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Omega-3 and omega-6 fatty acids have distinct effects on endothelial fatty acid content and nitric oxide bioavailability

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ABSTRACT

Treatment with high dose icosapent ethyl (IPE), an ethyl ester of the omega-3 fatty acid eicosapentaenoic acid (EPA), significantly reduced ischemic events in patients with either cardiovascular disease (CV) or diabetes plus other risk factors (REDUCE-IT) but the mechanism is not well understood. We compared the effects of EPA, docosahexaenoic acid (DHA), and the omega-6 fatty acid arachidonic acid (AA) on bioavailability of nitric oxide (NO) and fatty acid composition. Human umbilical vein endothelial cells (HUVECs) were pretreated with EPA, DHA, or AA (10 μM). Cells were stimulated with calcium ionophore and NO and peroxynitrite ($ONOO^-$) were measured using porphyrinic nanosensors. Levels of EPA, DHA, AA and other fatty acids were measured by gas chromatography (GC). EPA treatment caused the greatest NO release (18%, $p < 0.001$) and reduction in $ONOO^-$ (13%, $p < 0.05$) compared to control; the [NO]/[$ONOO^-$] ratio increased by 35% ($p < 0.001$). DHA treatment increased NO levels by 12% ($p < 0.01$) but had no effect on $ONOO^-$ release. AA did not affect either NO or $ONOO^-$ release. Fatty acid treatments increased their respective levels in endothelial cells. EPA levels increased 10-fold to 4.59 mg/g protein ($p < 0.001$) with EPA treatment and the EPA/AA ratio increased by 10-fold ($p < 0.001$) compared to vehicle. Only EPA increased docosapentaenoic acid (DPA, omega-3) levels by 2-fold ($p < 0.001$). AA alone decreased the EPA/AA ratio 4-fold ($p<0.001$). These findings support a preferential benefit of EPA on endothelial function and omega-3 fatty acid content.

1. Introduction

Endothelial cells mediate vasodilation through the regulated release of nitric oxide (NO) in response to changes in blood flow and various pharmacologic interventions [1-5]. Beyond its effects on smooth muscle cell relaxation, NO regulates platelet and leukocyte adherence, hemostasis/thrombosis and fibrinolysis [6, 7]. In a highly coordinated fashion, endothelial cells also produces potent vasoconstrictors, including endothelin-1, angiotensin II, thromboxane A_2 (TXA_2), and prostaglandin H2. In atherosclerosis, endothelial cell dysfunction associates with abnormal vasomotor control and loss of NO bioavailability [8, 9]. NO is generated by three distinct dimeric nitric oxide synthase (NOS) enzymes that catalyze the oxidation of L-arginine into L-citrulline and NO. Many cell types express constitutively two of these enzymes, endothelial NOS (eNOS) and neuronal NOS (nNOS). By contrast, inducible NOS (iNOS) is transcriptionally regulated in response to inflammatory stimuli. At low levels of the substrates (L-arginine and/or O_2) or the absence of adequate co-factors like tetrahydrobiopterin (BH_4), eNOS will donate electrons to molecular oxygen and produce superoxide (O_2^-) rather than NO, a process known as eNOS uncoupling [10, 11]. NO is a major scavenger of O_2^- that generates the powerful oxidant peroxynitrite ($ONOO^-$) by a rapid, diffusion limited reaction [12, 13].

Individuals with well controlled LDL still have residual cardiovascular (CV) risk associated in part with elevated triglycerides (TGs) that may be lowered by omega-3 fatty acids (n3-FAs) [14, 15]. Along with their various metabolites, n3-FAs influence inflammation, thrombosis and vascular reactivity in models of atherosclerosis [16-18]. In patients with diabetes or receiving statins, eicosapentaenoic acid (EPA) improved arterial compliance in a manner that correlated with reduced biomarkers of inflammation [19-23]. Additionally, EPA has been shown to inhibit lipopolysaccharide (LPS)-induced expression of adhesion

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Abbreviations

NOS	nitric oxide synthase
n3-FA	omega-3 fatty acid
n6-FA	omega-6 fatty acid
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
AA	arachidonic acid
NO	nitric oxide
$ONOO^-$	peroxynitrite

molecules [24]. When combined with a statin, EPA improved NO bioavailability following exposure of endothelial cells to either oxLDL or high glucose. Docosahexaenoic acid (DHA) did not reproduce these effects of the EPA/statin combination either *in vitro* or *ex vivo* [25]. However, DHA reduced cytokine-induced proatherogenic and proinflammatory proteins in cultured human endothelial cells [26, 27]. Arachidonic acid (AA), an omega-6 FA (n6-FA), counters certain favorable effects of n3-FAs [16]. AA competes with n3-FAs for cyclooxygenase and lipoxygenase enzymes to form inflammatory metabolites such as the leukotriene LTB_4 [28, 29]. Thus, the EPA/AA ratio predicts CV risk [7].

The results of rigorous and sufficiently powered CV outcome trials with n3-FAs have generally not demonstrated benefit for lower dose (1 g/d) or mixed n3-FA formulations that include DHA [30-33]. By contrast, the Reduction of Cardiovascular Events with Icosapent Ethyl–Intervention Trial (REDUCE-IT), used highly purified EPA ethyl ester (icosapent ethyl (IPE)). EPA treatment (4 g/d) in high risk patients with elevated triglycerides significantly reduced the risk of CV events [34-37]. A composite of ischemic events was reduced by 25% ($p < 0.0001$) while total (first and subsequent) ischemic events fell by 32% ($p < 0.0001$) compared with placebo. The benefits of EPA did not entirely correlate with triglyceride lowering, leading to interest in pleiotropic benefits and potential differences from DHA [38-40]. This conclusion was further supported by the Effect of Vascepa on Progression of Coronary Atherosclerosis in Persons With Elevated Triglycerides on Statin Therapy (EVAPORATE) trial, which met the primary endpoint as evidenced by significant regression of low-attenuation plaque volume with EPA (as IPE) compared to patients on statins alone [41]. The positive findings of the EVAPORATE trial provide mechanistic support for the reduced ischemic events in REDUCE-IT.

Another trial that tested a mixture of EPA and DHA at a similar high dose as REDUCE-IT (4 g/d), called A Long-Term Outcomes Study to Assess Statin Residual Risk Reduction with Epanova in High Cardiovascular Risk Patients with Hypertriglyceridemia (STRENGTH), failed to show any reduction in CV events and was halted prematurely due to futility at the recommendation of the independent data-monitoring committee [42, 43]. The failure to show significant benefit in STRENGTH indicates that the addition of DHA may diminish the beneficial CV properties of EPA or that its benefits in REDUCE-IT result from the EPA formulation (IPE) and/or dosage. To elucidate potential differences among n3-FAs that may contribute to these differences in clinical outcomes, this study compared the separate effects of EPA, DHA, and AA on the endothelial release ratio of NO to $ONOO^-$, an indicator eNOS coupling efficiency in relation to cellular fatty acid changes in these and other fatty acids.

2. Materials and methods

2.1. Materials

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Inc. (Walkersville, MD). Cells were cultured in the recommended complete endothelial cell growth medium and maintained at 37 °C in a 95% air /5% CO_2 humidified incubator. Cells were supplied with fresh medium every other day and propagated by an enzymatic (trypsin) procedure. Cell culture medium also contained 5% fetal bovine serum (FBS). The fatty acids EPA, DHA, and AA were purchased from Sigma-Aldrich (St. Louis, MO) and solubilized in redistilled ethanol under nitrogen atmosphere. The various acid stock solutions were stored at –20 °C until use.

2.2. Endothelial function analysis

Concurrent measurements of NO, $ONOO^-$ and the $[NO]/[ONOO^-]$ ratio were performed with tandem electrochemical nanosensors following eNOS stimulation with calcium ionophore. Cells were incubated with EPA, DHA, or AA at 10 μM or equivolume vehicle in medium containing 5% FBS, which contains albumin, to facilitate in the delivery of the fatty acids to the cells. The 10 μM concentration was chosen based on pharmacokinetic analysis of EPA levels in plasma and red blood cells (RBCs) in patients receiving 4 g/d as used in REDUCE-IT and regulatory trials [44]. NO and $ONOO^-$ measurements were taken at multiple time points up to 24 h-incubation with the fatty acids using custom tandem porphyrinic nanosensors and aggregated as there was no significant time-dependent benefit with any of the treatments. The methods for NO, $ONOO^-$ and the $[NO]/[ONOO^-]$ ratio, including the calibration of the nanosensors and the orientation of the sensors relative to the endothelial cells, have been previously described [25, 45-47].

2.3. Cellular fatty acid and total protein analysis

To determine the effects of EPA, DHA, and AA treatment on fatty acid composition, cells designated for fatty acid and protein content analysis were treated in parallel with the endothelial function analysis. Cells were incubated with each treatment for 8 h and then lysed for fatty acid analysis (10×10^6 cells in each sample). Total protein content in 20 μL aliquots from the cell lysates was measured using a Thermo Pierce™ BCA protein assay kit and quantified using absorbance at 562 nm. The remaining cell lysate sample was dried in a speed vacuum and the total fatty acid pool was derivatized using methanol with 14% boron trifluoride to form fatty acid methyl esters (FAME). Samples were then vortexed and heated at 100 °C for 10 min to aid in the derivatization reaction. A hexane/water solution was then added and the FAME were extracted in the hexane phase for analysis. Fatty acid content was determined using gas chromatography (GC) via a Shimadzu GC-2010 Gas Chromatograph with a Supelco SP-2560, 100-m fused silica capillary column (0.25 mm internal diameter, 0.2 μm film thickness). To determine the identity of the fatty acids in the samples, sample spectra were compared to spectra of the fatty acid standards. Fatty acid content was calculated as μg/mL in each sample and then normalized to the amount of protein per sample (mg/g total protein).

2.4. Statistical analyses

Data were presented as the mean ± *S*.E.M. for (N) separate samples of treatment groups ($N = 5$–16 for nitric oxide analysis, $N = 3$–4 for fatty acid analysis). Differences between groups were analyzed using ANOVA followed by Student-Newman-Keuls multiple comparisons *post hoc* analysis (for comparisons between three or more groups) or unpaired, two-tailed Student's T-test for comparisons between two groups. Alpha error was set to 0.05 in this study.

3. Results

3.1. Endothelial function analysis

The results of the endothelial function analysis are summarized in **Figs. 1** and **2**. The comparative effects of each treatment on nitric oxide

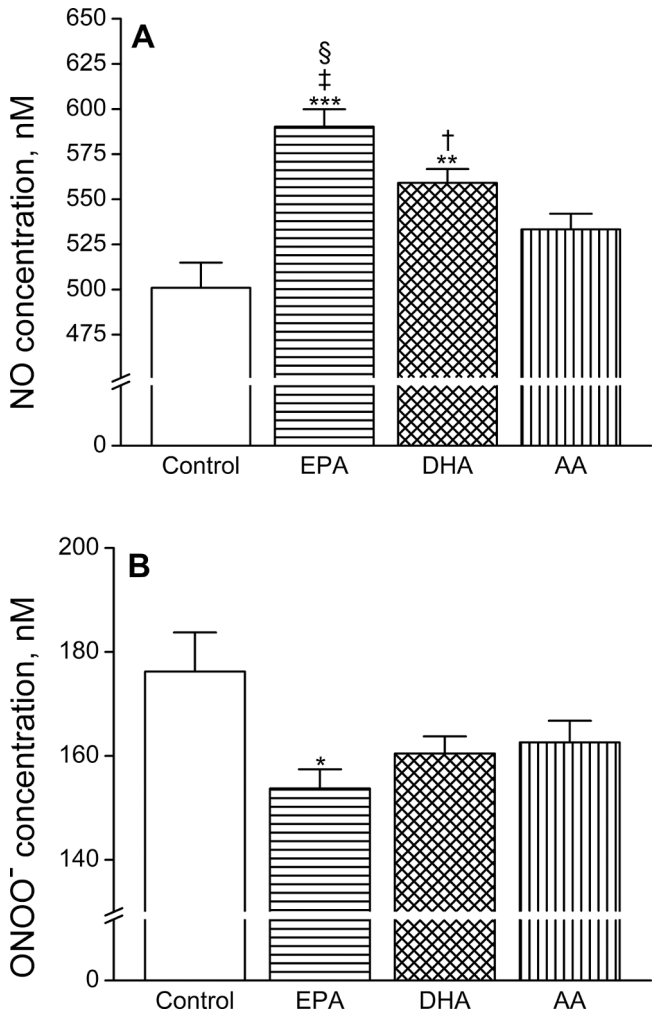

Fig. 1. A-B. Comparative Effects of EPA, DHA, and AA on Maximal (A) NO and (B) $ONOO^-$ Concentration from HUVECs. All treatments were delivered to cells in the presence of 5% FBS. NO Stats: $^{***}p < 0.001$ versus control; $^{**}p < 0.01$ versus control; $^{‡}p < 0.001$ versus AA; $^{†}p < 0.05$ versus AA; $^{§}p < 0.05$ versus DHA (Student-Newman-Keuls Multiple Comparisons test; overall ANOVA: $p < 0.0001$, $F = 10.984$). $ONOO^-$ Stats: $^{*}p < 0.05$ versus control (Student-Newman-Keuls Multiple Comparisons test; overall ANOVA: $p = 0.0437$, $F = 2.909$). Values are mean ± SEM.

release are summarized in Fig. 1**A**. EPA caused a significant increase in NO release compared to control (18%, 590 ± 10 versus 501 ± 14 nM, $p < 0.001$), compared to DHA (6%, 590 ± 10 versus 559 ± 8 nM, $p < 0.05$), and compared to AA (11%, 590 ± 10 versus 533 ± 9 nM, $p < 0.001$). AA failed to cause a significant increase in NO release compared to control. DHA caused a significant, 12% increase in NO release ($p < 0.01$) compared to control.

The comparative effects of each treatment on $ONOO^-$ release are summarized in Fig. 1**B**. EPA was the only treatment to cause a significant decrease in $ONOO^-$ release compared to control (13%, 154 ± 4 versus 176 ± 8 nM, $p < 0.05$). Both DHA (9%) and AA (7%) failed to match the significant decrease in $ONOO^-$ release seen following EPA treatment.

The above effects on NO and $ONOO^-$ release contribute to the overall effects on the [NO]/[$ONOO^-$] ratio, a key indicator of eNOS function. Since EPA and DHA were the only treatments to affect NO and $ONOO^-$ release separately, we compared the effects of these FAs on the [NO]/[$ONOO^-$] ratio, as summarized in Fig. 2**.** Treatment with EPA caused the largest and most significant increase in this ratio compared with control (35%, 3.87 ± 0.11 versus 2.86 ± 0.15, $p < 0.001$). The beneficial

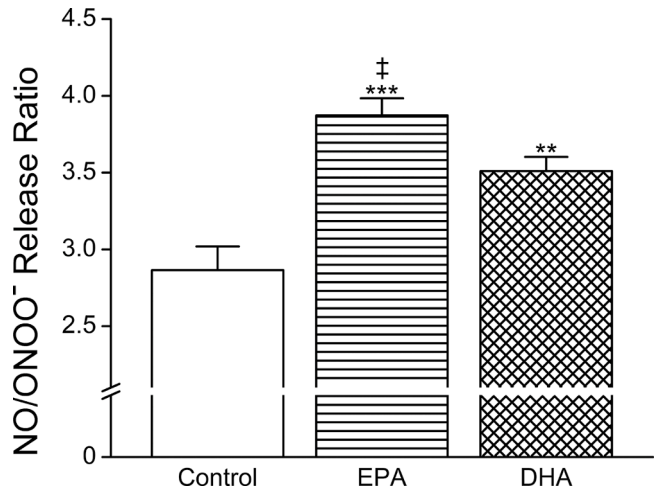

Fig. 2. Comparative Effects of EPA and DHA on the [NO]/[$ONOO^-$] Concentration Ratio from HUVECs. All treatments were delivered to cells in the presence of 5% FBS. $^{***}p < 0.001$ versus control; $^{**}p < 0.01$ versus control; $^{‡}p < 0.05$ versus DHA (Student-Newman-Keuls Multiple Comparisons test; overall ANOVA: $p < 0.0001$, $F = 12.886$). Values are mean ± SEM.

effect of EPA on the [NO]/[$ONOO^-$] release ratio was also significantly greater than DHA ($p < 0.05$). Treatment with DHA resulted in a 23% increase in the ratio compared with control ($p < 0.01$).

3.2. Cellular fatty acid analysis

Treatment of cells with EPA, DHA, or AA significantly modified the FA content of endothelial cells. The average measurements of fatty acids in the control and treated cell lysates from four separate cell cultures (~10×10^6 cells each) are summarized in Figs. 3 and 4 and Table 1. Treatment with each fatty acid increased their respective levels compared to control: EPA caused a 10-fold increase in EPA levels (0.44 ± 0.02 to 4.59 ± 0.26 mg/g protein, $p < 0.001$), DHA caused a 2.9-fold increase in DHA levels (4.10 ± 0.45 to 11.86 ± 0.85 mg/g, $p < 0.001$), and AA caused a 2-fold increase in AA levels (9.38 ± 0.50 to 18.02 ± 0.76 mg/g protein, $p < 0.001$). EPA treatment, but not DHA or AA, caused a 2-fold increase in cellular levels of the n3-FA docosapentaenoic acid (DPA, 22:5 n3; 3.24 ± 0.20 to 8.72 ± 0.51 mg/g protein, $p < 0.001$). DHA treatment resulted in a 2.5-fold increase in EPA levels to 1.12 ± 0.08 mg/g protein, though this was less than the increase observed with EPA ($p < 0.001$). In addition to the increase in AA levels, only AA treatment caused a 4-fold increase in the omega-6 fatty acid docosatetraenoic acid (DTA, 22:4 n6) from 2.14 ± 0.13 to 8.78 ± 0.34 mg/g protein ($p < 0.001$). EPA treatment increased the EPA/AA ratio 10-fold (Fig. 4) compared to control (0.047 ± 0.003 to 0.52 ± 0.042, $p < 0.001$), while DHA treatment increased the EPA/AA ratio 2.6-fold (0.12 ± 0.01, $p < 0.05$) and AA treatment decreased the EPA/AA ratio 4-fold (0.012 ± 0.001, $p < 0.0001$). The FA treatments significantly modulated a number of other cell FAs to a lesser extent, especially AA, as reviewed in Table 1.

4. Discussion and conclusions

The key finding of this study is that endothelial cells treated with n3-FAs (EPA, DHA) and the n6-FA (AA) have differential effects on NO bioavailability and fatty acid levels. EPA treated cells had significantly greater NO release with a concomitant reduction in release of $ONOO^-$. AA did not significantly alter either NO or $ONOO^-$ release while DHA only increased NO. Consistent with these results, EPA-treated cells showed a significantly greater improvement in the [NO]/[$ONOO^-$] concentration ratio compared with DHA or control. The different effects of the FAs on eNOS coupling correlated with distinct effects on the FA

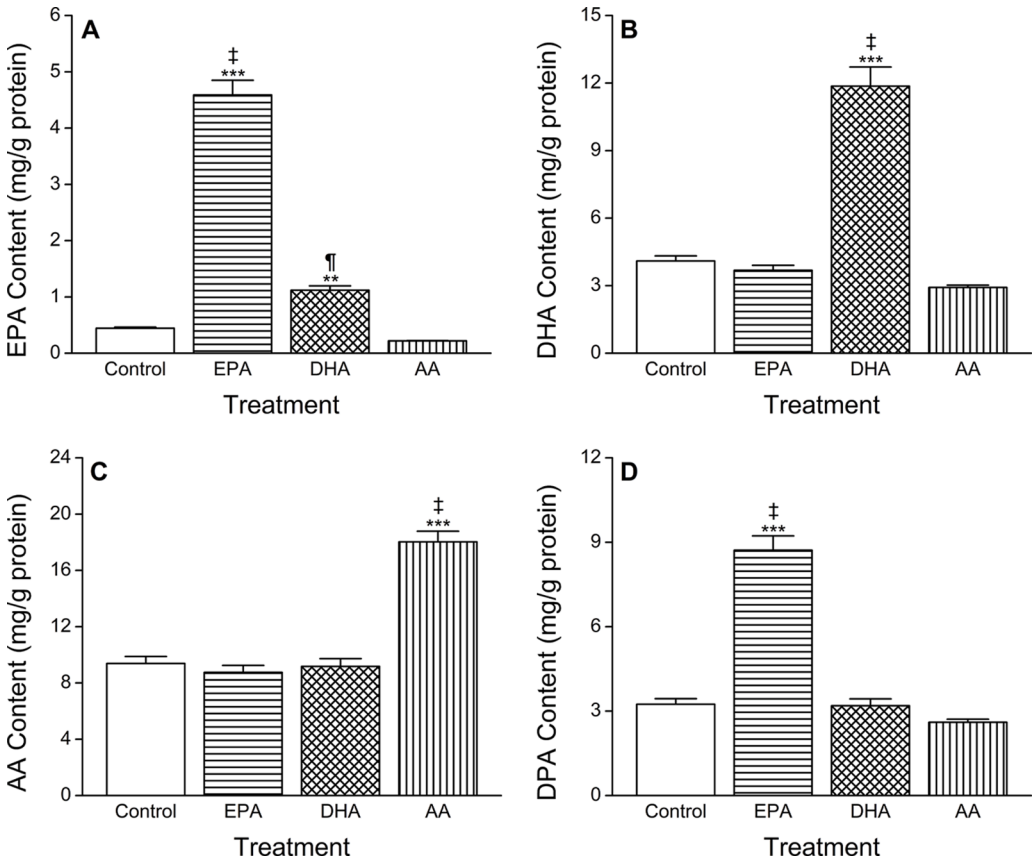

Fig. 3. A-D. Comparative Effects of EPA, DHA, and AA Treatment on (A) EPA, (B) DHA, (C) AA, and (D) DPA Levels from HUVECs. A: $^{***}p < 0.001$ versus control; $^{**}p < 0.01$ versus control; $^{‡}p < 0.001$ versus DHA and AA; $^{¶}p < 0.01$ versus AA (Student-Newman-Keuls Multiple Comparisons Test; overall ANOVA: $p < 0.0001$, $F = 222.51$). B: $^{***}p < 0.001$ versus control; $^{‡}p < 0.001$ versus EPA and AA (Student-Newman-Keuls Multiple Comparisons Test; overall ANOVA: $p < 0.0001$, $F = 83.638$). C: $^{***}p < 0.001$ versus control; $^{‡}p < 0.001$ versus DHA and EPA (Student-Newman-Keuls Multiple Comparisons Test; overall ANOVA: $p < 0.0001$, $F = 57.851$). D: $^{***}p < 0.001$ versus control; $^{‡}p < 0.001$ versus DHA and AA (Student-Newman-Keuls Multiple Comparisons Test; overall ANOVA: $p < 0.0001$, $F = 88.500$). Values are mean ± SEM ($N = 4$).

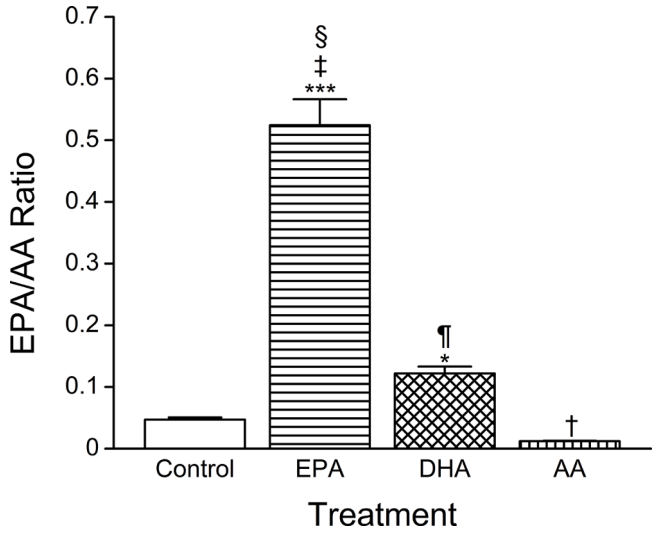

Fig. 4. Comparative Effects of EPA, DHA, and AA Treatment on the EPA/AA Ratio from HUVECs. $^{***}p < 0.001$ versus control; $^{*}p < 0.05$ versus control; $^{‡}p < 0.001$ versus DHA; $^{§}p < 0.001$ versus AA; $^{¶}p < 0.01$ versus AA (Student-Newman-Keuls Multiple Comparisons Test; overall ANOVA: $p < 0.0001$, $F = 117.89$). Values are mean ± SEM ($N = 4$). $^{†}p < 0.0001$ versus control (Unpaired, two-tailed Student's T-Test; $t = 11.068$, df = 6).

content of these cells. Treatment with each fatty acid significantly increased the respective cellular levels of that fatty acid. EPA significantly increased endothelial cell levels of EPA and its immediate metabolic product DPA by 10-fold and 2-fold, respectively, while not increasing DHA or AA compared to control. DHA treatment did not change DPA levels while AA treatment significantly reduced it. The conversion of EPA to DPA occurs via elongase enzymes which add 2 carbons to the acyl chain [48, 49]. EPA is eventually converted to DHA through DPA via one more elongase, a Δ6-desaturase, and a final step of β-oxidation which occurs in the peroxisome. Thus, it is possible that at this time point (8 h), the EPA added exogenously was primarily converted as far as DPA if changed at all. These n3-FAs may contribute more to the beneficial endothelial effects observed with EPA treatment as compared to DHA and especially AA which had no significant effect of eNOS coupling. We have previously shown that EPA, and to a lesser extent DPA, possess superior antioxidant effects as compared to DHA and AA in model membranes and lipoprotein particles [50, 51].

The conversion of EPA to DPA, along with other n3-FAs, occurs in parallel with the formation of various n6-FAs, including AA. Just as DPA is the immediate biosynthetic product of EPA, AA is converted to DTA by the same elongase [49]. We observed that treatment with AA, but not DHA or EPA, increased cellular levels of both AA and DTA, suggesting that the rate of conversion of EPA to DPA and AA to DTA are similar despite their different effects on endothelial function.

Imbalances in the EPA/AA ratio that favor more AA than EPA are associated with increased CV risk [7, 52]. The n3-FAs EPA and DHA generate anti-thrombotic metabolites thromboxane A3/Prostacyclin (PGI3) while AA forms thromboxane A2, a platelet activator that contributes to atherothrombosis [53]. The n3-FAs also compete with AA for cyclooxygenase (COX) enzymes that synthesize the thromboxanes, thus reducing formation of these pro-aggregatory and vasoconstrictor metabolites. As seen in the MARINE trial, treatment with icosapent ethyl (IPE, 2–4 g/d) significantly increased the EPA/AA ratio which was associated with increased plasma EPA levels [54]. In our study, we observed a large and significant increase in the EPA/AA ratio with EPA treatment. This increase appears to be driven primarily by the increase in cellular EPA levels as there was no decrease in AA levels following EPA treatment. By contrast, AA treatment decreased the EPA/AA ratio which was associated with a significant increase in AA. In addition to the

Table 1
Summary of Fatty Acid Levels in HUVECs Following Treatment with EPA, DHA, and AA.

Fatty Acid	Treatment			
	Control	EPA	DHA	AA
	(mg/g protein)	(mg/g protein)	(mg/g protein)	(mg/g protein)
14:0	4.13 ± 0.19	3.92 ± 0.19	3.94 ± 0.16	3.32 ± 0.14*
16:0	34.38 ± 1.48	31.72 ± 1.49	31.79 ± 1.21	27.69 ± 1.12*
trans-16:1n7	0.55 ± 0.02	0.52 ± 0.02	0.49 ± 0.02	0.40 ± 0.02*
cis-16:1n7	4.65 ± 0.21	4.57 ± 0.23	4.36 ± 0.22	3.50 ± 0.16*
18:0	22.31 ± 0.94	20.91 ± 0.98	20.95 ± 0.84	18.46 ± 0.73*
trans-18:1n9	0.61 ± 0.05	0.60 ± 0.03	0.57 ± 0.02	0.55 ± 0.02
cis-18:1n9	41.81 ± 2.10	38.78 ± 1.96	37.88 ± 1.96	31.83 ± 1.36*
trans-18:2n6	1.63 ± 0.07	1.33 ± 0.08*	1.32 ± 0.05*	1.09 ± 0.05*
cis-18:2n6	1.99 ± 0.09	2.27 ± 0.12	2.25 ± 0.12	1.81 ± 0.08
18:3n3	0.05 ± 0.01	0.02 ± 0.003*	0.02 ± 0.005*	0.004 ± 0.001*
18:3n6	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.15 ± 0.01
20:0	1.07 ± 0.01	0.76 ± 0.05*	0.87 ± 0.05*	0.77 ± 0.04*
20:1n9	0.86 ± 0.04	0.70 ± 0.03*	0.66 ± 0.03*	0.64 ± 0.03*
20:2n6	0.23 ± 0.02	0.24 ± 0.02	0.21 ± 0.01	0.19 ± 0.01
20:3n6	2.17 ± 0.10	2.10 ± 0.11	2.07 ± 0.12	2.06 ± 0.08
20:4n6	9.38 ± 0.50	8.75 ± 0.50	9.17 ± 0.55	18.02 ± 0.76*
20:5n3	0.44 ± 0.02	4.59 ± 0.26*	1.12 ± 0.08*	0.22 ± 0.01*
22:0	1.78 ± 0.11	1.20 ± 0.08*	1.33 ± 0.06*	1.22 ± 0.06*
22:4n6	2.14 ± 0.13	2.15 ± 0.12	1.95 ± 0.13	8.78 ± 0.34*
22:5n6	0.40 ± 0.03	0.31 ± 0.02*	0.38 ± 0.03	0.53 ± 0.03*
22:5n3	3.24 ± 0.20	8.72 ± 0.51*	3.19 ± 0.25	2.60 ± 0.11*
22:6n3	4.10 ± 0.23	3.68 ± 0.22	11.86 ± 0.85*	2.92 ± 0.10*
24:0	1.28 ± 0.04	0.92 ± 0.05*	1.02 ± 0.05*	0.95 ± 0.05*
24:1n9	0.62 ± 0.02	0.57 ± 0.03	0.49 ± 0.03*	0.45 ± 0.03*

Results shown as mean ± SEM. Statistical analysis were only carried out between each individual fatty acid treatment and control to improve clarity of the table using unpaired, two-tailed Student's *t*-test. Statistical indicators: *$p < 0.05$ vs control.

increase in cellular DPA and EPA, the increase in the EPA/AA ratio associated with EPA treatment may explain the superior endothelial benefits observed with EPA.

In cells, polyunsaturated fatty acids (PUFAs) are primarily found esterified to the *sn*-2 position of membrane phospholipids. Along with the headgroup composition, phospholipid fatty acids have complex effects on membrane biophysical characteristics, including fluidity, lipid domains, width and rates of oxidation that vary among different cell types as well as with age and various disease-like conditions. In particular, EPA modifies the lipid composition of membrane caveolae and the subcellular distribution of eNOS [55]. EPA changes the lipid fluidity of the caveolae following membrane phospholipid enrichment. There is also displacement of caveolin-1, an inhibitor of eNOS, with EPA incorporation to soluble fractions that allow activation. The comparative effects of various long chain fatty acids, including n6-FAs and n3-FAs, on lipid oxidation and the organization of cholesterol have also been recently characterized [56-59]. Despite similar structures, EPA and DHA have distinct effects on membrane structure, cholesterol distribution, oxidation rates and lipid dynamics [56-60]. Differences between EPA and DHA on rates of oxidation were reproduced in atherogenic ApoB-containing lipoproteins as well as ApoA-containing particles [61, 62].

Loss of normal vasodilation correlates with CV risk and all-cause mortality independent of traditional risk factors [63]. EPA produces favorable effects on arterial compliance and vasomotor control in patients with CV disease or its risk factors, including those with diabetes or receiving statins [19-22]. The benefits of EPA may be due to improved NO bioavailability, a potent vasodilator. This study used nanosensors to measure release kinetics of NO and $ONOO^-$, along with their ratio as an indication of eNOS coupling efficiency in increasing NO bioavailability and reducing $ONOO^-$ - a main component of nitroxidative stress. Previous studies showed that the improvement in NO bioavailability with EPA in these cells did not relate to changes in eNOS expression and were enhanced in combination with a statin [25]. The changes in NO bioavailability with EPA and a statin were observed under conditions of oxidative stress and hyperglycemia and not reproduced with DHA or other TG-lowering agents. These findings were extended using *ex vivo* experiments in rat glomerular endothelium [25].

Inadequate levels of substrates such as L-arginine and/or cofactors like BH_4 leads to uncoupling of dimeric eNOS. Instead of producing NO, uncoupled eNOS generates O_2^- that subsequently reacts quickly with NO to create $ONOO^-$. In addition to molecular oxygen, O_2^- reacts with BH_4 to further reduce eNOS function. Increased cellular levels of $ONOO^-$ secondary to eNOS uncoupling ultimately disrupts the redox balance and contributes to endothelial damage [64, 65].

These findings have important clinical implications when reviewing outcome trials for n3-FAs consisting of EPA only as compared to mixed EPA/DHA formulations. In the open-label Japan EPA Lipid Intervention Study (JELIS), purified EPA (1.8 g/d) combined with statin treatment resulted in a 19% relative reduction in major coronary events compared with statin treatment alone ($p = 0.011$) [66]. The multinational, blinded REDUCE-IT study (4 g/d of purified EPA as IPE) demonstrated a 25% relative reduction in first ischemic events, including a significant 20% reduction in death from CV causes. However, STRENGTH (4 g/d of a mixture of EPA and DHA) failed to reduce ischemic events in a similar patient population despite similar reductions in triglycerides. The contrasting results of REDUCE-IT and STRENGTH indicate important differences in clinical outcomes with n3-FA formulations. As mentioned previously, the EVAPORATE trial showed significant, favorable effects on measures of plaque volume and composition on noninvasive computed tomography with IPE versus placebo, including regression of vulnerable plaques [41]. These results were consistent with the open-label Combination Therapy of Eicosapentaenoic Acid and Pitavastatin for Coronary Plaque Regression Evaluated by Integrated Backscatter Intravascular Ultrasonography (CHERRY) trial which used invasive intravascular ultrasound [67].

Limitations of this analysis include uncertainty about whether these results apply directly to humans as this *in vitro* analysis used cultured cells. Additionally, the total fatty acid analysis included both delivered free fatty acids and those already incorporated into cellular phospholipids, though we do not differentiate among these in our measurements. These results support further investigations using different sources of endothelial cells under various disease-like conditions as well as *in vivo* experiments.

In conclusion, we observed pronounced differences in endothelial NO bioavailability following treatment with EPA, DHA, and AA, along with differential changes in fatty acid content. EPA treated cells had significantly greater [NO]/[$ONOO^-$] concentration ratio as compared to control, DHA, and AA. Treatment with EPA increased cellular EPA levels 10-fold, DPA levels 2-fold, and the EPA/AA ratio 10-fold, while DHA and AA treatment increased their respective levels without increases in DPA. These findings support a preferential benefit of EPA on endothelial cell function that correlates with increased EPA, DPA and EPA/AA ratio that may help explain differences in clinical outcomes among recent n3-FA trials.

Summary

Introduction – We compared the effects of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA) on endothelial cell (EC) function, including nitric oxide (NO) release, and fatty acid composition. Loss of endothelial NO bioavailability is implicated in inflammation and atherosclerosis.

Materials and Methods – Human ECs were treated with equimolar EPA, DHA, or AA (10 μM) or vehicle. At multiple time points, NO and peroxynitrite ($ONOO^-$) were measured by porphyrinic nanosensors. Cellular fatty acid composition was determined by GC–MS.

Results – EPA significantly increased the [NO]/[$ONOO^-$] release ratio compared to vehicle, DHA and AA. Each fatty acid treatment increased their respective levels in the cells. Only EPA treatment also increased cellular levels of DPA and the EPA/AA ratio.

Conclusions – These findings support a preferential benefit of EPA on endothelial function and fatty acid content that may contribute to atheroprotection.

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CRediT authorship contribution statement

Samuel C.R. Sherratt: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Hazem Dawoud:** Methodology, Investigation. **Deepak L. Bhatt:** Writing – original draft, Writing – review & editing, Supervision. **Tadeusz Malinski:** Methodology, Resources, Supervision, Project administration. **R. Preston Mason:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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