Comparative anti-inflammatory effects of plant- and marine-derived omega-3 fatty acids explored in an endothelial cell line

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Ella J. Baker[1], Carina A. Valenzuela[1,2], Camila O. De Souza[1,3], Parveen Yaqoob[4], Elizabeth A. Miles[1] and Philip C. Calder[1,5]

[1]School of Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, United Kingdom;
[2]School of Nutrition, Faculty of Pharmacy, University of Valparaíso, Playa Ancha, Valparaíso, Chile;
[3]Department of Cell and Developmental Biology, University of Sao Paulo, Sao Paulo, Brazil;
[4]School of Chemistry, Food and Pharmacy, University of Reading, Reading RG6 6AP, United Kingdom;
[5]NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust and University of Southampton, Southampton, United Kingdom.

Corresponding author: Dr Ella Baker, Human Development and Health, Faculty of Medicine, University of Southampton, IDS Building, MP887 Southampton General Hospital, Southampton SO16 6YD, United Kingdom.
E.Baker@soton.ac.uk

Running title: Plant- and marine-derived omega-3 fatty acids

Highlights:

- Marine-derived fatty acids (EPA and DHA) had potent anti-inflammatory effects in EA.hy926 endothelial cells.
- Plant-derived fatty acids (α-linolenic and stearidonic acids) modulated some inflammatory responses in EA.hy926 endothelial cells.
- Of the two plant-derived fatty acids, stearidonic acid had greater anti-inflammatory effects.

Abbreviations used: ALA, alpha-linolenic acid; COX, cyclooxygenase; CVD, cardiovascular disease; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; DMEM, Dulbecco's Modified Eagle Medium; EC, endothelial cell; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; MFI, median fluorescence intensity; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PE, phycoerythrin; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acid; RANTES, regulated on activation, normal T cell expressed and secreted; RCT, randomised control trial; SDA, stearidonic acid; TNF-α, tumor necrosis factor alpha; VLC, very long-chain; n-3, omega 3; WHO, World Health Organisation.

Abstract

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) lower risk of cardiovascular disease. The primary source of EPA and DHA is fatty fish. Plant-derived alpha linolenic acid (ALA) and stearidonic acid (SDA) could provide sustainable land-based alternatives, but their functionality is underexplored. Omega-3 fatty acids (n-3 FAs) may influence atherogenic processes through changing endothelial cell (EC) function and lowering inflammation. This study compared effects of marine- and plant-derived n-3 FAs on EC inflammatory responses. EA.hy926 cells were exposed to ALA, SDA, EPA or DHA prior to stimulation with tumor necrosis factor (TNF)-α. All FAs were shown to be incorporated into ECs in a dose-dependent manner. SDA (50 μM) decreased both production and cell-surface expression of intercellular adhesion molecule (ICAM)-1; however EPA and DHA resulted in greater reduction of ICAM-1 production and expression. EPA and DHA also significantly lowered production of monocyte chemoattractant protein 1, interleukin (IL)-6 and IL-8. ALA, SDA and DHA (50 μM) all reduced adhesion of THP-1 monocytes to EA.hy926 cells. DHA significantly decreased nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)p105 gene expression and phosphorylated NFκBp65 protein. Both EPA and DHA (50 μM) significantly decreased cyclooxygenase (COX)-2 protein. Thus, both marine-derived n-3 FAs, particularly DHA, had potent anti-inflammatory effects in this EC model. Of the plant-derived n-3 FAs, SDA showed the greatest inhibition of inflammation. Although neither ALA nor SDA reproduced the anti-inflammatory effects of EPA and DHA in this model, there is some potential for SDA to be a sustainable anti-inflammatory alternative to the marine n-3 FAs.

Key words: Omega-3, inflammation, cytokine, endothelial cell, atherosclerosis, plant and marine-derived fatty acids

1 Introduction

Epidemiological studies and randomised controlled trials (RCTs) have demonstrated an association between consumption of very long chain (VLC) omega-3 (n-3) polyunsaturated fatty acids (PUFAs) and long-term health benefits, including lowering risk of cardiovascular disease [1-4]. Atherosclerosis, the build-up of fatty plaques within the blood vessel wall, leads to cardiovascular disease, and it is widely accepted that atherosclerosis is an inflammatory disease [5-9]. Thus, the protective effects of VLC n-3 PUFAs towards cardiovascular disease might be due to their well described anti-inflammatory actions [10-12]. The bioactive VLC n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to modulate a number of inflammatory processes through changes in eicosanoid production and through reduction in production of other inflammatory mediators, including chemokines, cytokines and adhesion molecules [10-13]. These effects involve incorporation of EPA and DHA into cell membranes and modulation of key signalling pathways, such as nuclear factor kappa-light chain enhancer of activated B cells (NFκB) activation [10-13].

The main dietary source of EPA and DHA is fatty fish, and several expert bodies recommend consuming one or more portions of fatty fish per week for long-term health benefits [14]. However, current stocks of fatty fish are rapidly declining [15] and are not likely to meet the needs of humans for VLC n-3 PUFAs [16]. It is also important to note that much of the population in many countries does not regularly consume fatty fish [16, 17]. This is due to many factors, including dislike of the taste and smell, cost, concerns over contaminants, such as mercury, or lifestyle choice, such as vegetarianism. Therefore, there is a need for alternative sources of fatty acids (FAs) to achieve the health benefits of EPA and DHA. Plant-derived n-3 PUFAs, including alpha-linolenic acid (ALA) and stearidonic acid (SDA), may be land-based sustainable n-3 PUFAs with health benefits, but their functionality has been underexplored [18].

ALA is a metabolic precursor of EPA and DHA, whilst SDA is an intermediate in the same pathway. Sources of ALA include green plant tissue, various nuts (e.g. walnuts), some seeds (e.g. flaxseed) and some vegetable oils (e.g. rapeseed, soybean and flaxseed oils), while SDA is found in Echium oil, and has been increased in soybean oil by genetic modification [19]. ALA is the predominant n-3 PUFA consumed by those who do not regularly eat fatty fish or

take VLC n-3 PUFA supplements. Typical intakes of ALA in adult western populations range from 0.5 to 2.3 g/d [20-22]. Consumption of SDA is much lower than that of ALA; however SDA intake is difficult to estimate since there are few commonly consumed sources of this FA and it is not typically found in nutrient databases [18]. Bioactivity of ALA may relate to its conversion to EPA, although this appears limited in humans [18]. Since SDA is the product of the rate limiting step of the pathway of conversion of ALA to EPA and DHA, it seems to be a better substrate for synthesis of these VLC n-3 PUFAs [18] and so may offer superior bioactivity to ALA.

The development of atherosclerosis is closely linked to endothelial cell (EC) function and responses and endothelial dysfunction is recognised as one of the first steps in the development of atherosclerotic plaques [23, 24]. The functional responses of ECs may be studied in cell culture and in this regard, human umbilical vein ECs (HUVECs) have been widely used as a model [13, 25-27] . Many studies using ECs of different origins, including HUVECs, describe anti-inflammatory effects of EPA and DHA, including lowering of surface expression of adhesion markers, reduced monocyte adhesion, and decreased inflammatory marker expression, at the levels of both protein and messenger RNA [27-32]. These data suggest that EPA and DHA may reduce atherogenesis by lowering endothelial inflammation. HUVECs offer a model system with which to explore the potential anti-inflammatory effects of ALA and SDA compared to those of EPA and DHA; there are few studies of ALA and no studies of SDA on inflammatory responses of ECs. Therefore, this study aimed to compare the effects of ALA and SDA to those of EPA and DHA in a model of endothelial inflammation using EA.hy926 cells, an immortalised HUVEC cell line.

2 Materials and methods

2.1 Endothelial cell culture

EA.hy926 cells (ATCC, LGC standards, Middlesex, UK) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine-penicillin-streptomycin solution and 1% HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine); medium and supplements were purchased from Sigma-Aldrich (Gillingham, UK). Cultures were maintained at 37°C in humidified 95% air and 5% CO_2. Cells were seeded at a density of 32,000 cells per cm^2 in 96 well plates (for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, mediator production,

and adhesion), six well plates (for flow cytometry, real-time PCR and western blotting) or T25 flasks (for gas chromatography).

2.2 FA treatment

ALA, SDA, EPA and DHA (all from Sigma-Aldrich) were prepared as 50 mM, 25 mM or 10 mM stocks in 100% ethanol. Experimental FA concentrations were achieved by diluting stock solutions in complete medium to 10, 25 and 50 µM in a final concentration of 0.1% ethanol.

2.3 Cell viability

Cell viability was assessed using the MTT assay which measures cellular mitochondrial activity. EA.hy926 cells were incubated in the presence of FAs at concentrations of 0, 10, 25 or 50 µM for 48 hr, and further exposed to complete medium with or without tumor necrosis factor (TNF)-α (1 ng/mL final concentration) for 24 hr. After incubation, supernatant was removed and replaced with DMEM containing 0.05 mg/mL MTT (Sigma-Aldrich) (100 µL/well) and samples incubated at 37^{o}C for 4 hr. Supernatants were removed (75 µL) and 75 µL of dimethylsulphoxide (Sigma-Aldrich) added. Absorbance was measured at 540 nm on a plate reader. The effects of FAs and TNF-α on cell viability were normalised to control (0.1% ethanol) (i.e. no FA or TNF-α) cultures (100%).

2.4 FA composition of cells

The FA composition of EA.hy926 cells after culture with various FAs was determined using gas chromatography. Total lipid was extracted from EA.hy926 cells using chloroform/methanol (2:1 vol/vol) as described elsewhere [33]. Lipid extracts were dried under nitrogen. FAs were released from these isolated lipids and simultaneously methylated by heating with 2% sulphuric acid in methanol at 50^{o}C for 2 hr. The FA methyl esters were extracted into hexane and then separated and analysed by gas chromatography using conditions described by Fisk et al. [33]. FAs were identified by comparison of retention times with those of analytical standards and are expressed as µg/10^6 cells.

2.5 Measurement of inflammatory mediator concentrations

The concentrations of inflammatory mediators (interleukin (IL)-6, IL-8, intercellular adhesion molecule (ICAM)-1, regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein (MCP)-1) in the medium of EA.hy926 cells were determined simultaneously using human magnetic luminex screening assay kits (R&D

Systems, Minneapolis, MN). Assays were conducted in accordance with the instructions from the manufacturer. Plates were analysed on a calibrated Bio-Plex 200 analyser using Bio-Plex software (version 6.1, Bio-Rad Laboratories Inc., Berkeley, CA). Lower limits of detection (pg/mL) were: IL-6, 1.7; IL-8, 1.8; MCP-1, 9.9; RANTES, 1.8; ICAM-1, 87.9.

2.6 Cell surface ICAM-1 expression

Surface expression of ICAM-1 (also known as CD54) on EA.hy926 cells was determined using flow cytometry. Cells were exposed to FAs at 25 and 50 μM for 48 hr followed by stimulation with TNF-α (1 ng/mL) for 6 hr. Following this, cells were detached, centrifuged and stained with phycoerythrin (PE)-conjugated monoclonal anti-human CD54 (BD Biosciences, San Jose, CA) diluted in staining solution (2% bovine serum albumin in PBS) for 30 min. Mouse IgG1 (PE) isotype was used as a negative control. After staining, cells were analysed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). A total of 10,000 events were collected. Percentage positive cells (gated cells) and median fluorescence intensity (MFI) were measured.

2.7 RNA isolation, cDNA synthesis and Real-Time PCR

Changes in relative gene expression were analysed by RT-PCR. Cells were exposed to FAs for 48 hr followed by stimulation with TNF-α (1 ng/mL) for 6 hr. Taqman Gene Expression Primers (ThermoFisher Scientific, Waltham, MA) were used to determine the expression of inhibitor of nuclear factor kappa B kinase subunit beta (IKKβ) (Hs00233287_m1), NFκB subunit 1 (p105) (Hs00765730_m1), and peroxisome proliferator activated receptor alpha (PPARα) (Hs00945536_m1) in EA.hy926 cells after treatment. Total RNA was extracted using the ReliaPrep RNA cell Miniprep System (Promega, Southampton, UK). RNA quantity and quality were analysed by NanoDrop. Analysis of RNA using Agilent Bioanalyzer (RNA Total Eukaryote 2100 Nano) was performed to determine RNA RIN scores. cDNA was synthesised from total RNA using GoScript Reverse Transcriptase (Promega). Housekeeping reference genes were determined using a geNorm Kit (Primerdesign, Camberley, UK). Quantification of relative gene expression was analysed using B2M (Hs00187842_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02786624_g1) and RPL13A (Hs04194366_g1) as housekeeping genes.

2.8 Protein extraction and Western blotting

The expression of NFκBp65, phosphorylated NFκBp65, cyclooxygenase (COX)-1, COX-2 and GAPDH was assessed by Western blotting. Cells were exposed to FAs at 50 µM for 48 hr followed by stimulation with TNF-α (1 ng/mL) for 1 hr (NFκB) or 16 hr (COX-1 and COX-2). Protein was extracted from cells using RIPA buffer. Protein quantification was determined using the BCA assay (Pierce, ThermoFisher Scientific) following the manufacturer's instructions. Then, samples were diluted and a total of 30 µg of protein loaded onto pre-cast 10% sodium dodecyl sulphate gels (Obtiblot, Abcam, Cambridge, UK) alongside a Prism Ultra Protein Ladder (10-245 kDa). Gels were run for 1.5-2 hr and proteins transferred onto a nitrocellulose membrane which was probed with antibodies (Abcam). Western blots were quantified using ImageJ software and relative quantification values are presented as the ratio of each protein band relative to the loading control (GAPDH).

2.9 Monocyte adhesion to EA.hy926 cells

Adhesion of monocytic THP-1 cells to EA.hy926 cells was determined using the Vybrant Cell Adhesion Assay Kit (ThermoFisher Scientific). EA.hy926 cells were exposed to FAs at 25 and 50 µM for 48 hr followed by stimulation with TNF-α (1 ng/mL) for 6 hr. After stimulation, calcein labelled THP-1 cells were incubated with EA.hy926 cells for 1 hr at 37^{o}C. Non-adherent THP-1 cells were removed by gentle washing, 100 µL PBS added to each well and co-cultures read on the Glomax Discover System (Promega). THP-1 monocyte adhesion was measured as a percentage of control (TNFα stimulated cells). Images of fluorescence-labelled THP-1 monocytes bound to EA.hy926 cells were taken with a Nikon Elipse Ti using NIS elements software (version 4.30).

2.10 Statistics/Data Analysis

Data are presented as mean ± SEM and were analyzed by one or two-way analysis of variance (one or two-way ANOVA), as appropriate, followed by Tukey's post-hoc tests for determining pairwise differences. Analyses were performed using GraphPad Prism 6.0. Differences were considered significant when $p<0.05$.

3 Results

3.1 Results of MTT assay: Effects of FAs on EA.hy926 cell viability

Cell mitochondrial activity was measured using the MTT assay and viability was calculated as a % of control (cells in DMEM plus supplements). Exposure to TNF-α at 1 ng/mL for 24 hr had no effect on cell viability. FA treatment for 48 hr, followed by 24 hr TNF-α (1 ng/mL) exposure, had a concentration-dependent effect on EA.hy926 viability. Figures 1 A-D show a significant reduction in viability of cells cultured with ALA ($p<0.05$), SDA ($p<0.05$), and DHA ($p<0.0001$) at 100 μM compared to stimulated control cells (<60%, <70%, <25% viability, respectively). At 50 μM, viability of cells exposed to ALA, SDA, EPA or DHA was >75%. Based on these results, a maximum concentration of 50 μM for each FA was chosen for further experiments.

3.2 FA incorporation into EA.hy926 cells

FA incorporation into EA.hy926 cells was determined by gas chromatography. After 48 hr FA treatment, each FA was incorporated into EA.hy926 cells in a concentration-dependent manner, with the appearance of specific elongation products. ALA treatment (10 and 50 μM) led to significant concentration-dependent increases of ALA in EA.hy929 cells ($p<0.01$ and $p<0.0001$, respectively) (Figure 2A). ALA treatment also lead to a significant increase in docosapentaenoic acid (DPA) at 10 ($p<0.001$) and 50 μM ($p<0.05$) (Figure 2A); the modest enrichment in EPA was not significant (Figure 2A). Incubation with SDA at 50 μM increased SDA levels significantly ($p<0.05$) and also increased levels of 20:4n-3 ($p<0.0001$) and of both EPA and DPA (both $p<0.0001$) (Figure 2B). Incubation of EA.hy926 cells with EPA at 50 μM significantly increased EPA content of the cells ($p<0.0001$) (Figure 2C), and there was a significant concentration-dependent increase in DPA after incubation with EPA at 10 and 50 μM ($p<0.0001$) (Figure 2C). Incubation with EPA did not enrich the cells with DHA. Incubation of EA.hy926 cells with DHA at 10 and 50 μM significantly increased DHA content of the cells ($p<0.0001$) (Figure 2D). There was also a significant increase in DPA after incubation with DHA at both 10 and 50 μM ($p<0.05$), suggesting retroconversion of DHA (Figure 2D). Increased content of n-3 PUFAs was not accompanied by changes in the content of the n-6 PUFAs linoleic, dihomo-γ-linolenic or arachidonic (data not shown). Rather the content of palmitic, stearic and oleic acids was decreased (data not shown).

3.3 Effects of FAs on inflammatory mediators produced by EA.hy926 cells

TNF-α stimulation (24 hr at 1ng/mL) led to markedly increased concentrations of all inflammatory mediators examined in the medium of EA.hy926 cells (data not shown); this increase in concentration is considered to reflect increased production. FA exposure had differential effects depending on the individual FA and on FA concentration (Figures 3 A-E).

ALA did not significantly affect the production of any of the mediators studied, although at 50 μM ALA showed a tendency to decrease production of soluble ICAM-1 (Figure 3A) and MCP-1 (Figure 3B). SDA significantly decreased production of soluble ICAM-1 ($p<0.05$) (Figure 3A) and tended to decrease MCP-1 production when used at 50 μM (Figure 3B).

Of the FA tested, DHA had the greatest inhibitory effect on inflammatory mediator production compared to control (TNF-α stimulated EA.hy926 cells). DHA significantly decreased production of all five mediators measured: soluble ICAM-1 ($p<0.0001$), MCP-1 ($p<0.0001$), IL-6 ($p<0.0001$), IL-8 ($p<0.01$) and RANTES ($P<0.001$) (Figures 3A-E). The effects of DHA were clearly concentration dependent and evident even at 10 μM in many cases. EPA also had significant inhibitory effects (at 50 μM), decreasing production of four of the five mediators examined (soluble ICAM-1 ($p<0.0001$), MCP-1 ($p<0.0001$) IL-6 ($p<0.0001$) and IL-8 ($p<0.05$) (Figures 3A-D)).

When comparing ALA or SDA to EPA and DHA, the marine-derived n-3 FAs had a significantly greater inhibitory effect on each of the mediators secreted (Figures 3A-E).

3.4 Effects of FAs on expression of ICAM-1 on EA.hy926 cells

Incubation of EA.hy926 cells with TNF-α significantly up-regulated cell surface ICAM-1 expression (Figure 4). ALA treatment at both concentrations used (25 or 50 μM) had no effect on the % of cells expressing ICAM-1 (Figure 5 A,B). However, ALA treatment at 50 μM significantly decreased the level of ICAM-1 expression on positive cells (i.e. MFI) ($p<0.01$) (Figure 5D) compared to stimulated control cells. SDA treatment had a greater effect on level of expression (MFI), significantly decreasing ICAM-1 expression at both 25 μM and 50 μM ($p<0.01$, $p<0.0001$, respectively) (Figure 5C-D), whereas only the highest concentration of SDA (50 μM) lead to a significant decrease of % positive cells. EPA treatment at 50 μM significantly decreased both the % of cells expressing ICAM-1 and level of expression (MFI)

(Figures B,D). DHA had the greatest inhibitory effect on ICAM-1, decreasing both the % positive cells and MFI at both 25 and 50 μM ($p<0.0001$, $p<0.0001$) (Figures 5A-D).

3.5 Effects of FAs on THP-1 adhesion to EA.hy926 cells

Adhesion of calcein-labelled THP-1 cells to EA.hy926 cells significantly increased as a result of TNF-α stimulation of the ECs (data not shown). Figures 6A-E show images of fluorescence-labelled THP-1 monocytes bound to EA.hy926 cells with and without FA exposure. Both ALA and SDA reduced adhesion of THP-1 cells when used at 50 μM (Figure 7). ALA and SDA reduced adhesion of THP-1 cells by ~23% and ~30% respectively ($p<0.05$, $p<0.01$) compared to stimulated control cells. When used at 50 μM, DHA had the most potent effect on THP-1 cell adhesion to EC monolayers, inhibiting this by ~40% ($p < 0.0001$; Figure 7). In contrast, EPA treatment at this concentration had no effect on adhesion (Figure 7).

3.6 Effects of FAs on expression of inflammation-related genes in EA.hy926 cells

ALA treatment at 50 μM resulted in a significant increase in the relative gene expression of NFκBp105 ($p<0.05$) and a tendency to increase IKKβ expression ($p<0.051$) (Figures 8A,B). DHA at both 25 μM and 50 μM increased relative expression of the gene for IKKβ ($p<0.05$, $p<0.01$, respectively) (Figure 8B). DHA (50 μM) also increased PPARα gene expression compared to stimulated control cells ($p<0.05$; Figure 8C). Neither SDA nor EPA at 25 or 50 μM had any significant effects on the expression of the genes examined (Figures 8A-C).

The marine-derived n-3 FAs had significantly different effects on relative expression of the genes examined compared to the plant-derived n-3 FAs (Figures 8A-C). Pre-treatment with ALA or SDA at 25 μM resulted in significantly higher NFκBp105 gene expression compared to EPA at 25 μM ($p<0.01$, $p<0.05$); this effect was also seen with ALA treatment compared to EPA at 50 μM ($p<0.05$) (Figure 8A). PPARα gene expression after treatment with ALA at both 25 and 50 μM was significantly lower than that after DHA treatment at 25 and 50 μM ($p<0.05$, $p<0.001$, respectively) (Figure 8C). SDA treatment resulted in significantly lower relative gene expression of PPARα compared to DHA at 25 μM ($p<0.01$) and both EPA and DHA at 50 μM ($p<0.05$, $p<0.0001$, respectively) (Figure 8C).

3.7 Effects of FAs on COX-1, COX-2 and NFκBp65 protein expression in EA.hy926 cells

Pre-treatment with ALA (50 μM) had a tendency to increase the ratio of phosphoNFκBp65/NFκBp65 ($p=0.06$) (Figure 9A), but had no significant effect on COX-1 or

COX-2 protein expression (Figure 9B). Treatment with SDA had no effect on COX-1, COX-2 or phosphoNFκBp65/NFκBp65 ratio (Figure 9A,B,C).

DHA significantly decreased the ratio of phosphoNFκBp65/NFκBp65 ($p<0.01$) and the overall quantities of both phosphoNFκBp65 and NFκBp65 (Figure 9A). DHA (50 µM) also significantly decreased COX-2 protein levels compared to control ($p<0.01$) (Figure 9C). EPA treatment also lead to significantly lower levels of COX-2 protein than in control cells ($p<0.05$) (Figure 9C). However, EPA had no effect on the phosphoNFκbp65/NFκBp65 ratio (Figure 9A). Neither DHA nor EPA treatment had any significant effect on the level of COX-1 protein (Figure 9B).

ALA and SDA treatments led to significantly higher ratios of phosphoNFκBp65/NFκBp65 protein in stimulated EA.hy926 cells compared to DHA ($p<0.001$, $p<0.001$ respectively) (Figure 9A). Pre-treatment with ALA also produced a significantly higher ratio of phosphoNFκBp65/NFκBp65 compared to EPA treatment ($p<0.05$) (Figure 9A).

Comparing protein levels of COX-2 after exposure to plant-derived n-3 FAs to EPA and DHA showed a significant difference between SDA treatment and treatment with DHA ($p<0.001$) (Figure 9C).

No significant differences were observed when comparing protein levels of COX-1 after treatment with any of the FA (Figure 9B).

4 Discussion

The functionality of plant n-3 PUFAs has been underexplored, this study examines the potential of plant-derived n-3 PUFAs as sustainable alternative sources to marine derived n-3 PUFAs with respect to modulation of inflammatory processes. A number of studies have examined the actions of EPA and DHA on EC inflammation and function [25-28, 30, 34-36], whereas only few have described effects of ALA [37-40] and none have described the effects of SDA in this context. There is no previous research which has compared the functions of marine derived n-3 PUFAs to those of plant derived n-3 PUFAs in ECs. The current study confirms the ability of DHA and EPA to decrease inflammatory responses of ECs, with DHA being more potent than EPA. Furthermore, the study shows very limited effects of ALA and SDA, although SDA did elicit some modest anti-inflammatory actions. The finding suggest that ALA and SDA are unlikely to be able to exert anti-inflammatory actions on ECs.

This study identified a broad range of anti-inflammatory effects of the marine-derived n-3 PUFA, DHA, in cultured EA.hy926 cells exposed to TNF-α, a potent inflammatory stimulus. EA.hy926 cells are derived from HUVECs, which are commonly used to study EC biology. HUVECs respond to inflammatory stimuli in a similar way to other human ECs, including those derived from the aorta and coronary artery [13]. At a concentration of 25 μM, DHA decreased production of four cytokines and chemokines (IL-6, MCP-1, IL-8 and RANTES), all known to be involved in initiation and growth of the atherosclerotic plaque, and decreased cell surface expression and secretion of an adhesion molecule (ICAM-1) known to be involved in the early interaction between ECs and infiltrating monocytes. At 25 μM, DHA also increased expression of the gene for IKKβ. At a concentration of 50 μM, the effects of DHA on all of the above outcomes were even more pronounced, and at this concentration, DHA also decreased the ability of EA.hy926 cells to bind THP-1 monocytes, phosphorylation of NFκBp65 and intracellular COX-2 protein levels, while increasing the expression of the PPARα gene. DHA also tended to decrease COX-1 protein, although this effect was not significant. These observations are in accordance with the proposed anti-inflammatory action of DHA through inhibition of the NFκB pathway and activation of the PPAR pathway that will result in a reduction in pro-inflammatory cytokines, chemokines, adhesion molecules and enzymes at both the mRNA and protein levels [10, 11]. PPARα agonists have been shown to exhibit anti-inflammatory effects in endothelial cells, including decreased IL-6, IL-8 and ICAM-1 [41-43]. Similar to findings reported here, Lin et al. describe DHA treatment of EA.hy926 cells lead to PPARα activation and consequent reduction of ICAM-1 expression in EA.hy926 cells [42]. Lee et al. also demonstrated that DHA decreased NFκB activation in macrophages stimulated with lipopolysaccharide through maintenance of IκBα levels and that this resulted in decreased levels of COX-2 and IL-1α protein [44].

In the current study, EPA also exerted anti-inflammatory effects. The effects of EPA on IL-6, IL-8 and ICAM-1 production were like those of DHA. However, EPA had weaker effects than DHA on MCP-1 production, cell surface expression of ICAM-1 and COX-2 protein levels and did not affect RANTES production or THP-1 monocyte binding. In accordance with these weaker effects, EPA did not influence IKKβ or PPARα gene expression or phosphorylation of NFκBp65. These observations suggest that overall EPA has weaker anti-inflammatory actions than DHA, which also agrees with the earlier work of Lee et al. [44] in macrophages.

Anti-inflammatory effects of plant-derived n-3 PUFAs are much less well explored compared to those of marine-derived n-3 PUFAs. In the current study, ALA had very limited anti-inflammatory effects, only decreasing cell surface ICAM-1 expression and THP-1 binding when used at 50 μM. However, this latter observation may be important and demonstrates the close link between expression of ICAM-1 on the EC surface and the ability of ECs to bind monocytes. ALA treatment also had a tendency to reduce the level of COX-1 protein. It is suggested that ALA might exert anti-inflammatory effects as a result of its metabolic conversion to EPA and DHA [18]. Incubation of EA.hy926 cells with ALA did not result in appearance of either EPA or DHA, although there was a small increment in cell DPA content. The lack of appearance of EPA and DHA may explain the lack of anti-inflammatory actions in ECs incubated with ALA. In contrast, incubation of EA.hy926 cells with SDA resulted in a dose-dependent increase in EPA content; once again an increase in DPA, but not in DHA, was detected. SDA also had limited anti-inflammatory effects: it decreased secretion of ICAM-1, cell surface ICAM-1 expression and THP-1 binding at 50 μM. These may be effects exerted by ALA and SDA themselves or might be due to their metabolic products. Both ALA and SDA, and also EPA, enriched the cells in DPA and it is possible that DPA modulates ICAM-1 expression in ECs which in turn affects binding of monocytes. The effects of DPA were not explored in the current study. However, what is clear from these observations is that neither ALA nor SDA can mimic the anti-inflammatory actions of EPA and DHA. Thus, the order of anti-inflammatory potential of these n-3 PUFAs is:

$$\text{ALA} \leq \text{SDA} <<< \text{EPA} < \text{DHA}$$

The FA composition changes that are observed here suggest that these cells have an intact pathway of metabolism from ALA to DPA, but cannot convert DPA further to DHA. This is consistent with observations in many other cell types and in humans *in vivo* [18]. Synthesis of DHA from DPA requires peroxosomal β-oxidation and there is a report that ECs from rats do not express peroxisomal fatty acid β-oxidation activity [45]. Future work should address this pathway in more detail in human ECs.

It is noteworthy than none of the n-3 PUFAs, including EPA and DHA, altered the n-6 PUFA content of the EA.hy926 cells. This is in contrast to what is often observed in other cell types involved in inflammatory processes where n-3 PUFAs are incorporated at the expense of n-6

PUFAs [10, 11]. The reason for this is not clear but may reflect a difference in fatty acid handling or phospholipid metabolism in endothelial cells.

A number of previous studies have examined the effects of marine-derived n-3 PUFAs on inflammatory responses of ECs. De Caterina et al. [34] reported that DHA (10 μM) decreased gene expression, protein levels and cell surface expression of several adhesion molecules and decreased IL-6 production by cytokine-stimulated human saphenous vein ECs and also decreased binding of THP-1 monocytes to the ECs. Chen et al. [36] cultured human coronary artery ECs with 10 or 50 μM EPA or DHA and stimulated the cells with oxidised low-density lipoprotein. They reported that both EPA and DHA decreased ICAM-1 gene expression and protein levels at both concentrations and that EPA and DHA had similar effects. They also demonstrated decreased adhesion of monocytes to the ECs treated with either EPA or DHA. Goua et al. [26] identified that both EPA and DHA at 25 μM decreased expression of ICAM-1 on the surface of HUVECs stimulated with TNF-α. Wang et al. [46] incubated human aorta ECs with 25 to 160 μM EPA or DHA and stimulated the ECs with TNF-α. Both n-3 PUFAs decreased surface expression of vascular cell adhesion molecule 1, but only DHA decreased ICAM-1 expression. DHA, but not EPA, reduced THP-1 binding to the ECs, which is in accordance with the findings of the current study and is consistent with ICAM-1 being important in the binding of THP-1 monocytes to ECs. Huang et al. [31] reported that both EPA and DHA at 100 μM decreased mRNA, protein and cell surface expression of both vascular cell adhesion molecule 1 and ICAM-1 in lipopolysaccharide treated human aorta ECs with the effect of DHA being greater than that of EPA. Both EPA and DHA decreased THP-1 monocyte binding to the ECs and the effects of both n-3 PUFAs were related to inhibition of the NFκB pathway. Lee et al. [47] reported that several concentrations of both EPA and DHA decreased COX-2 protein in HUVECs with a greater effect of DHA. Massaro et al. [48] showed 50% reduction in intracellular COX-2 protein in IL-1α stimulated human saphenous vein endothelial cells after DHA treatment at 25 μM. Similarly, DHA (10 and 40 μM) decreased COX-2 protein levels in rat brain microvascular ECs [49]. The results of the current study are in general agreement with these earlier studies. However, the current study not only extends the earlier findings by examining a broader range of inflammatory outcomes (several cytokines and chemokines, adhesion molecule secretion and cell surface expression, monocyte binding, intracellular inflammatory protein levels (phosphorylated

NFκB and COX-2), and inflammatory pathway gene and protein expression) than any of the earlier studies, but includes a detailed comparison of marine-derived n-3 FAs with plant-derived n-3 PUFAs.

As indicated, there are few previous studies examining the anti-inflammatory effects of plant-derived n-3 PUFAs, especially SDA. Shen et al. [37] described no changes in IL-6 production after treatment of HUVECs with ALA at 50 and 200 μM followed by stimulation with lipopolysaccharide. In contrast, Bork et al. [38] described reduced secretion of ICAM-1 after treatment of EA.hy926 cells with ALA at 100 μM. The current study did not explore the effects of FAs at this concentration since there was evidence of toxicity. However, there are other reports of decreased ICAM-1 expression and secretion after ALA treatment of HUVECs at 50 μM [37, 39]. The current finding of decreased ICAM-1 surface expression with 50 μM ALA is in agreement with these earlier reports. Wang et al. [50] described no effects on nuclear NFκB quantities in porcine ECs after treatment with ALA (20 μM), while there are reports of no effect of ALA treatment on COX-2 expression in vascular endothelial growth factor-stimulated HUVECs [40] or in porcine ECs [50]. SDA treatment (100 μM) of 3T3-L1 adipocytes stimulated with lipopolysaccharide had no effect on MCP-1 production but increased IL-6 production [51]. The effects of ALA or SDA treatment on monocyte binding to ECs have not been previously explored and are therefore novel.

In summary, the current study describes multiple anti-inflammatory effects of EPA and DHA in cultured human ECs and suggests that DHA is more potent than EPA. It also describes limited anti-inflammatory effects of ALA and SDA in this model, but demonstrates that SDA may be more potent than ALA. This might relate to better metabolism of SDA to longer chain more unsaturated n-3 FAs.

Strengths of the current study include confirmation of the FA concentrations added to the cell cultures; confirmation of the incorporation of the n-3 FAs into the cells; evaluation of the effect of different concentrations of FAs for several of the experiments; integrated examination of the effects of the FAs on aspects of cell signalling, gene expression, protein expression, secreted proteins and binding of ECs to monocytes; and the novel comparison of plant- and marine-derived n-3 PUFAs. Although we studied the expression of genes encoding IKKβ, NFκB p105 and PPARα, activated NFκB p65, and the intracellular and secreted concentrations of a range of inflammatory proteins, we did not assess expression

of the genes encoding the latter. One limitation of the study is that it examined the effects of only a single period of exposure of the FAs to the ECs (48 hr). Furthermore the study used only a single inflammatory stimulus, TNF-α, and it would be interesting to examine other stimuli such as lipopolysaccharide or IL-6. It is also worth noting that the FAs were compared at matching concentrations, as required in a controlled study, but that these FAs show markedly different dietary intakes, blood concentrations and cell levels. In general, among the four fatty acids studied here, intake of ALA is greater than intakes of DHA and EPA which are greater than intake of SDA [18]. Blood and cell levels of SDA are very low and cell levels of ALA are low [18]. In most cells and in blood, DHA exceeds EPA [52, 53]. One study conducted in young healthy adults reported mean plasma SDA concentrations of 0.2 µmol/L; in the same study mean plasma ALA, EPA and DHA concentrations were 50.4, 40.3 and 88.8 µmol/L, respectively [54]. Increasing consumption of any of these FAs, including SDA, can lead to increases in their blood concentrations [52, 55, 56]. Lastly, the EA.hy926 EC line was used; although this cell line maintains many of the innate properties of primary ECs [57], it may have some differences in properties or responses. However, the observed effects of EPA and DHA in the current study are similar to those reported for other types of EC [25-28, 30, 34-36].

It is concluded that although plant-derived n-3 PUFAs cannot mimic the anti-inflammatory actions of EPA and DHA, SDA may still provide health benefits to humans, especially those consuming plant-based diets. The context of the current research is the initiation and progression of atherosclerotic plaques that predispose to coronary heart disease and other cardiovascular diseases. The findings support a protective role of marine n-3 PUFAs [1-4] through their anti-inflammatory actions.

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Figure legends

Figure 1. Viability of EA.hy926 cells after incubation for 48 hr with DMEM containing 0.1% of ethanol (Control; CTL) or fatty acids (ALA (A), SDA (B), EPA (C) and DHA (D)) at 10, 25, 50 or 100 µM followed by incubation with or without TNF-α (1 ng/mL) for 24 hr. Bars are mean ± SEM of 9 samples from 3 experiments. Two-way ANOVA with Tukey's post hoc test: Fatty acid vs CTL (unstimulated (DMEM)), *p < 0.05; Fatty acid vs CTL (stimulated (TNF-α 1 ng/mL)), $p < 0.05.

Figure 2. Incorporation of fatty acids (ALA, SDA, EPA and DHA) and appearance of elongation products after incubation of EA.hy926 cells for 48 hr with DMEM containing 0.1% of ethanol (Control) or fatty acids (ALA (A), SDA (B), EPA (C) and DHA (D)) at 10 µM or 50 µM. Bars are mean ± SEM of 9 samples from 3 experiments. One-way ANOVA with Dunnett's post hoc test: Fatty acid vs Control, *p < 0.05.

Figure 3. Concentrations (pg/mL) of ICAM-1 (A), MCP-1 (B), IL-6 (C), IL-8 (D) and RANTES (E) in the medium of EA.hy926 cells incubated for 48 hr with DMEM containing 0.1% of ethanol (Control, CTL) or fatty acids (ALA, SDA, EPA or DHA) at 25 µM or 50 µM, followed by incubation with TNF-α (1 ng/mL) for 24 hr. Bars are mean ± SEM of 9 samples from 3 experiments. Two-way ANOVA with Tukey's post hoc test: Fatty acid vs Control, *p < 0.05; Fatty acid vs DHA, $p < 0.05; Fatty acid vs EPA #p < 0.05.

Figure 4. Flow cytometry plots for ICAM-1 cell surface analysis. Gating of stimulated but unstained EA.hy926 cells, side scatter height (SSC-H) vs forward scatter height (FSC-H) (A). Gated unstimulated (control, TNF-α (-)) and stimulated (TNF-α (+)) PE-conjugated ICAM-1 antibody stained cells (B).

Figure 5. Cell surface expression of ICAM-1 in EA.hy926 cells incubated for 48 hr with DMEM containing 0.1% of ethanol (Control, CTL) or fatty acids (ALA, SDA, EPA or DHA) at 25 µM or 50 µM, followed by incubation with TNF-α (1 ng/mL) for 6 hr. Data are expressed as % difference from control (A,B) and as % of control (C,D). Bars are mean ± SEM of 9 samples from 3 experiments for % gated (A, B) and MFI (C, D). One-way ANOVA with Tukey's post

hoc test: Fatty acid vs CTL, *p < 0.05; Fatty acid vs DHA, $p < 0.05; Fatty acid vs EPA #p < 0.05.

Figure 6. Adhesion of THP-1 cells to EA.hy926 cells incubated for 48 hr with DMEM containing 0.1% of ethanol (Control (A)) or fatty acids (ALA (A), SDA (B), EPA (C) or DHA (D)) at 50 µM, followed by incubation with TNF-α (1 ng/mL) for 6 hr and 1 hr co incubation with calcein labelled THP-1 cells. Attached THP-1 cells were visualised by fluorescence microscope Nikon Elipse Ti at a magnitude of 10 x under transmitted light.

Figure 7. Adhesion of THP-1 cells to EA.hy926 cells incubated for 48 hrs with DMEM containing 0.1% of ethanol (Control, CTL) or fatty acids (ALA, SDA, EPA or DHA) at 25 µM (A) or 50 µM (B), followed by incubation with TNF-α (1 ng/mL) for 6 hr and 1 hr co incubation with calcein labelled THP-1 cells. Data are expressed as % of control. Bars are mean ± SEM of 9 samples from 3 experiments. One-way ANOVA with Tukey's post hoc test: Fatty acid vs CTL, *p < 0.05; Fatty acid vs DHA, $p < 0.05.

Figure 8. Gene expression of NFκB p105 (A), IKKβ (B) and PPARα (C) in EA.hy926 cells incubated for 48 hr with DMEM containing 0.1% of ethanol (Control, CTL) or fatty acids (ALA, SDA, EPA or DHA) at 25 µM or 50 µM, followed by incubation with TNF-α (1 ng/mL) for 6 hr. Bars are mean ± SEM of 9 samples from 3 experiments. One-way ANOVA with Tukey's post hoc test: Fatty acid vs CTL, *p < 0.05; Fatty acid vs DHA, $p < 0.05; Fatty acid vs EPA, #p < 0.05.

Figure 9. Protein expression of phosphoNFκBp65 and NFκBp65 (A), COX-1 (B) and COX-2 (C) in EA.hy926 cells incubated for 48 hr with DMEM containing 0.1% of ethanol (Control, CTL) or fatty acids (ALA, SDA, EPA or DHA) at 50 µM, followed by incubation with TNF-α (1 ng/mL) for 1 or 16 hr. Data are normalised to loading control (GAPDH). Bars are mean ± SEM of 9 samples from 3 experiments. One-way ANOVA with Tukey's post hoc test: Fatty acid vs CTL, *p < 0.05; Fatty acid vs DHA, $p < 0.05; Fatty acid vs EPA, #p < 0.05.

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