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Effects of Dietary Decosahexaenoic Acid (Dha) on eNOS in Human Coronary Artery Endothelial Cells

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Abstract

Endothelial dysfunction occurs in heart disease, and may reduce functional capacity via attenuations in peripheral blood flow. Dietary DHA may improve this dysfunction, but the mechanism is unknown. We determined if DHA enhances expression and activity of eNOS in cultured human coronary artery endothelial cells (HCAEC). HCAEC from 4 donors were treated with 5 nM, 50 nM, or 1 μ M DHA for 7 days to model chronic DHA exposure. A trend for increased expression of eNOS and phospho-eNOS was observed with 5 and 50 nM DHA. DHA also enhanced expression of two proteins instrumental in activation of eNOS; phospho-Akt (5 and 50 nM) and HSP90 (50 nM and 1 μ M). VEGF-induced activation of Akt increased NO_x in treated (50 nM DHA) vs. untreated HCAEC (9.2 ± 1.0 vs. 3.3 ± 1.1 μ mol/ μ g protein/ μ l). Findings suggest that DHA enhances eNOS and Akt activity, augments HSP90 expression, and increases NO bioavailability in response to Akt kinase activation

Keywords

phospho-eNOS; Akt kinase; phospho-Akt; Hsp90; 3-nitrotyrosine; cell culture

Endothelial dysfunction plays a prominent role in heart disease where it contributes to vasoconstriction, increased vascular resistance and reduced peripheral perfusion.^{1,2,3} These changes can lead to reductions in functional capacity that are characterized by attenuations in blood flow and oxygen delivery to active skeletal muscle⁴ and the heart.⁵ Attenuations in blood flow may be due, in part, to endothelial dysfunction associated with a decrease in the bioavailability of endothelial nitric oxide (NO), a major mediator of vascular tone.² Decreases in NO bioavailability have been linked to decreases in endothelial NO synthase (eNOS) expression,⁶ reductions in eNOS activity,⁷ and enhanced degradation of NO by reactive oxygen species.⁸ Thus, interventions that enhance the expression or activity of

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eNOS may increase the bioavailability of NO in pathophysiological conditions (e.g., heart disease) leading to improvements in endothelial function and functional capacity.

Dietary supplementation with omega-3 polyunsaturated fatty acids may represent one such intervention. Decosahexaenoic acid (DHA), in particular, has protective effects on the cardiovascular system.⁹ It also enhances endothelium-dependent vasorelaxation of aortic rings via augmented release of NO,¹⁰ and increases IL-1 β -provoked NO production in vascular smooth muscle cells.¹¹ One possible mechanism underlying these effects of DHA is increased expression of vascular eNOS. Moreover, DHA may enhance activation of eNOS to stimuli such as vascular endothelial growth factor (VEGF) or shear stress. This effect could occur, at least in part, via increases in expression of two important endothelial proteins, Akt kinase and heat shock protein 90 (Hsp90), that synergistically participate in post-transcriptional activation of eNOS.¹² Accordingly, DHA-induced increases in expression of Akt and Hsp90 may enhance NO production and blood flow. That is because both Akt and Hsp90 are activated by shear stress,^{13,14} an important stimulus for NO production during exercise.¹⁵

Based on these observations, we tested the hypotheses that: 1) treatment of human coronary artery endothelial cells (HCAEC) with DHA enhances expression and activity of eNOS and Akt and expression of Hsp90; and 2) stimulation of eNOS via activation of Akt and Hsp90 increases NO production.

Methods

Endothelial Cell Culture

Cultured HCAEC from 5 donors (three male and two female) were purchased from Cambrex Bioproducts (East Rutherford, New Jersey). All experiments were conducted at an early passage number as previously described.¹⁶

Treatment Protocols

HCAEC from 2 male and 2 female donors were grown to 70-80% of confluence¹⁶ (5-7 passages) and then divided into 2 groups; one control group (untreated cells maintained in media for 7 days), and one group treated for 7 days with DHA. This long term in vitro incubation was done to simulate in vivo effects of an increase in dietary DHA. Concentrations of DHA were selected that are within a range of plasma levels that are nutritionally achievable.^{20,21} Thus, DHA concentrations were 5 and 50 nM, and 1 μ M. Concentrations of 50 and 100 μ M were also tested. Cells from each group were analyzed for expression of eNOS, phospho-eNOS, Akt, phospho-Akt, Hsp90, and β -actin. An ELISA assay kit was used to measure 3 nitrotyrosine (Cell Sciences), an index of oxidative stress and endothelial damage caused by peroxynitrite, and basal cGMP production according to the manufacturer's instructions (Assay Designs). Lactate dehydrogenase (LDH) release was measured as an index of cell necrosis using a kit (Sigma) as previously described.¹⁷

Western Blotting

At least 6 blots of different HCAEC from each group (Control, 5 nM, 50 nM, and 1 μ M DHA) were analyzed from each donor for each antibody. Western blotting was performed as previously described.¹⁶ Blots were incubated with antibodies to eNOS (1:1000, BD Biosciences), Akt (1:1000, Cell Signaling.), Hsp90 (1:1000, Cell Signaling), and β -actin (1:20,000, Novus Biologicals). Phospho-blots were blocked with 5% Milk and developed for phospho-eNOS (serine-1177, 1:500, Cell Signaling) and phospho-Akt (serine 473, 1:400, Cell Signaling). B-actin was used as a loading control. Blots were scanned and density analyzed as previously described.¹⁶ Sample densities were normalized to beta actin and then to control (untreated) samples on each blot for analysis. For phospho-blots, samples were normalized to total density for respective protein and then to control values for the same blot.

Measurement of NO_x

In 2 groups of HCAEC (untreated and treated for 7 days with 50nM DHA) obtained from a male donor, changes in NO production were assessed as a functional correlate of increased expression eNOS. NO_x was measured as an index of NO. NO_x is a combined measurement of NO and its metabolites NO₂⁻ and NO₃⁻. Since peroxynitrite is also metabolized to NO₂⁻ and NO₃⁻, increases in NO_x could be misleading. Consequently, on day 7, cells in each group were treated with serum starved media for 2 hrs before media collection for initial measurement of NO_x. Then, the media was replaced and cells were incubated for 20 min with new serum starved media that included cell permeable polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) (300 U/ml of culture medium, Sigma). Media was then collected for initial measurement of NO_x. Subsequently, this protocol was repeated in each group with the exception that VEGF was added to the media to stimulate NO production via activation of Akt.¹⁸ Aliquots of HCAEC culture medium were aspirated and transferred to centrifuge tubes. All samples were centrifuged at 1500 $\times$ g for 1 min before transferring 40 μ l of the supernatant to a purge vessel containing 0.8% vanadium chloride in 1 N HCL at 95° C. These conditions resulted in the conversion of NO_x to NO^{*}, which was carried with N₂ into a Sievers chemiluminescence NO analyzer (Model 280). Standard curves were generated daily using 0.1 to 10 μ M NaNO₃.

Parallel studies were conducted with both untreated and DHA treated cells (50 nM, 7 days), which were serum starved for 2 hr and then left untreated or incubated with VEGF (1 nM) for 20 min in serum free media. Lysates were collected and analyzed for AKt, phospho-Akt, eNOS, and phospho-eNOS.

Data Analysis

Quantification of expression of phosphorylation of eNOS (at serine-1177) and of Akt (at serine 473) were used as indices of the activity of these two enzymes after normalization to total Akt or total eNOS. Data were compared by ANOVA followed by a Dunn's post-hoc analysis. For normalized data, an ANOVA on Ranks was used. When only two means were compared, the Student's paired t or unpaired t test was used. Results are expressed as mean $\pm$ SEM. Statistical significance was accepted at P<0.05.

Results

Effects of DHA Treatment on Akt Activity and Akt Expression

Chronic *in vitro* treatment of HCAEC with DHA induced significant increases in phospho-Akt of 1.8 fold in response to the 5 and 50 nM concentrations of this fatty acid (Figure 1, Panel A). In addition, the 1 μ M concentration increased phospho-Akt expression in 3 of 4 donors. However, DHA treatment did not increase expression of Akt at any concentration (Figure 1, panel B). In fact, it caused small decreases at concentrations of 50 nM and 1 μ M.

Initial studies using DHA concentrations of 50 and 100 μ M in male and female HCAEC caused cell death within 24-48 hr that was characterized by disruption of cell membranes as seen under light microscopy.

Effects of DHA Treatment on eNOS, cGMP, and 3-Nitrotyrosine

Seven days of DHA incubation *in vitro* had no statistically significant effects on eNOS activation or expression (i.e., phospho-eNOS expression) in HCAEC (Fig. 2). Still, compared to control values, eNOS activation was higher in all 4 donors in response to the 5 nM concentration of DHA and in 3 out of 4 donors in response to 50 nM DHA.

Basal cGMP production, which was measured as an index of eNOS activation, was higher only in cells treated with 5 nM DHA (Figure 2C). This finding was consistent with the trend for increased eNOS activation at the same dose.

There was no increase in LDH release after 7 days of treatment with 5 nM, 50 nM, or 1 μ M DHA (0.24 ± 0.04 , 0.30 ± 0.07 , and 0.17 ± 0.03 , units/mg protein, respectively) compared with control cells (0.33 ± 0.05 units/mg protein, $p > 0.05$). Similarly, when we selectively examined the effects of 50 nM of DHA on effects on 3-nitrotyrosine concentrations compared with untreated HCAEC, no differences were observed (64.1 ± 3.0 vs. 60.9 ± 3.1 pM/ μ g protein).

DHA Treatment and Expression of Hsp90

Expression of Hsp90 was augmented by 7 days of DHA treatment at concentrations of 50 nM and 1 μ M (Fig. 3, Panel A). The 5 nM dose also increased Hsp90 expression in all 4 donors, but this increase was not statistically significant.

Effects of VEGF-activation and DHA on NOx Production by HCAEC

Treatment of HCAEC with 50 nM DHA for 7 days increased Akt but not eNOS activity (Figures 4A and 4B). Similarly VEGF stimulation increased Akt activity in both untreated and DHA treated cells. but had no affect on the activity of eNOS (Figures 4A and 4B). VEGF stimulation of DHA pre-treated cells was the only intervention that led to higher eNOS activity (Figure 4B).

Stimulation of untreated male HCAEC for 20 min with VEGF caused a modest increase ($\sim 40\%$) in the mean concentration of NOx in the media (Fig. 4). Conversely, cells incubated for 7 days with the concentration of DHA (50 nM) that augmented both eNOS activity and HSP90 expression caused much larger VEGF-induced increases in NOx concentrations. In this case, 20 min of VEGF incubation increased the mean NOx level by ~ 3 fold (Fig. 4).

Discussion

We found that the 5 nM and 50 nM of DHA caused significant increases in Akt activity and that the 50 nM and 1 μ M concentration enhance expression of HSP90. A trend for DHA to enhance the expression of eNOS was also observed. Levels of this enzyme were increased in all 4 donors at the 5 nM concentration and in 3 of 4 donors at the 50 nM concentration. These responses are noteworthy because the interaction of eNOS, Akt, and HSP90 plays a major role in the synthesis of NO.^{7,14,19} Moreover, the 50 nM concentration of DHA is within a range produced by nutritional supplementation with this fatty acid (i.e., nM to low μ M concentrations).^{20,21}

Effects of DHA on eNOS expression and Akt activity appeared to be dose dependent since they were seen at 5 and 50 nM, but not at 1 μ M DHA. Consequently, the ability of DHA to enhance eNOS expression may be limited to a narrow range of concentrations. In fact, higher concentrations of this polyunsaturated fatty acid were toxic to the cells, as concentrations of 50 and 100 μ M caused cell death within 24-48 hrs. This outcome is consistent with observations that 24-48 hr incubations with higher concentrations of DHA can cause deregulation of eNOS activation (20-80 μ M)²² or cell death (50-200 μ M).²³ Our results also imply that the threshold concentration for any effects of chronic activation of eNOS by DHA is in the low nM range.

Akt is an important mechanism for activation of eNOS in endothelial cells.^{24,25} It enhances eNOS activity by phosphorylation of this enzyme at the amino acid residue serine 1177.²⁴ Akt is activated when it is phosphorylated via phosphatidylinositol-3-OH kinase (PI3 kinase).²⁴ The activity of Akt can be increased by stimuli such as shear stress and VEGF,^{19,24} leading to increased NO production.²⁵ Thus, DHA-induced increases in the expression of eNOS, and in the activity of eNOS and Akt kinase, could result in greater production of NO in response to a given level of stimulation (e.g., shear stress or VEGF). Our findings support this likely outcome in that DHA treatment augmented VEGF-induced activation of Akt and eNOS and concomitant production of NOx. This augmentation of Akt activity may be relevant from a functional perspective because its activation maintains NO activation,²⁴ an effect that would be important in the regulation of blood flow.

Hsp90 is an important signaling factor involved in post-translational regulation of eNOS activity²⁶ that associates with eNOS in quiescent endothelial cells. Stimulation of these cells (e.g., via shear stress) intensifies the interaction between Hsp90 and eNOS and increases eNOS activity and NO release.^{15,27} Akt also binds to Hsp90 such that the interaction of these three proteins plays an integral role in the regulation of NO.²⁷ Thus, the DHA-induced increases in HSP90 expression we found may indicate an rise in NO bioavailability.

DHA-induced increases in eNOS expression and activity may promote endothelial dysfunction, which could potentially occur in response to high dietary supplementation with fish oil capsules containing this fatty acid. Consequently, activation of greater levels of eNOS protein could lead to a higher production of NO. In turn, a greater production of peroxynitrite could occur if NO rapidly reacts with the superoxide ion ($O_2^{\cdot-}$).²⁸ This phenomenon might reduce NO bioavailability and its consequent effects. What is more,

peroxynitrite is a potent endogenous oxidizing and nitrating agent²⁹ that forms 3-nitrotyrosine, a substance that can cause oxidative stress, endothelial dysfunction and DNA damage.³⁰ Peroxynitrite-induced nitration can also inactivate and/or attenuate the activity of important antioxidant enzymes (e.g., magnesium superoxide dismutase)³¹ and other enzymes that regulate endothelial-induced vasodilation (e.g., PGI₂ synthase).³²

Although we did not measure peroxynitrite, the potential for DHA to induce these effects was assessed by comparing 3-nitrotyrosine production in DHA treated and untreated HCAEC. Since LDH release and expression of 3-nitrotyrosine levels in treated cells were no different than those in control cells, and cGMP was higher in treated cells (5 nM DHA), it is reasonable to assume that DHA-induced increases in eNOS lead to increases in NO bioavailability and not to cell damage, increased protein nitration, or elevated peroxynitrite production. This contention is further supported by the results of a study where rats were fed a diet rich DHA and EPA.³³ SOD activity and NADH-induced production of O₂⁻ in aortic segments were similar to rats fed a control diet, suggesting that peroxynitrite production was also similar between the two groups.

Another important finding of this study was that treatment of HCAEC with 50 nM DHA, which increased expression of HSP90 and Akt activity, and tended to increase eNOS activity, augmented NOx production in response to acute VEGF stimulation. VEGF increases the activity of eNOS via enhanced Akt-induced phosphorylation at the serine-1177 position on the eNOS protein,³⁴ which might explain how DHA enhanced NOx under these circumstances. The fact that activation of eNOS can augment production of cGMP and NOx³⁵ suggests that our VEGF-induced elevations in NOx were indicative of enhanced NO bioavailability.

NOx measurements reflect concentrations of stable end products of NO metabolism (i.e., NO₂⁻ and NO₃⁻)³⁶ However, some NO may combine with O₂⁻ to form peroxynitrite, which can quickly decompose to peroxynitrous acid and then isomerize rapidly to NO₃⁻.³⁶ To limit this potential contamination of NOx, we treated each group with PEG-SOD because it enhances the metabolism of O₂⁻, which tends to reduce the formation of peroxynitrite from NO and O₂⁻. Unlike native SOD, it is resistant to enzymatic breakdown and has a much longer half-life. Therefore, NOx measurements under these conditions more accurately reflect NO production.

In summary, chronic treatment of HCAEC with low physiological concentrations of DHA enhanced the activity of Akt and expression of HSP90, important proteins that activate eNOS in response to shear stress. In addition, a trend for this fatty acid to increase eNOS activity was found. In DHA treated HCAEC, acute activation of Akt by VEGF enhanced the activity of eNOS and caused nearly a 3.0 fold increase in NOx production. This response occurred in the absence of differences in indices of cell damage or effects of peroxynitrite. Although these in vitro findings are preliminary, and further in vivo studies are necessary, they do suggest that chronic exposure to DHA can enhance NO bioavailability in HCAEC via the eNOS and Akt pathways.

Clinical Implications

DHA treatment may enhance NO bioavailability under physiological conditions. Akt is instrumental in eNOS activation during shear stress,²⁴ and this stimulus plays a major role in the endothelial release of NO¹⁶ that contributes to exercise-induced coronary vasodilation.³⁷ Moreover, endothelial dysfunction in pathological conditions such as heart failure, atherosclerosis, and hypertension is due, at least in part, to reduced production and/or increased inactivation of NO via oxidative stress.^{38,39} Thus, DHA-induced increases in NO bioavailability may augment endothelial function and blood flow in patients with cardiovascular disease such that functional capacity and exercise intolerance are improved. This response is suggested by the observation that dietary supplementation with fish oils containing DHA improves endothelial function in heart failure patients.⁴⁰ Therefore, there is a need for additional studies of HCAEC from donors with normal and impaired endothelial function.

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Figure 1.

Line graph depicting mean values of phospho/total Akt (Panel A) and total Akt/beta actin expression (Panel B) in HCAEC in response to 7 days of incubation with specific concentrations of DHA, and in untreated controls (Ctl). Representative Western blots of phospho/total Akt, total Akt kinase and β -actin expression are also shown in the right hand panel. * $p < 0.05$, group mean vs. control (Ctl)

Figure 2.

Line graph depicting mean values of phospho/total eNOS (Panel A) and total eNOS/beta actin (Panel B) expression in HCAEC after 7 days of incubation with specific concentrations of DHA, and in untreated controls (Ctl). Shown in Panel C is a representative Western blot of phospho/total eNOS, total eNOS, and β -actin expression. Panel D represents a line graph of mean values of cGMP after 7 days of incubation with specific concentrations of DHA and in untreated controls (Ctl). * $p < 0.05$, group mean vs. control (Ctl)

Figure 3.

Mean values of Hsp90 expression in untreated control cells (Ctl) and in response to 7 days of incubation with specific concentrations of DHA are illustrated in the top panel. Western blots representing the expression of Hsp90 and β -actin in response to DHA incubation are shown in the bottom panel. * $p < 0.05$, group mean vs. control (Ctl)

Figure 4.

Bar graphs summarizing increases in phospho/total Akt (Panel A) and phospho/total eNOS (Panel B) in control (Ctl) and DHA treated male HCAEC cells, and in Ctl and DHA treated cells stimulated with 1 nM of VEGF. Shown in Panel C are representative Western blots of Akt, phospho-Akt, eNOS, and phospho-eNOS in Ctl and DHA treated cells, and in Ctl and DHA treated cells stimulated with 1 nM of VEGF. Panel D depicts increases in NOx concentration (n=6) in response to VEGF stimulation (1 nM) in untreated male HCAEC cells and those incubated with 50 nM of DHA for 7 days. *P<0.05, vs. Ctl. †P<0.05, vs. DHA.