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Effects of fatty acids on endothelial cells: Inflammation and monocyte adhesion

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ABSTRACT

Background: Diet is known to have an important impact on cardiovascular health. n-3 Fatty acids (FAs), found in high quantity in fish oil, have demonstrated beneficial effects in patients with coronary artery disease. The role of n-6 FAs remains more controversial. The objective of this study was to examine the effect of arachidonic acid (AA), an n-6 FA, and eicosapentanoic acid (EPA), an n-3 FA, on the interaction between monocytes and endothelial cells (ECs).

Design: We used a cellular model of ECs (EA.hy.926) and monocytes (human leukemic myelomonocytic U937). Confluent ECs were treated with AA or EPA, in the presence of tumor necrosis factor-alpha (TNF-α) or vehicle alone for either 4 or 24 h. Adhesion of monocytes to the endothelial monolayer was performed. For gene expression, reverse transcription, followed by real-time quantitative polymerase chain reaction, was performed.

Results: There was a significant increase in adhesion of monocytes to the endothelial monolayer in the presence of n-6 FAs, both in the presence and in the absence of TNF-α at 4 and 24 h. The adhesion of monocytes to the endothelial monolayer was decreased with n-3 FAs at 24 h. Intercellular adhesion molecule 1, vascular cell adhesion molecule 1, E-Selectin, Interleukin 6, and TNF-α were significantly increased in ECs treated with n-6 FAs.

Conclusions: We conclude that AA increases inflammation and enhances the ability of ECs to bind monocytes *in vitro*. EPA leads to a decrease in the ability of EA.hy.926 to bind monocytes, although the effect appears more modest. Taken together, these data indicate that the n-6 FA AA could potentiate inflammation and early events of atherosclerosis.

1. Introduction

There is significant evidence that polyunsaturated fatty acids (PUFAs), particularly n-3 fatty acids (FAs), affect coronary artery disease (CAD) [1–3] and possibly other atherosclerotic syndromes, such as peripheral arterial disease [4]. Secondary prevention trials have established that fish oil and n-3 FA supplementation lead to a decrease in total mortality, cardiovascular death, sudden cardiac death, and nonfatal cardiovascular events in patients with CAD [1–3,5]. Mechanisms

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postulated for their clinical benefits include their actions as endogenous 3-hydroxy-3-methylglutaryl-coenzymeA reductase and angiotensin-converting-enzyme enzyme inhibitors, antiarrhythmics, antihypertensive, anti-atherosclerotic, anti-inflammatory, cytoprotective, and cardioprotective agents [6]. It is unclear whether n-6 FAs play a similar protective role or, conversely, may lead to atherosclerosis [7].

Atherosclerosis is an inflammatory disease process in which arterial walls become thickened with lipid because of accumulation of lipid-loaded macrophages and smooth muscle cells [8,9]. One of the earliest events in atherosclerosis is binding and infiltration of monocytes through the endothelium. Monocyte–endothelial interactions are therefore key to the development of atherosclerosis. Monocyte binding is facilitated by adhesion molecule expression (vascular cell adhesion molecule 1 [VCAM-1], intercellular adhesion molecule 1 [ICAM-1], and selectins) on the endothelial cells (ECs), which occurs as a result of inflammatory activation of the endothelium [10]. It is likely that n-3 and n-6 FAs affect the binding of monocytes to the endothelium, but several studies report discordant results [11]. Furthermore, the effects of PUFAs on adhesion molecule expression remain controversial, leaving a significant gap in knowledge as to the specific effects of n-3 and n-6 FAs on endothelial activation and subsequent monocyte binding [11].

We studied the effects of n-3 and n-6 FAs on ECs with regard to the following: 1) inflammatory activation, 2) adhesion molecules expression, and 3) monocyte binding, using a cellular model of EA.hy.926 ECs and U937 monocytes. We hypothesized that n-6 FAs would lead to an increase in inflammation, adhesion molecule expression, and subsequently, monocyte binding and that these effects are mediated through the cyclooxygenase (COX) pathway. We further hypothesized that n-3 FAs would have the opposite effects to those of n-6 FAs in view of their overall beneficial clinical effects. This experimental design would allow us to better understand the effects of PUFAs on ECs and possibly uncover some of the molecular and cellular mechanisms involved in their cardiovascular actions.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) and RPMI-1640 medium were purchased from Fisher Scientific (Pittsburg, PA). Penicillin–Streptomycin and L-glutamine were purchased from Mediatech (Herdon, VA). RNase inhibitor, MultiScribe Reverse Transcriptase, and SyBr Green Master Mix were purchased from Applied Biosystems (Foster City, CA). Oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Tumor necrosis factor-alpha (TNF-α) was purchased from R&D systems (Minneapolis, MN). Arachidonic acid (AA) (n-6 FA) and eicosapentanoic acid (EPA) (n-3 FA) were obtained from Cayman Chemical (Ann Arbor, MI). Vybrant cell adhesion assay kit and Hoechst stain for nuclear staining were obtained from Invitrogen (Eugene, OR). Albumin was purchased from Sigma (St. Louis, MO). Phosphate saline buffer solution (PBS) was from the University of California San Francisco Cell Culture Facility (San Francisco, CA).

2.2. Cell culture

The cellular model used in this experiment included EA.hy.926 (for ECs) and U937 monocytes. EA.hy.926 cells are a fusion of human umbilical vein ECs and the A549 epidermal carcinoma line, which retains many of the characteristics of primary ECs [12]. The EA.hy.926 cells were a kind gift from Dr. Cora-Jean S. Edgell from the University of North Carolina, Chapel Hill [12]. U937 cells are a human monocyte cell line and were obtained from the American Type Cell Collection [13]. The ECs were maintained in a 37°C/5% CO_2 incubator in DMEM with 10% FBS, 2 mM L-glutamine, 1% (v/v) penicillin–streptomycin–neomycin antibiotic mix, 4.5 g/L D-Glucose, and 1% (v/v) amphotericin. The monocytes were maintained in a 37°C/5% CO_2 incubator in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 1% (v/v) penicillin–streptomycin–neomycin antibiotic mix. Medium was changed every 2 d. Cell concentration was adjusted to 5×10^5 cells/mL at each medium change. Cell counts were performed with a hemacytometer (Improved Neubauer, Reichert, NY).

2.3. Treatment of ECs for adhesion assays

Confluent ECs were trypsinized and plated at 5000 cells per well in a 96-well plate, then grown to confluency before experimental treatments. FA and cytokine treatments were performed as follows: ECs were incubated with either 5 μg/mL EPA, 5 μg/mL AA, 100 ng/mL TNF-α, vehicle alone or a combination of these for 4 or 24 h before the adhesion assay. The dose of TNF-α was selected based on dose–response curves leading to the greatest activation of the ECs based on adhesion molecule expression. The doses of FAs were selected based on the human physiological dose range and dose–response curves performed in our laboratory. Vehicle contained DMEM with 2% FBS, 2 mM L-glutamine, 1% (v/v) penicillin–streptomycin–neomycin antibiotic mix, 4.5 g/L D-Glucose, 1% (v/v) amphotericin, and 1.25 mg/mL FA-free albumin. Monocytes were labeled with Calcein-AM [14] according to the Molecular Probes Vybrant cell adhesion assay kit manufacturer's instructions and washed twice with PBS. Labeled monocytes were resuspended in vehicle with 0.1% FBS and incubated for 20 min with ECs. The 96-well plate was gently washed one or two times with PBS. Fluorescence per well (Ex 488 nm, Em 515 nm) was measured using a Fluoroscan II plate reader.

To investigate a possible pathway for the actions of PUFAs (i.e., the COX pathway), experiments with the n-6 FA AA were performed in the presence of a COX-2 inhibitor. For pretreatment with the COX-2 inhibitor, adhesion assays were repeated using the same conditions mentioned above, with pretreatment for 1 h with indomethacin (50 uM) before treatment with the n-6 FA.

2.4. Ribonucleic acid isolation

Ribonucleic acid (RNA) was isolated using RNeasy Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. For RNeasy Mini kit RNA isolation, cells were seeded in

six-well plates with DMEM media supplemented with 10% FBS, 2 mM L-glutamine, 1% (v/v) penicillin–streptomycin–neomycin antibiotic mix, 4.5 g/L D-Glucose, and 1% (v/v) amphotericin until they reached confluence. Once confluent, media was changed to DMEM supplemented with 2% FBS, whereas all other supplements remained the same. The cells were then treated with the different FAs and TNF-α (5 μg/mL EPA, 5 μg/mL AA, 100 ng/mL TNF-α, a combination of AA and TNF-α, or vehicle alone) for a period of 4 h. On removal of media, the ECs were washed two times with PBS and then 350 uL of RNA lysis buffer (supplied in kit) were added to each well. Cells were scrapped off the plate with a cell lifter. The lysate was then placed into a QIA shredder homogenizer (QIAGEN, Valencia, CA), and the flow through was isolated using the Qiagen RNeasy Mini kit and processed as per manufacturer's instruction. The samples were then stored at −80°C until further analysis.

2.5. Reverse transcription

RNA (0.3 ug) was added to 30 uL reverse transcriptase (RT) reaction buffer containing 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM deoxyribonucleotide triphosphate molecules, 2.5 μM oligo d(T) primer, 2.5 U/μL of MultiScribe, and 20 U/μL of RNase inhibitor. The RT reaction was incubated at room temperature for 10 min, 42°C for 30 min, inactivated at 99°C for 5 min, and cooled at 5°C for 5 min.

2.6. Real-time quantitative RT-polymerase chain reaction

Complementary deoxyribonucleic acid (2 μL) from the RT reaction was added to 20 μL real-time quantitative polymerase chain reaction (qPCR) mixture containing 10 μL of 2x SYBR Green PCR Master Mix and 12 pmol oligonucleotide primers. PCRs were performed in a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The thermal profile was 50°C for 2 min and 95°C for 10 min to activate the Taq polymerase, followed by 50 amplification cycles, consisting of denaturation at 95°C for 1 min 40 s, annealing at 63°C for 1 min 10 s, and elongation at 72°C for 1 min 40 s. Fluorescence was measured and used for quantitative purposes. At the end of the amplification period, melting curve analysis was performed to confirm the specificity of the amplicon. RNA samples were normalized to *cyclophilin (CPHI)* internal standard. Relative quantification of gene expression was calculated by using the $2^{-(\text{Ct gene T}-\text{Ct CPHI T})-(\text{Ct gene 0 h}-\text{Ct CPHI 0 h})}$ equation, where "C_t gene T" represents the calculated threshold cycle (C_t) of a time point of each sample other than 0 h or each treatment other than control. Relative gene abundance was calculated using $2^{(\text{Ct gene T}-\text{Ct CPHI T})}$. Some primer sequences have been previously used [15–17] and are presented in Table 1. All data derived using qRTPCR were from independent biological samples ($n = 4$).

2.7. Fluorescence microscopy

Confluent ECs were trypsinized and plated on a coverslip in 96-well plates, then grown to confluency. FAs and cytokine treatments were performed as described above for the 96-well plate assay. Calcein-AM–labeled monocytes were incubated with ECs for 30 min, before the coverslips were gently dipped in PBS four times. ECs and monocytes were imaged using Zeiss Axioscop Fluorescent Microscope (Carl Zeiss, Germany) and an Orca-ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ).

2.8. Statistical considerations

Data were assessed for normality and outliers. Means and standard deviations (SDs) are presented. Analyses of variance were used to compare the means of the different treatment groups. The *P* values presented were corrected for multiple-hypothesis testing using the Bonferroni method. Statistical analyses were performed using Stata/SE 12 (StataCorp, College Station, TX).

3. Results

3.1. n-6 INCREASES whereas n-3 decreases the adhesion of monocytes to the ECs

To test the hypothesis that PUFAs affect the adhesion of monocytes to the endothelial monolayer, we studied the effects of 4 and 24 h of exposure of n-3 and n-6 PUFAs on monocyte adhesion to ECs. Four hours of FA treatment led to significant changes in adhesion of monocytes to ECs, both in the nonactivated (without TNF-α; $P < 0.0001$ for the overall group effect) and in the activated conditions (with TNF-α; $P < 0.0001$ for the overall group effect) (Fig. 1a). In the nonactivated state, treatment with n-6 FA led to a significant increase in the adhesion of monocytes to ECs compared with baseline ($P < 0.001$). Although the effect of n-3 FA on adhesion was not significant at baseline, the difference between n-3 and n-6 FA responses on monocytes adhesion was significant ($P < 0.001$). In the activated state, there also was a significant increase in the adhesion of monocytes to ECs with n-6 compared with baseline ($P < 0.001$). There was no significant

Table 1 – Primers used for qRTPCR in ECs.

Primer	Forward primer 5'→3'	Reverse primer 5'→3'
E-Selectin	ACCTCCACGGAAGCTATGACT	CAGACCCACACATTGTTGACTT
ICAM-1	GTGGTAGCAGCCGCAGTC	GGCTTGTGTGTTCGGTTTCA
IL-6	GCTGAAAAAGATGGATGCTT	GGCTTGTTCCTCACTACTCTC
TNF-α	TCAGATCATCTTCTCGAACCCC	ATCTCTCAGCTCCACGCCAT
VCAM-1	AATGGGAATCTACAGCACCTTT	ATATCCGTATCCTCCAAAAACT

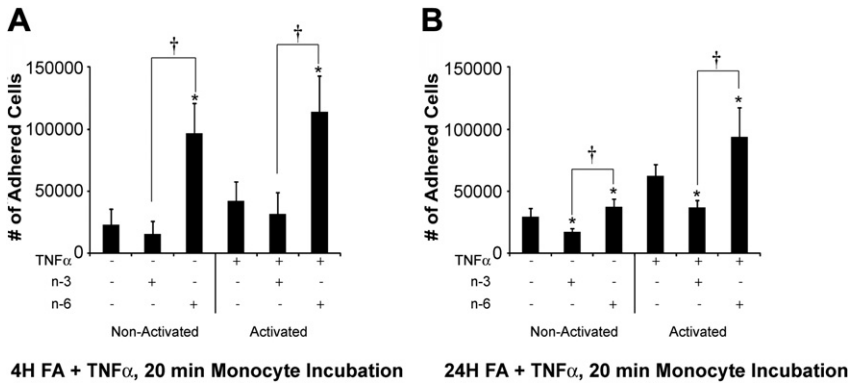

Fig. 1 – Monocyte adhesion after treatment of ECs with n-3 and n-6 FAs for 4 h (A) and 24 h (B). To assess the effects of FA on ECs' ability to bind monocytes, ECs were grown to confluency and treated with n-3 and n-6 FAs (5 ug/mL) with or without TNF-α (100 ng/mL) for 4 and 24 h. At 4 h, in the absence or presence of TNF-α, adhesion of monocytes to the endothelial monolayer is higher in the n-6 FA-treated cells than in the nontreated or n-3 FA-treated cells. This remains true at 24 h. Means ± SD ($n = 8$). *$P \le 0.05$ for n-3 or n-6 treatment compared with the control without FA. †$P \le 0.001$ between n-3 and n-6 FA-treatment groups.

difference between n-3 FAs and TNF-α compared with TNF-α alone ($P = 0.98$). Significant response difference persisted for ECs treated with n-3 and n-6 FAs in the presence of TNF-α ($P < 0.001$).

Interestingly, longer treatment periods of ECs with FAs (24 h) also led to significant changes in adhesion of monocytes to ECs, both in the nonactivated ($P < 0.0001$ for the overall group effect) and in the activated conditions ($P < 0.0001$ for the overall group effect) (Fig. 1b). In the nonactivated state, there was a significant increase in the adhesion of monocytes to the EC monolayer in the presence of n-6 FA ($P = 0.03$) and a significant decrease in monocyte adhesion in the presence of n-3 FA ($P = 0.001$). The response between n-3 and n-6 FAs was significantly different ($P < 0.001$). In the activated state, n-3 FAs led to a significant decrease ($P = 0.02$) and n-6 FAs led to a significant increase ($P = 0.003$) in monocyte adhesion to ECs, compared with TNF-α alone. The response between n-3- and n-6-treated FAs also was significantly different in the activated state ($P < 0.001$).

Although the direct effects of n-3 FA on monocyte adhesion (decrease in adhesion) are more pronounced with a longer treatment period, our results suggest that n-6 FAs lead to an increase in monocyte adhesion to the endothelial monolayer in a manner that is less dependent on time of exposure. This increased binding of the monocytes to ECs with n-6 FA also was observed with fluorescence microscopy (Fig. 2).

3.2. *The effects of n-6 FA on ECs are mediated through the COX pathway*

To assess if the cellular effects of n-6 FAs are mediated through the COX pathway, ECs were exposed to indomethacin, a COX-2 inhibitor, in the presence and absence of the n-6 FA AA. Pretreatment with indomethacin at the dose of 50 uM significantly decreased the binding of monocytes to the endothelial monolayer in the presence of AA ($P = 0.001$) (Fig. 3). Indomethacin alone did not have a significant impact on the adhesion of monocytes to ECs ($P = 1.00$).

3.3. *An increase in inflammatory and adhesion molecule gene expression could mediate the effects of n-6 on ECs*

To investigate molecular mechanisms involved in the effects of PUFAs, gene expression in ECs was investigated, in the presence or absence of PUFAs and with and without activation with TNF-α (Fig. 4). The primers used are described in Table 1.

Treatment with FAs led to significant changes in gene activation (Fig. 4). At baseline, in the absence of TNF-α, there was a significant increase in ICAM-1, VCAM-1, E-Selectin as well as interleukin 6 (IL-6) and TNF-α with n-6 FAs. The gene activation response after treatment with PUFAs (in the absence of TNF-α) differed between n-3 and n-6 FAs for ICAM-1, VCAM-1, E-Selectin, IL-6, and TNF-α.

In the presence of the activator TNF-α, only n-6 FAs led to significant changes in gene activation. This was characterized by an increase in ICAM-1 and VCAM-1, as well as an increase in IL-6. Similarly, the pattern of gene activation was significantly different between n-3 and n-6 FAs in the presence of TNF-α.

Overall, this suggests that n-6 FAs lead to an activation of ECs, characterized by an increase in gene expression of inflammatory mediators, that is, IL-6 and TNF-α and an increase in adhesion molecules. These responses differ from the response to n-3 FAs.

4. Discussion

Monocyte–endothelial interactions and inflammation are central to the initiation of atherosclerosis. Our data demonstrate that treatment of ECs with the n-6 FA AA leads to an increase in monocyte adhesion to ECs, likely mediated by an increase in ICAM-1, VCAM-1, and E-Selectin. n-6 FAs also appear to increase inflammatory markers in the ECs. With regard to this finding, to our knowledge, this is the first study demonstrating a direct increase in IL-6 and TNF-α gene expression with the n-6 FA AA in endothelial-type cells.

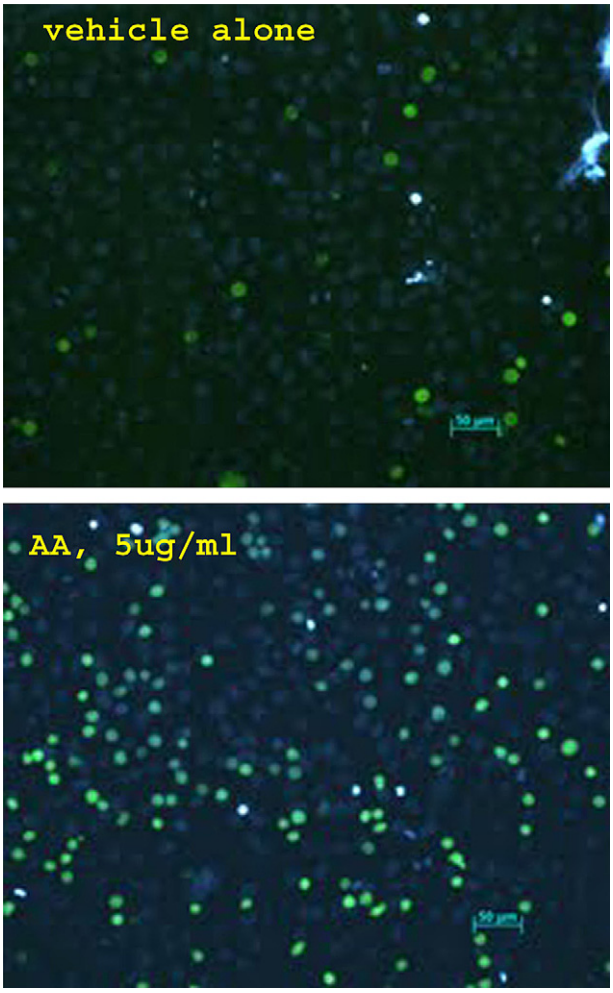

Fig. 2 – Monocyte adhesion to ECs at fluorescence microscopy after treatment with n-6 FAs. Confluent ECs were incubated with 5 μg/mL AA (n-6 FA) or vehicle alone for 5 h before a 30-min incubation with Calcein-AM–labeled U937 cells. The coverslips were gently dipped in PBS to remove unbound U937 cells and then imaged by fluorescence microscopy. ECs were labeled with Hoeschst stain (blue), and U937 cells were labeled with Calcein-AM (green). Fluorescence microscopy demonstrates an increase in monocytes binding to ECs after treatment with n-6 FA.

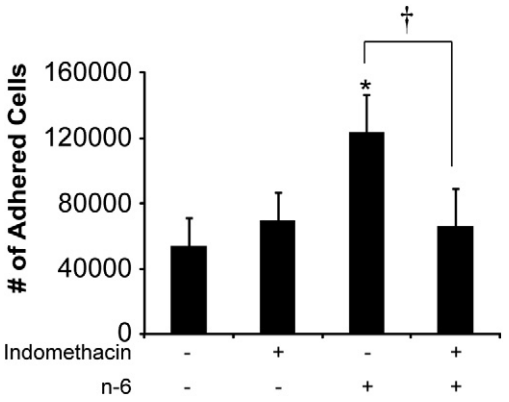

Fig. 3 – Effect of pretreatment of ECs with indomethacin, a COX-2 inhibitor, on monocyte adhesion. To evaluate if the cellular effects of n-6 FA are mediated through the COX pathway, ECs were exposed to indomethacin, a COX-2 inhibitor, at the dose of 50 uM in the presence and absence of the n-6 FA AA. Indomethacin decreased the adhesion of monocytes to the n-6-treated endothelial monolayer. Indomethacin alone did not affect adhesion. Means ± SD ($n = 8$). *$P \leq 0.001$ for n-6-treated endothelial monolayer compared with no treatment. †$P = 0.001$ for n-6-treated endothelial monolayer compared with n-6-treated endothelial monolayer after pretreatment with indomethacin.

Furthermore, the COX pathway appears central to these interactions. Conversely, n-3 FA appears to decrease adhesion of monocytes to ECs, but this effect is more modest. These findings have important clinical implications, particularly in resolving the controversy concerning the association of specific PUFAs in the diet (i.e., n-6 FAs and n-3 FAs) on atherosclerotic syndromes [7,18].

4.1. *Effects of n-6 FAs on monocyte adhesion to the endothelial monolayer and on inflammation*

In this experiment, we have consistently demonstrated an increase in monocyte–EC adhesion after treatment with n-6 FAs. This effect appears to be mediated by an increase in ICAM-1, VCAM-1, and E-Selectin. In previous literature, these interactions were more controversial. Reissig *et al.* [19] reported that linoleic acid (LA), a precursor of AA, led to a decrease in monocyte–endothelial adhesion. Others have reported no changes [20]. Consistent with our results, some authors have reported a stimulatory effect of n-6 FA on adhesion molecule expression and adhesion of monocytes to ECs [21–23]. Dichtl *et al.* [21] demonstrated that LA led to an increase in VCAM-1 protein and mRNA, which was mediated through nuclear factor kappa-beta (NF-KB) signaling. Findings of Toborek *et al.* [22] also support this with an increase in NF-KB, VCAM-1, and ICAM-1 after stimulation of ECs with LA.

AA is the precursor for an extensive array of eicosanoids (20-carbon FA metabolites), including all the two-series prostaglandins, thromboxane A_2, prostacyclin (PGI_2), the four-series leukotrienes, and a variety of cytochrome P-450 metabolites. These compounds are bioactive and several mediate inflammatory responses, stimulate platelet aggregation, and produce vasoconstriction. Although these are all essential metabolic functions, in excess and unopposed, they can promote atherosclerotic disease and thrombus formation. Our findings provide evidence that AA directly impacts production of inflammatory mediators in the ECs with an increase in IL-6 and TNF-α.

4.2. *Enzymatic involvement: COX-2*

In the present study, we demonstrated that pretreatment with indomethacin led to less monocyte adhesion to ECs in the presence of AA, pointing toward the importance of COX-2 in the mechanisms of action of AA. Indomethacin sits in the catalytic pocket of COX-2, thus inhibiting the conversion

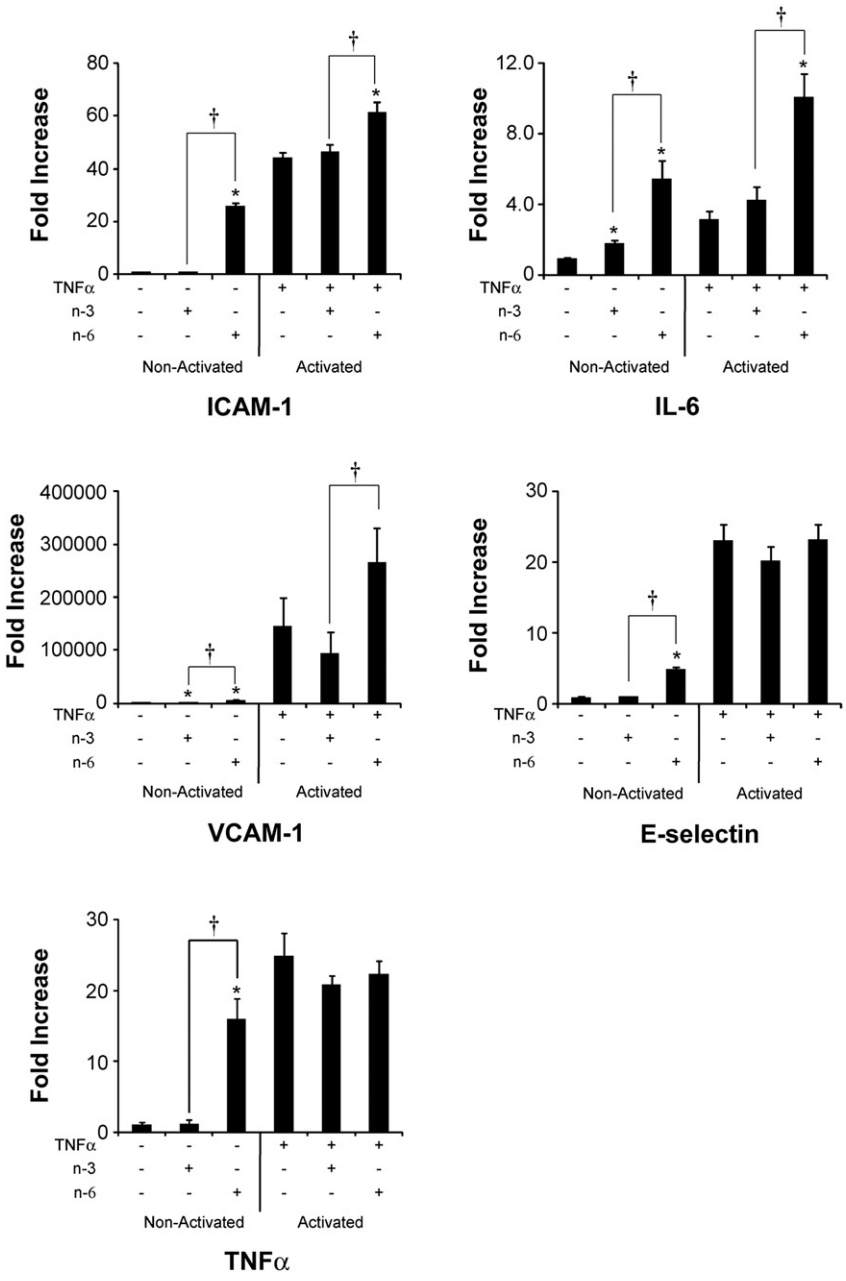

Fig. 4 – Gene expression in ECs after treatment of n-3 and n-6 FAs using qRTPCR analysis. To assess molecular mechanisms involved in the alterations in monocyte binding to treatment with FAs (4 h), qRTPCR analysis of gene expression was done. Treatment of ECs with FA in both the activated (with TNF-α) and the nonactivated states shows a marked increase in expression of adhesion molecules and inflammatory mediators in n-6 compared with n-3 FA. Means ± SD ($n = 4$). *$P \le 0.05$ for n-3 or n-6 treatment compared with the control without FA. †$P \le 0.05$ between n-3 and n-6 FA-treatment groups.

of the n-6 FA AA, to its by-products prostaglandins and lipoxygenases and other inflammatory mediators. The inability of AA to make its downstream products in the presence of indomethacin is most likely the cause of its consequent inability to influence adhesion. Although indomethacin led to a decrease in monocyte adhesion to ECs in the presence of n-6 FAs, the clinical relevance of these findings need not to be overstated as several large studies have demonstrated adverse effects of COX-2 inhibition in cardiovascular disease (reviewed in [24]). No recommendation can be made based on our findings with regard to COX-2 inhibition clinically; however, our findings support the mechanism of action of AA on monocyte–endothelial adhesion through the COX pathway.

Some of the biological effects of PUFA are believed to be related to their incorporation into cell membrane phospholipids. There is evidence that in the presence of n-3 FA, prostaglandin D3 replaces prostaglandin D2 [25], leading to competitive inhibition at the COX-2 level. This replaces AA with the prostanoid derivatives of EPA, which are potentially less prothrombotic and vasoconstrictive than the AA

derivatives [26]. The present study provides further evidence that effects of PUFA on monocyte–endothelial interactions are mediated by the COX-2 enzyme. The effects of PUFA could lead to alterations in the NF-KB [27–29] system and peroxisome proliferator-activated receptor-alpha [30]. Future investigations will include studies of the AA-induced expression of chemokines/cytokines and downstream products that are changed by the presence of nonsteroidalanti-inflammatory drugs.

4.3. *Effects of n-3 FA on monocyte adhesion to the endothelial monolayer*

Our study confirms prior work demonstrating that treatment with EPA leads to a decrease in adhesion of monocytes to ECs [31–35]. Our study further elucidates the effects of PUFA on adhesion molecule expression. We demonstrated that treatment of n-3 or n-6 FA had relatively differential effects on adhesion molecules, providing further evidence that the actions of PUFA are related to changes in adhesion molecule expression (ICAM-1, VCAM-1, and E-Selectin) and cytokine expression (i.e., IL-6 and TNF-α).

De Caterina and Libby [32] demonstrated that a decrease in monocyte adhesion to ECs with n-3 FA was related to a reduction in VCAM-1, E-Selectin, ICAM-1, IL-6, and IL-8. Wang *et al.* [36], in human aortic ECs treated with EPA and decosahexaenoicacid (DHA), demonstrated a decrease in VCAM-1 by cell-surface enzyme-linked immunoabsorbent assay, which was confirmed by protein analysis of the cell lysates. Although other pieces of evidence support alterations in VCAM-1, E-Selectin, and ICAM-1 with PUFA treatment [29,33,37], some conflicting evidence negates such changes in adhesion molecule gene expression [38,39]. Taken together, our findings confirm most data presently available, supporting a beneficial effect of n-3 FA on reduction in leukocyte adhesion to the endothelium, steps that are critical in early atherogenesis. This parallels findings from Thies *et al.* [40] demonstrating that n-3 FAs modulate the cellular and structural composition of the atherosclerotic plaque, in a manner to reduce rupture or ulceration.

4.4. *Clinical implications of the present findings*

Our findings add to existing knowledge suggesting a protective effect of n-3 FAs at the cellular level as relates to monocyte–endothelial interactions. They support the recent recommendations from the American Heart Association (AHA) promoting a diet rich in n-3 FAs. The AHA suggests that the general population should aim to consume fish, especially oily fish, at least twice a week [41]. For patients with documented CAD, recommended consumption is of $\sim$1 g of EPA + DHA/d, preferably from fish high in EPA and DHA, as this is associated with a reduced risk of both sudden death and death from CAD [42,43]. Hence, our findings provide further evidence for the importance of nutrition in addition to optimal medical therapy in patients suffering from cardiovascular disease.

Despite the evidence of beneficial effects of n-3 FAs, definitive evidence that consumption of n-6 FAs for primary and secondary prevention of atherosclerosis is less clear. The AHA has recently recommended that consumption of n-6 FA be increased to 5%–10% of energy intake to reduce CAD risk that may potentially be related to lower intake [44]. Inconsistencies in the evidence base have rendered these recommendations controversial [7,45–47]. Our findings suggest a cautionary approach to n-6 dietary advice, in view of a possible increase in inflammation and monocyte binding at the level of the endothelial monolayer. Further evidence from translational studies also supports this. For example, Ambring *et al.* [48] studied n-6:n-3 PUFA ratios in healthy subjects while on a Swedish diet or a Mediterranean diet high in fish and flaxseed oil. The Mediterranean diet led to a lower n-6:n-3 ratio and a lower total number of circulating leukocyte and platelets. Ferrucci *et al.* [49] assessed the relationship between plasma PUFAs and circulating inflammatory markers in a community-based sample and found that the total n-3 PUFAs were independently associated with lower levels of pro-inflammatory markers and higher anti-inflammatory markers independent of confounders. A study by Dwyer *et al.* [50] demonstrated that dietary AA significantly enhances the apparent atherogenic effect of genotype, whereas increased dietary intake of n-3 FAs blunted this effect. Furthermore, the large cardiovascular trials have demonstrated clinical benefits to increasing the dose of n-3 PUFAs in the diet [1–3]. Based on the concentration of PUFAs in human serum [51], we would hypothesize that the levels used in this study should be looked at as a pharmacological effect of a physiological dose, which correlates with evidence from the literature.

4.5. *Limitations*

The cell lines used in this study are not primary human EC lines, which could lead to different responses compared with primary human EC lines. Additionally, the adhesion experiments were performed in static conditions, which presents another level of complexity as to how our results could be interpreted for human vasculature. Dose–response curves for n-6 and n-3 FAs have been performed by the authors in previous work for osteoblasts, epithelial cells, ECs, prostate cancer, and colorectal cancer cells and were not repeated here. The best concentration for cellular response has been in the 5 μg/mL range for both types of FAs for all cell types. Furthermore, this concentration remains within human physiological range, estimated to be on average 16.1 mg/100 mL in Japanese males and 21.1 mg/100 mL in Dutch males sera for AA and 3.7 mg/100 mL and 1.0 mg/100 mL for EPA in Japanese and Dutch sera, respectively [51]. These factors as well as general limitations when comparing *in vitro* with *in vivo* studies restrict the direct applicability to clinical settings. Nonetheless, the findings provide a clearer understanding of the effects of PUFAs on the monocyte–endothelial interactions and inflammation.

5. Conclusions

We conclude that AA increases binding of monocytes to the ECs through a mechanism that is related to an increased expression of ICAM-1, VCAM-1, and E-Selectin, whereas EPA likely decreases the binding of monocytes to ECs. Equally important is the direct effect of AA in inducing inflammation

through an increase in IL-6 and TNF-α, a novel finding. Taken together, these data indicate that PUFAs have the ability to alter monocyte adhesion to ECs, strengthening the evidence of the importance of dietary PUFAs on early events in atherosclerosis.

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REFERENCES

[1] Burr ML, Fehily AM, Gilbert JF, Rogers S, Holliday RM, Sweetnam PM, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet 1989;2:757.
[2] Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet 1999;354:447.
[3] Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet 2007;369:1090.
[4] Grenon SM, Hughes-Fulford M, Rapp J, Conte MS. Polyunsaturated fatty acids and peripheral artery disease. Vasc Med 2012;17:51.
[5] Marik PE, Varon J. Omega-3 dietary supplements and the risk of cardiovascular events: a systematic review. Clin Cardiol 2009;32:365.
[6] Das UN. Essential fatty acids and their metabolites could function as endogenous HMG-CoA reductase and ACE enzyme inhibitors, anti-arrhythmic, anti-hypertensive, anti-atherosclerotic, anti-inflammatory, cytoprotective, and cardioprotective molecules. Lipids Health Dis 2008;7:37.
[7] Ramsden CE, Hibbeln JR, Majchrzak SF, Davis JM. n-6 fatty acid-specific and mixed polyunsaturate dietary interventions have different effects on CHD risk: a meta-analysis of randomised controlled trials. Br J Nutr 2010;104:1586.
[8] Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685.
[9] Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res 2002;91:281.
[10] Osterud B, Bjorklid E. Role of monocytes in atherogenesis. Physiol Rev 2003;83:1069.
[11] Ringseis R, Eder K. Fatty acids and signalling in endothelial cells. Prostaglandins Leukot Essent Fatty Acids 2010;82:189.
[12] Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci U S A 1983;80:3734.
[13] Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565.
[14] Braut-Boucher F, Pichon J, Rat P, Adolphe M, Aubery M, Font J. A non-isotopic, highly sensitive, fluorimetric, cell-cell adhesion microplate assay using calcein AM-labeled lymphocytes. J Immunol Methods 1995;178:41.
[15] Hatton JP, Pooran M, Li CF, Luzzio C, Hughes-Fulford M. A short pulse of mechanical force induces gene expression and growth in MC3T3-E1 osteoblasts via an ERK 1/2 pathway. J Bone Miner Res 2003;18:58.
[16] Boonyaratanakornkit JB, Cogoli A, Li CF, Schopper T, Pippia P, Galleri G, et al. Key gravity-sensitive signaling pathways drive T cell activation. Faseb J 2005;19:2020.
[17] Hughes-Fulford M, Sugano E, Schopper T, Li CF, Boonyaratanakornkit JB, Cogoli A. Early immune response and regulation of IL-2 receptor subunits. Cell Signal 2005;17:1111.
18 Deckelbaum RJ, Torrejon C. The omega-3 fatty acid nutritional landscape: health benefits and sources. J Nutr 2012;142(3): 587S.
[19] Reissig D, Rassoul F, Salvetter J, Wagner O, Richter V. Effect of fatty acids on expression of endothelial leukocyte adhesion molecules. Eur J Nutr 2003;42:224.
[20] Holthe MR, Andersson Y, Lyberg T. Lack of proinflammatory effects of free fatty acids on human umbilical cord vein endothelial cells and leukocytes. Acta Obstet Gynecol Scand 2005;84:672.
[21] Dichtl W, Ares MP, Jonson AN, Jovinge S, Pachinger O, Giachelli CM, et al. Linoleic acid-stimulated vascular adhesion molecule-1 expression in endothelial cells depends on nuclear factor-kappaB activation. Metabolism 2002;51:327.
[22] Toborek M, Lee YW, Garrido R, Kaiser S, Hennig B. Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells. Am J Clin Nutr 2002;75:119.
[23] Reiterer G, Toborek M, Hennig B. Quercetin protects against linoleic acid-induced porcine endothelial cell dysfunction. J Nutr 2004;134:771.
[24] Amer M, Bead VR, Bathon J, Blumenthal RS, Edwards DN. Use of nonsteroidal anti-inflammatory drugs in patients with cardiovascular disease: a cautionary tale. Cardiol Rev 2010; 18:204.
[25] Tull SP, Yates CM, Maskrey BH, O'Donnell VB, Madden J, Grimble RF, et al. Omega-3 fatty acids and inflammation: novel interactions reveal a new step in neutrophil recruitment. PLoS Biol 2009;7:e1000177.
[26] Dyerberg J, Bang HO, Stoffersen E, Moncada S, Vane JR. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? Lancet 1978;2:117.
[27] Wang L, Lim EJ, Toborek M, Hennig B. The role of fatty acids and caveolin-1 in tumor necrosis factor alpha-induced endothelial cell activation. Metabolism 2008;57:1328.
[28] Chen W, Esselman WJ, Jump DB, Busik JV. Anti-inflammatory effect of docosahexaenoic acid on cytokine-induced adhesion molecule expression in human retinal vascular endothelial cells. Invest Ophthalmol Vis Sci 2005;46:4342.
[29] Goua M, Mulgrew S, Frank J, Rees D, Sneddon AA, Wahle KW. Regulation of adhesion molecule expression in human endothelial and smooth muscle cells by omega-3 fatty acids

and conjugated linoleic acids: involvement of the transcription factor NF-kappaB? Prostaglandins Leukot Essent Fatty Acids 2008;78:33.
[30] Sethi S, Ziouzenkova O, Ni H, Wagner DD, Plutzky J, Mayadas TN. Oxidized omega-3 fatty acids in fish oil inhibit leukocyte-endothelial interactions through activation of PPAR alpha. Blood 2002;100:1340.
[31] Weber C, Erl W, Pietsch A, Danesch U, Weber PC. Docosahexaenoic acid selectively attenuates induction of vascular cell adhesion molecule-1 and subsequent monocytic cell adhesion to human endothelial cells stimulated by tumor necrosis factor-alpha. Arterioscler Thromb Vasc Biol 1995;15:622.
[32] De Caterina R, Libby P. Control of endothelial leukocyte adhesion molecules by fatty acids. Lipids 1996;31:S57.
[33] De Caterina R, Cybulsky MI, Clinton SK, Gimbrone MA Jr, Libby P. The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells. Arterioscler Thromb 1994;14:1829.
[34] De Caterina R, Massaro M. Omega-3 fatty acids and the regulation of expression of endothelial pro-atherogenic and pro-inflammatory genes. J Membr Biol 2005;206:103.
[35] Yamada H, Yoshida M, Nakano Y, Suganami T, Satoh N, Mita T, et al. In vivo and in vitro inhibition of monocyte adhesion to endothelial cells and endothelial adhesion molecules by eicosapentaenoic acid. Arterioscler Thromb Vasc Biol 2008;28:2173.
[36] Wang TM, Chen CJ, Lee TS, Chao HY, Wu WH, Hsieh SC, et al. Docosahexaenoic acid attenuates VCAM-1 expression and NF-kappaB activation in TNF-alpha-treated human aortic endothelial cells. J Nutr Biochem 2011;22:187.
[37] De Caterina R, Cybulsky MA, Clinton SK, Gimbrone MA Jr, Libby P. Omega-3 fatty acids and endothelial leukocyte adhesion molecules. Prostaglandins Leukot Essent Fatty Acids 1995;52:191.
[38] Mayer K, Merfels M, Muhly-Reinholz M, Gokorsch S, Rosseau S, Lohmeyer J, et al. Omega-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. Am J Physiol Heart Circ Physiol 2002;283:H811.
[39] Schaefer MB, Wenzel A, Fischer T, Braun-Dullaeus RC, Renner F, Dietrich H, et al. Fatty acids differentially influence phosphatidylinositol 3-kinase signal transduction in endothelial cells: impact on adhesion and apoptosis. Atherosclerosis 2008;197:630.
[40] Thies F, Garry JM, Yaqoob P, Rerkasem K, Williams J, Shearman CP, et al. Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet 2003;361:477.
[41] Lichtenstein AH, Appel LJ, Brands M, Carnethon M, Daniels S, Franch HA, et al. Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. Circulation 2006;114:82.
[42] Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation 2002;106:2747.
[43] Wang C, Harris WS, Chung M, Lichtenstein AH, Balk EM, Kupelnick B, et al. n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. Am J Clin Nutr 2006; 84:5.
[44] Harris WS, Mozaffarian D, Rimm E, Kris-Etherton P, Rudel LL, Appel LJ, et al. Omega-6 fatty acids and risk for cardiovascular disease: a science advisory from the American Heart Association Nutrition Subcommittee of the Council on Nutrition, Physical Activity, and Metabolism; Council on Cardiovascular Nursing; and Council on Epidemiology and Prevention. Circulation 2009;119:902.
[45] Fritsche KL. Too much linoleic acid promotes inflammation-doesn't it? Prostaglandins Leukot Essent Fatty Acids 2008; 79:173.
[46] Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med (Maywood) 2008;233:674.
[47] Kris-Etherton P, Fleming J, Harris WS. The debate about n-6 polyunsaturated fatty acid recommendations for cardiovascular health. J Am Diet Assoc 2010;110:201.
[48] Ambring A, Johansson M, Axelsen M, Gan L, Strandvik B, Friberg P. Mediterranean-inspired diet lowers the ratio of serum phospholipid n-6 to n-3 fatty acids, the number of leukocytes and platelets, and vascular endothelial growth factor in healthy subjects. Am J Clin Nutr 2006;83:575.
[49] Ferrucci L, Cherubini A, Bandinelli S, Bartali B, Corsi A, Lauretani F, et al. Relationship of plasma polyunsaturated fatty acids to circulating inflammatory markers. J Clin Endocrinol Metab 2006;91:439.
[50] Dwyer JH, Allayee H, Dwyer KM, Fan J, Wu H, Mar R, et al. Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. N Engl J Med 2004; 350:29.
[51] Hirai K, Horiuchi R, Ohno Y, Higuchi H, Asano Y. Lower eicosapentaenoic acid and higher arachidonic acid levels in Sera of young adults in the Netherlands than in Japan. Environ Health Prev Med 2000;5:60.