

After 48-hour treatment with ω -3PUFAs, RMEC monolayers were placed in 0.1% PS medium for 2 hours and then stimulated for 15 minutes with VEGF (10 ng/mL). Cells were directly scraped in 1× LDS loading buffer, sonicated, and heated at 95°C for 5 minutes. Equivalent volumes of sample were resolved in 9% SDS-PAGE according to the protocol described. Blots were probed with polyclonal anti-phospho-eNOS antibody (1:1000; Cell Signaling), followed by incubation with anti-rabbit HRP secondary antibody (1:1000; Santa Cruz Biotechnology). After stripping (Stripping Buffer; Pierce), membranes were re-probed with anti-eNOS. Band densitometry was performed as described, and results are expressed as phosphorylated eNOS/total eNOS.

Statistical Analysis

All results obtained are from three to six independent experiments from independent RMEC isolations, with each data point performed in triplicate, and are expressed as mean $\pm$ SEM. Statistical analysis was evaluated by one-way ANOVA and the Tukey-Kramer multiple comparison post hoc test. $P < 0.05$ was considered significant.

RESULTS

Effect of ω -3 PUFA Supplementation on Vascular Sprout Formation in RMECs

EPA and DHA supplementation caused a dose-dependent decrease in sprout formation that was maximal at 50 μ M (Sup-

plementary Fig. S1, <http://www.iovs.org/content/51/12/6815/suppl/DC1>). Direct comparisons at a concentration of 50 μ M revealed that EPA and DHA caused a profound decrease in sprout growth from the first to the second layer of basement membrane matrix compared with controls (Fig. 1). In contrast, ω -6 PUFA arachidonic acid (AA) and saturated PA (Supplementary Fig. S1) had no effect on sprout formation. Furthermore, VEGF treatment in control wells significantly increased sprout formation compared with non-VEGF-treated wells. In DHA- and EPA-treated cells, the pattern of inhibition was similar to that of ω -3PUFA-only treatment, suggesting an inability to respond to VEGF.

Analysis of Wound-Healing Ability in RMECs

At the lowest concentration (10 μ M), DHA, but not EPA, increased wound recovery, after which there was an incremental decrease that reached a maximum at a concentration of 50 μ M (Supplementary Figs. S2, S3). This dose-dependent decrease was also apparent after VEGF treatment. When a direct comparison was performed, EPA reduced wound recovery more potently than did DHA (Fig. 2). In addition, EPA showed a greater increase in response to additional VEGF than did DHA, but this did not reach statistical significance. In agreement with the sprout formation assay, AA had no significant effect on wound healing. For all subsequent experiments, a concentration of 50 μ M was used to make direct comparisons between DHA and EPA.

Measurement of Apoptosis in RMECs following ω -3 PUFA Treatment

TUNEL assay was conducted to determine whether the decrease in tube formation and wound-healing ability with 50 μ M

FIGURE 1. Effect of ω -3 PUFA treatment on vascular sprout formation in RMECs. Vascular tube formation was determined using a three-dimensional model of angiogenesis in which RMECs were plated in basement membrane matrix, forming a first layer and covered after 24 hours by a second layer of basement membrane matrix containing EPA, DHA, and AA in the presence and absence of VEGF. After 24 hours of incubation, the cells were fixed, and sprouts entering the second layer were counted. (A) Representative images of endothelial sprouts crossing the interface from the first to the second layer are shown for each treatment. Results of at least five independent experiments are expressed as the percentage of vascular sprouts with respect to control cells. (B) Direct comparison of the effects of DHA and EPA (50 μ M) in nonstimulated and VEGF-stimulated RMECs. * $P < 0.05$ vs. control; + $P < 0.05$ vs. control + VEGF; & $P < 0.05$ vs. DHA.

FIGURE 2. Comparison of the effects of EPA and DHA on endothelial wound healing in RMECs. Cells were treated with PUFA for 48 hours before wounding and were allowed to recover in medium without or with VEGF for 12 hours. (A) Representative images. (B) Results shown are from five independent experiments and are expressed as the percentage of wound area recovered after 12 hours with respect to control cells. * $P < 0.05$ vs. control; $^+P < 0.05$ vs. control + VEGF; $\&P < 0.05$ vs. EPA.

ω -3 PUFA was the result of increased apoptotic cell death. The results showed that after ω -3 or ω -6 PUFA treatment, there was no significant change in the number of cells showing evidence of apoptotic DNA fragmentation (Fig. 3).

Endothelial Cell Proliferation following ω -3 PUFA Treatment

Endothelial wound healing involves several cellular mechanisms, including migration and proliferation. Therefore, we sought next to investigate the effect of ω -3 PUFA supplementation on the proliferative potential of RMECs. The addition of VEGF to control cells significantly stimulated proliferation; however, treatment of wounded RMEC monolayers with 50 μ M EPA or DHA significantly reduced the numbers of proliferating cells in control and VEGF-treated cultures (Fig. 4). This anti-proliferative effect was much more pronounced in the DHA-treated cells. AA, in contrast, had no effect on RMEC proliferation.

Effect of DHA and EPA Treatment on Superoxide Levels in Endothelial Cells

VEGF and PMA stimulation showed a rapid increase in superoxide production (Supplementary Fig. S4) that was inhibitable with SOD and apocynin, indicating the specificity of the reaction for $O_2^{\cdot-}$. PUFA supplementation with ω -3, but not ω -6, significantly reduced superoxide production (Fig. 5). In addition, there was a significant increase in the magnitude of the effect with DHA treatment compared with EPA.

Measurement of Basal and VEGF-Stimulated NO Production following PUFA Treatment

The basal amount of nitrite produced was unaffected by pre-loading with EPA but was not stimulated further by VEGF treatment. DHA, in contrast, significantly increased basal nitrite accumulation, which again was not increased further on VEGF treatment, suggesting that EPA and DHA treatment prevents VEGF-stimulated NO production (Fig. 6A). Nitrite production was inhibitable with L-NAME. Western blot analysis for all NOS isoforms confirmed that the DHA-induced increase in NO accumulation was not due to alterations in protein expression (Fig. 6B) or to upregulation of the other NOS isoforms. Flk-1 expression was similarly unaffected. There was a tendency toward increased basal P-eNOS in DHA-treated cells (Fig. 6C). However, this did not reach statistical significance, possibly because of the presence of 5% PS in our experiments, which might have reduced the amplitude of any measurable changes in P-eNOS. Because of this limitation, we performed an estimation of VEGF-stimulated P-eNOS compared with the levels in the absence of supplemental VEGF and demonstrated that this was indeed blunted in EPA- and DHA-treated cells, in agreement with the nitrite results (Fig. 6C, Supplementary Fig. S5).

Localization of eNOS in Caveolae Microdomains following Exposure to DHA and EPA

After subcellular fractionation, caveolae were found to be mainly localized to fractions 3 to 5, as indicated by the pres-

A **B**

FIGURE 3. Effect of ω -3 PUFA on apoptosis in RMEC monolayer after 48 hours. (A) Representative images are shown of each treatment along with a DNase I-treated positive control. (B) Results are expressed as percentage of TUNEL-positive cells (green) with respect to the total number of PI-stained (red) cells.

ence of Cav-1 (Fig. 7). The expression of eNOS was greater in fractions 3 to 5 in control and saturated fatty acid control (PA)-treated cells compared with EPA- and DHA-treated cells. In contrast, eNOS expression was greater in fractions 6 to 9 in EPA- and DHA-treated RMECs compared with controls, demonstrating a definite shift in eNOS localization from the caveolae-rich (light buoyancy density) fractions to the noncaveolar (heavier buoyancy density) fractions in EPA- and DHA-treated cells.

DISCUSSION

Disturbances to the nitroso-redox balance can have a deleterious effect on vascular integrity and angiogenic signaling, resulting in vaso-oblivation and pathologic intravitreal neovascularization. Pharmacologic or dietary interventions that could correct this imbalance may have a major beneficial effect on vasoproliferative diseases. Thus, our aim here was to compare the effects of the ω -3 PUFAs DHA and EPA on NO and ROS signaling pathways in RMECs.

Many *in vivo* studies have been performed with the use of dietary supplementation and a combination of PUFAs. EPA and DHA, however, are likely to have differential effects. Therefore, it was important to directly compare the effects of EPA and DHA. To do this, a concentration of 50 μ M was chosen because this was found to have maximal effect on vascular tube formation and scratch-wound assay. Indeed, this concentration is comparable to plasma levels found in populations consuming high dietary fish intake.³⁷ At this concentration, EPA and DHA significantly suppressed endothelial cell proliferation, migration, and tubule formation under basal conditions. Also, VEGF stimulation did not cause any further increase in any of these parameters, suggesting an altered responsiveness to VEGF signaling. These observations are in agreement with previous findings demonstrating that EPA¹ and DPA² decreased serum-induced endothelial cell migration, proliferation, and tube-forming activity.

An integral component of VEGF signaling in vascular endothelial cells is the production of ROS from the NADPH oxidase, which acts as a second messenger to stimulate proliferation and angiogenesis.^{11,13} Therefore, we next examined the possibility that the decrease in angiogenic signaling after ω -3 PUFA treatment was attributed to decreased signaling through ROS

production. In agreement with others,¹² we³⁸ have previously shown that the NADPH oxidase is a major source of ROS production in RMECs; therefore, it is likely that any effects of ω -3 supplementation on the production of this free radical are mediated through this enzyme.^{12,38} Indeed here we showed that EPA and DHA decreased the levels of O_2^- in RMECs, implying that these fatty acids exert a negative effect on NADPH oxidase activity as reported previously in macrovascular endothelial cells.³⁹ Several vascular pathologic conditions, among them diabetes, are associated with the overproduction of ROS⁴⁰; our findings suggest that EPA and DHA could have therapeutically beneficial effects in such diseases.

In addition to reducing O_2^- , it is important that the bioavailability of NO be maintained for it to exert its antiapoptotic and pro-survival effects.⁴¹ NO is also known to play a pivotal role in VEGF-mediated angiogenic signaling by acting through VEGFR2 (Flk-1).⁴² Therefore, we investigated the effect of ω -3 PUFA supplementation on NO production. We found that supplementation with DHA caused a paradoxical increase in basal nitrite levels without altering the concentration of any of the NOS isoforms; VEGF treatment did not increase this further. In contrast, EPA treatment produced no observable increase in basal or VEGF-stimulated nitrite. Moreover, this was not associated with decreased VEGFR2 expression, as described in other studies.² The nitrite result was further examined by investigating the phosphorylation status of Ser1177 on eNOS, which is required for VEGF-mediated EC migration.³⁴ We did detect an increase in basal levels of P-eNOS in the DHA-treated cells, though this did not reach statistical significance. In our hands, RMECs required a minimum of 5% serum to ensure survival for the 48 hours of PUFA treatment, whereas experiments quantifying levels of phosphorylated proteins are often performed in either the complete absence of serum or reduced serum concentrations (1%–2%). It is, therefore, possible that this was responsible for the marginal effect on P-eNOS status we observed in the DHA-treated cells. However, in agreement with the nitrite findings, P-eNOS status in the presence of additional VEGF was unaltered, again suggesting a decrease in VEGF responsiveness.

Increased basal nitrite production and absence of VEGF responsiveness are characteristic phenomena observed in Cav-1 knockout mice because of the absence of negative regulation by Cav-1 and a structural lack of caveolae. Therefore,

FIGURE 4. Effects of DHA and EPA on proliferation in wounded RMEC monolayers treated without or with VEGF for 12 hours. Proliferation in PUFA-treated cellular monolayers was determined by the incorporation of BrdU. Positive cells were visualized by fluorescence microscopy and were expressed as a percentage of the total number of cells. (A) Representative images with BrdU-positive green fluorescent cells are shown; all cells are PI labeled (red). (B) Results are expressed as the percentage of proliferating cells with respect to control. * $P < 0.05$ vs. control; $^+P < 0.05$ vs. control + VEGF; & $P < 0.05$ vs. DHA.

given this similarity with our findings, we next investigated whether the ω -3 PUFAs had any effect on caveolar protein localization. The localization of eNOS to caveolae is known to be dependent on posttranslational acylation. This localization enables the clustering of cell surface receptors with downstream effector molecules. In addition, caveolae are enriched in caveolin-1, which binds to eNOS, maintaining it basally inactive.¹⁷ ω -3 PUFAs can be incorporated into the acyl recognition domains in place of the usual straight-chain saturated palmitoyl moiety. The presence of these highly kinked ω -3 unsaturated fatty acids prevents the docking of such acylated proteins into cholesterol-rich lipid rafts and caveolae, with consequences for downstream signaling.^{19,31,43,44} Indeed, some studies have shown that in macrovascular HUVECs, ω -3 PUFAs can disrupt eNOS caveolar localization.^{26,27} There are significant differences between macrovascular and microvascular endothelial cells that could potentially alter their responsiveness to ω -3 PUFA treatment.²⁸ Therefore, we felt it important to investigate the effect of ω -3 PUFA treatment on the localization of eNOS in RMECs. To examine the spatial distribution of eNOS within the plasma membrane, we subfractionated caveolae according to their buoyancy density in a sucrose gradient. In control cells, eNOS was also localized in the lighter buoyancy density Cav-1-containing fractions, whereas on supplementation with ω -3 PUFAs, the eNOS was shifted to non-caveolar fractions. This shift was dependent on the presence of unsaturated fatty acid because the saturated fatty acid control, palmitic acid, of equivalent length did not cause a similar

shift. Thus, this finding demonstrates that the spatial coupling of eNOS signaling is as important in RMECs as it is in macrovascular cells. Furthermore the colocalization of Cav-1 with eNOS is essential for basal inhibition with Cav-1; therefore, the observed dissociation of eNOS away from Cav-1 is likely to be partially responsible for the increase in basal NO production after DHA treatment. Indeed, this was also observed in Cav-1 knockout mice, which showed an increase in basal NO production because of the absence of negative regulation by Cav-1 and a structural lack of caveolae. In addition to the increase in basal NO, the displacement of eNOS from caveolar localization is likely to have displaced eNOS from close proximity with VEGFR2, which is also concentrated in these microdomains, leading to the observed decrease in VEGF signal transduction.¹⁵ Again, this is similar to findings in Cav-1-depleted animals, which, in addition, to increased basal levels of NO, display reduced responsiveness to VEGF, impaired endothelial cell migration, and reduced angiogenesis, demonstrating the uncoupling of VEGFR activation with eNOS stimulation.^{45,46}

Our results also revealed significant differences between EPA and DHA with regard to their redox-balancing potencies, which correlated with differences in their ability to modulate migration and proliferation. Wound healing is the sum of two separate processes: migration and proliferation. Therefore, if EPA, compared with DHA, had a large inhibitory effect on wound healing and a lesser effect on proliferation, this implies that EPA had its most significant inhibitory effect on migration. The converse would be true for DHA, with DHA having a larger

FIGURE 5. EPA and DHA decreases superoxide anion release in RMECs. Superoxide anion release was detected by loading cells with DHE and was fluorescence quantified in a microplate reader. EPA and DHA treatment inhibited basal and VEGF-stimulated superoxide release. Results are expressed as the percentage of fluorescence at 592 nm with respect to control and are the mean of at least six independent experiments. * $P < 0.05$ vs. control; + $P < 0.05$ vs. control + VEGF; & $P < 0.05$ vs. DHA; ~ $P < 0.05$ vs. DHA + VEGF.

effect on proliferation than EPA. With regard to their redox-balancing potencies, DHA produced a more pronounced increase in NO and a corresponding decrease in O_2^- compared

with EPA. This higher NO/ O_2^- ratio generated after DHA treatment correlated with a higher migratory potential compared with EPA. Thus, in agreement with a role for NO in stimulating migration,⁴⁷ this suggests that there was a higher proportion of NO in the DHA-treated cells to stimulate migration. In contrast, EPA demonstrated a higher O_2^- /NO ratio and a higher proliferative potential. Indeed, a high ratio of O_2^- /NO is implicated as a contributory factor in disturbed angiogenesis in diabetes.^{48–50} Indeed strategies aimed at decreasing O_2^- or improving NO bioavailability can stimulate reparative angiogenesis.^{51,52} Together these findings suggest that DHA may have a therapeutic advantage over EPA for the treatment of vasoproliferative disorders. One possible explanation for this differential effect may be related to the presence of an additional unsaturated double bond in DHA that would increase the unsaturation index compared with EPA. DHA has been shown to have a higher anti-inflammatory potency and to have produced greater vasodilatation than EPA.^{29,53,54} Considering this, it is surprising that we did not observe any significant difference between the proportion of eNOS displaced from caveolae by the two fatty acids, but this might have been because of limits in the sensitivity of the assay. Alternatively, and in addition to their effects on membrane fluidity, ω -3 PUFAs have a high affinity for eicosanoid-synthesizing enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX).^{21,22} In agreement with this and in marked contrast to the effects observed with the ω -3 PUFAs, the ω -6 PUFA AA had no inhibitory effect

FIGURE 6. DHA increases nitric oxide production in RMECs basally and reduces VEGF responsiveness. Cells were preloaded with the various fatty acids for 48 hours before nitric oxide production was assessed. (A) Nitric oxide production was quantified as nitrite accumulation in supernatants by Griess assay and normalized to total cellular protein content. Direct comparison of EPA and DHA in nonstimulated and VEGF-stimulated RMECs. Results are expressed as nitrite per total protein content. (B) Representative Western blots of nNOS, iNOS, eNOS, Flk-1, and β -actin and (C) PeNOS accumulation in PUFA- and VEGF-treated RMECs. * $P < 0.05$ vs. control; & $P < 0.05$ vs. DHA; ~ $P < 0.05$ vs. DHA + VEGF.

FIGURE 7. ω -3 PUFA causes a shift in the localization of eNOS from caveolae microdomains in RMECs. Caveolae-rich fractions were isolated from treated cells by fractionation on a sucrose gradient, and the localization of eNOS investigated by Western blot analysis. Caveolin-1, a marker for caveolae, was predominantly localized to light buoyancy density fractions 3 to 5. eNOS was predominantly localized to caveolae in control and PA-treated cells and shifted to noncaveolae (heavier buoyancy density fractions 6–9) after treatment with EPA or DHA. β -COP, Golgi marker; PA, palmitic acid saturated fatty acid; C, control. Data are representative of three experiments ($n = 3$; $*P < 0.05$ vs. control). β -COP was predominantly localized to fractions 7 to 9.

on tube formation, migration, or proliferation and no significant modulation of the redox balance, demonstrating that our findings are indeed unique to ω -3 fatty acids. Indeed, as a precursor for proinflammatory eicosanoids derived from COX, LOX, or cytochrome P-450 epoxygenases, AA at moderate concentrations is proangiogenic.²³ ω -3 PUFAs, in opposition, can compete with AA for access to the active sites of these enzymes, thereby inhibiting AA activity and AA-stimulated angiogenesis.^{21,22} Thus, it is probable that the effects of EPA and DHA may be mediated by a reduction in AA-derived products. In addition to reducing the concentration of AA-derived eicosanoids, the same enzymes (LOX and COX) produce specific EPA- or DHA-derived bioactive eicosanoids that are less inflammatory, have fewer growth-promoting properties, and have more antiapoptotic properties and antiangiogenic activity than their ω -6 PUFA-derived counterparts.^{24,25} These metabolites, therefore, might also have contributed to the differential effects we observed between EPA and DHA.

Furthermore, our results suggest that ω -3 fatty acids at the physiologically relevant concentrations we used here would have multiple effects on RMECs—direct effects on membrane composition, a decrease in AA-derived eicosanoids, and possibly indirect effects through the increased production of DHA- and EPA-derived metabolites. It is possible that the significant effects we observed on caveolar protein composition could influence the activity of enzymes such as COX and LOX and could modulate their downstream signaling cascades. Future investigation into how these individual pathways converge will be important in further dissecting the role of ω -3 fatty acids in angiogenesis.

In summary, this study provides evidence that ω -3 PUFAs, in particular DHA, can improve the nitroso-redox balance by modulating the production of NO and O_2^- . In addition, they blunt angiogenic signaling, which we suggest is mediated in part by the displacement of eNOS from caveolae. Previous studies showing that dietary supplementation with ω -3 PUFAs decreased pathologic retinal neovascularization in an OIR model suggested that this beneficial effect was mediated, in part, by a decrease in TNF- α production from microglial cells.⁴ Here, we propose that in addition

to their effects on proinflammatory signaling, the beneficial effects described in this model may also be attributed to direct effects on RNS and ROS signaling in vascular endothelial cells. Our findings suggest that an increase in ω -3 PUFA-mediated NO bioavailability, combined with a decrease in VEGF responsiveness, would also contribute to improved vascular recovery and reduced NV tuft formation. Taken together, these studies provide evidence that the benefits of ω -3 PUFAs may be realized in maintaining the prosurvival effects of NO in the early degenerative phase of ischemic retinopathies while reducing the severity of VEGF-mediated signaling in the late proliferative phase.

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