Omega-3 fatty acids modulate collagen signaling in human platelets

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ABSTRACT

Dietary intake of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) results in cardioprotective benefits. However, the cellular and physiological bases for these benefits remain unclear. We hypothesized that EPA and DHA treatments would interfere with collagen-mediated platelet signaling. Thirty healthy volunteers received 28 days of 3.4 g/d EPA+DHA with and without a single dose of aspirin. Clinical hematologic parameters were then measured along with assays of collagen-stimulated platelet activation and protein phosphorylation. Omega-3 therapy led to a small but significant reduction in platelets (6.3%) and red blood cells (1.7%), but did not impair clinical time-to-closure assays. However, collagen-mediated platelet signaling events of integrin activation, z-granule secretion, and phosphatidylserine exposure were all reduced by roughly 50% after omega-3 incorporation, and collagen-induced tyrosine phosphorylation was significantly impaired. The diminished platelet response to collagen may account for some of the cardioprotective benefits provided by DHA and EPA.

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1. Introduction

Epidemiological studies from the 1970s uncovered the cardioprotective effects of a diet high in marine oils, with Greenland Inuits experiencing extremely low rates of acute myocardial infarction (MI) [1]. The main omega-3 fatty acids found in the Inuit diet, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have also been associated with up to 90% reductions in risk for primary cardiac arrest [2,3] and a lower death rate in coronary heart disease patients [4]. Significantly, randomized controlled studies in post-MI patients given daily doses of 850 mg of EPA and DHA showed significant reductions in both sudden death and total mortality, with the effects observable within 120 days of treatment [5]. Despite the evidence for clinical benefits of DHA and EPA, a biochemical mechanism by which these lipids exert their cardioprotective effects has remained elusive.

Several candidates for DHA and EPA cellular targeting have been suggested by studies, in which these fatty acids reduced tachycardia in a canine experimental MI model [6], reduced mortality after MI in rats [7], and lowered heart rate in cardiac patients [8]. Calcium signaling flux is also regulated by DHA and EPA levels [9], and DHA and EPA can also inhibit thromboxane activity by occupying the active site of cyclooxygenase [10] and the thromboxane A2 receptor [11]. Intriguingly, DHA and EPA incorporation into membranes disorders membrane rafts [12], indicating a potential for alterations in membrane-proximal signaling pathways. Effects on thromboxane production and membrane rafts suggest that platelet function can also be dramatically affected by DHA and EPA, as has been widely described for platelet aggregometry [13–16] and adhesion [17].

In this study, we sought to identify the cellular mechanisms behind this inhibition of platelet function. Collagen-mediated platelet signaling is dependent on the raft-bound GPVI receptor [18,19], and therefore this pathway is a likely target for modulation by DHA and EPA. Indeed, our previous findings with the whole blood aggregometry suggest that collagen-mediated platelet signaling could be inhibited by four weeks of treatment with prescription omega-3 acid ethyl esters (P-OM3, Lovaza, GlaxoSmithKline) [14]. We now describe the results of a follow-up study to examine the effects of P-OM3, alone and in combination with aspirin, on collagen-mediated platelet signaling in order to identify mechanisms by which EPA and DHA impact the platelet function.

2. Materials and methods

Study protocol: this was an open-label, four-week sequential therapy study, where the subjects served as their own controls, similar to our previous studies [14]. Blood was drawn at four separate visits: Day 1 (control), Day 2 (one day after a standard therapeutic 650 mg dose of aspirin), Day 29 (after 28 days of 4 g/d

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of P-OM3, delivering ~1.86 g/d EPA and ~1.5 g/d DHA), and Day 30 (after one day of combined P-OM3 and 650 mg aspirin treatment).

Subjects: two separate cohorts of fifteen healthy individuals each (non-smokers and not taking prescription medications or supplements) between the ages of 21 and 59 were recruited for this study. One group was studied in the summer of 2008, and the other in the spring of 2009. As the protocols were identical, the results from both studies were combined. A detailed questionnaire was given on the first visit to exclude subjects with a bleeding history or with allergies to fish, fish oils or aspirin. The same questionnaire was used in subsequent visits to determine if any bleeding diatheses developed during the study. Subjects who drank more than three alcoholic beverages a day, had ulcers or stomach bleeding, had liver or kidney disease, had congestive heart failure or heart disease, had high blood pressure, gout, asthma, arthritis, or nasal polyps were excluded from the study. The protocol was approved by the University of South Dakota/Sanford School of Medicine Institutional Review Board, and written consent was obtained from all subjects. Subjects were screened at each visit for complete blood counts to ensure safety, and a preliminary platelet function analyzer test with epinephrine/collagen cartridges (see below) was performed to screen for normal platelet function prior to commencement of treatment. All platelet function tests were carried out using blood drawn into sodium citrate and all functional protocols were carried out within one hour of blood draws.

Platelet aggregation: whole blood platelet function was analyzed at the Sanford Health Clinical Laboratory, using a platelet function analyzer (PFA)-100 system (Siemens, Deerfield, IL, USA). This assay measures anti-coagulated whole blood forced through agonist-coated apertures and measures the time to aperture closure facilitated by platelet-platelet aggregation without the contribution of fibrin formation. When closure times of >300 s were detected, a value of 300 s was assigned for purposes of statistical analysis. The PFA-100 test was done at each visit in the summer 2008 study (n=15), but only at screening in the spring 2009 study (n=15).

Platelet and fatty acid composition: fatty acid incorporation into cell membranes was determined as previously described [14]. Briefly, platelets were isolated by washing at high speed (1000g) and treated with 14% boron trifluoride–methanol. The fatty acid methyl esters were then analyzed by capillary gas chromatography and compared to the weighted standards of 22 fatty acids. Fatty acid levels are expressed as a percent of total fatty acids.

Flow cytometry: platelet-rich plasma (PRP) was collected following the centrifugation of whole blood (20 min at 200g) and 5 µl of PRP was added to microtubes containing HEPES-buffered saline, vehicle control or collagen (Chronologic, Havertown, PA, USA), final concentration of 13 µg/ml, determined in preliminary experiments as a half-maximal dose) and 5 µl of one of the following fluorescein isothiocyanate (FITC)-labeled fluorophores: PAC-1 monoclonal antibody (indicating GPIb/IIa integrin activation), anti-C6D2P monoclonal antibody (indicating α-granule secretion), or annexin-V (indicating phosphatidylserine exposure; BD Biosciences, San Jose, CA, USA). For annexin-V assays, 2.5 mM Ca²⁺ was included in the HEPES-buffered saline. Samples were allowed to incubate for 20 min in the dark at room temperature, then diluted with 0.2% paraformaldehyde and fixed for 10 min prior to analysis on an Accuri C6 flow cytometer (Ann Arbor, MI, USA) within 2 h post-fixation. Results were tabulated as mean fluorescence of the sample. Flow cytometry was performed only in the spring 2009 study.

Western blotting: prostaglandin I₂ (1 µg/ml) was added to PRP, which was subsequently spun at 1000g for 10 min to isolate platelets. Platelets were resuspended in Tyrode's buffer with 0.1% glucose and stimulated with 60 µg/ml collagen for 2 min, then lysed with 2x concentrated lysis buffer (300 mM NaCl, 20 mM Tris (pH 7.3), 2 mM EDTA, 2 mM EGTA, 2% Nonidet-P 40, 1% Octyl-β-glucopyranoside, 20 mM MgCl₂) with HALT protease/phosphatase inhibitor (Thermo Scientific, Rockford, IL, USA) and 10 µM pepstatin. Samples were lysed for 30 min at 4°C, then protein concentrations were equalized using a BCA protein assay kit (Thermo Scientific). Equal amounts of protein were loaded onto 4–20% Tris–glycine gradient gels (Invitrogen, Carlsbad, CA USA) and subsequently transferred to PVDF membranes. Membranes were blotted using pan-anti-phosphotyrosine antibody (clone 4G10, Millipore, Billerica, MA USA) or polyclonal anti-tyrosine phosphorylated (Y1217) phospholipase γ2 (PLCγ2, Cell Signaling Technology, Danvers, MA USA) and using anti-GAPDH as a loading control (Millipore). Bands were detected using an anti-mouse HRP-linked secondary antibody and Westpico substrate kit (Thermo Scientific) with detection on a C:Box 16-bit digital imaging station (Syngene, Frederick, MD, USA). All image adjustments were limited to brightness and contrast and applied to the entire image. Non-saturating western blot band intensities were quantified using GeneTools software (Syngene). These assays were performed on frozen platelet lysates from samples collected in the spring 2009 study.

Statistics: data were analyzed using the JMP software, version 8.0.2 (Cary, NC USA), using a 2 x 2 factorial design with a mixed model ANCOVA adjusted for age, sex, and BMI. The assumption of normality and equal variance was verified, and if needed, data were log or rank transformed. If an aspirin and P-OM3 interaction was observed at p > 0.2, it was removed from the statistical model. In some analyses, subject data was excluded as indicated in the text. Differences with p-value of < 0.05 were considered statistically significant.

3. Results

After four weeks of P-OM3 treatment, the omega-3 index of the platelet membrane rose from 1.80% to 4.80% (p < 0.0001; Fig. 1). The percentage of an EPA in the platelet membrane rose from 0.22% to 1.77% (p < 0.0001), and the percentage of DHA changed from 1.55% to 3.00% (p < 0.0001). P-OM3 reduced the abundance of arachidonic acid (AA) in the platelet membrane from 23.4% to 22.0% (p=0.0012); neither AA nor omega-3 levels were significantly affected by aspirin. These results clearly demonstrate that omega-3 supplementation can dramatically alter platelet lipid composition in a relative short time.

The effects of P-OM3 and aspirin on hematological parameters are presented in Table 1. Two of the 30 subjects were removed from the data set, one who showed signs of anemia at baseline (~30% hematocrit), and one whose platelet counts showed an unexplained fluctuation between Day 1 and 29 (>200 K/µl between visits). The overall results suggest mild, but statistically significant, variations in several hematologic endpoints. Mean platelet counts dropped by 6.3% with P-OM3 treatment (p < 0.0001), and erythrocyte counts dropped in response to aspirin (1.3%, p=0.04) and to P-OM3 (1.7%, p=0.007). Concurrently, hemoglobin (1.3%, p=0.02) and hematocrit levels (1.4%, p=0.02) dropped in response to aspirin treatment, while hemoglobin (2.1%, p=0.0002), but not hematocrit (0.8%, p=0.28), dropped after the P-OM3 supplementation. White blood cell counts were not affected by either treatment.

The effects of P-OM3 and aspirin on platelet function were assessed using a test commonly included in clinical medicine (PFA-100) in the 15 summer 2008 subjects. As expected, closure time of the collagen/epinephrine cartridge aperture significantly increased after aspirin treatment (Fig. 2, 91.6% increase, p < 0.0001), but the time-to-closure after P-OM3 treatment slightly, but significantly, decreased (10.4% decrease, p=0.03). There was no effect of P-OM3 on collagen/ADP cartridge aperture closure time, but there was a small increase in response to aspirin (5.1% increase, p = 0.0001). The decrease in closure time in response to P-OM3 is inconsistent with previous studies looking at the whole blood platelet aggregation [14], and may suggest that the PFA-100 test does not adequately gauge the overall effects of P-OM3 therapy in this time span. It may
also suggest that the risk for excessive bleeding in normal subjects taking P-OM3 is minimal as supported by the lack of bleeding episodes reported in study questionnaires (data not shown). Though no effect of P-OM3 on platelet function was evident through clinical testing, significant in vitro changes were observed in the platelet activation. In the 15 subjects who participated in the spring 2009 study, the platelet response to collagen was examined in three cytometric readouts: PAC-1 binding, P-selectin exposure, and annexin-V binding. Platelets from one subject did not respond to collagen stimulation at Day 1, and data from that subject was removed from the analysis, resulting in an \( n = 14 \) for each measurement. Compared to baseline, P-OM3 treatment decreased GPIIb/IIIa integrin activation in response to collagen as determined by PAC-1 binding (Fig. 3, \( p = 0.003 \)). Aspirin had no significant effects on the same measure. Collagen-mediated expression of the adhesive protein P-selectin (CD62P) showed a similar profile, with a reduction in cell surface expression after P-OM3 treatment (\( p = 0.017 \), but not with

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<th>Table 1</th>
<th>Changes in blood cell counts in response to P-OM3 and aspirin treatment.</th>
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<td>Study day</td>
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<td>Day 1</td>
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At each of the four visits, blood was drawn and immediately analyzed for complete blood counts in the clinical laboratory. Numbers in parentheses represent 95% confidence intervals, and the significance was determined as compared to baseline levels, and are as follows: main effects of P-OM3:

** \( < 0.005 \), * \( < 0.05 \); main effects of aspirin: † \( < 0.005 \), ‡ \( < 0.05 \). n = 28.

Fig. 2. PFA-100 assay closure rates before and after aspirin (Asp) and P-OM3 treatment. Time-to-closure assays were performed using epinephrine/collagen (Epi/Col) and ADP/collagen (ADP/Col) cartridges. All results of \( > 300 \) s, the upper limit for the instrument, were truncated at 300 s for the statistical analysis. All data were rank transformed to account for the lack of ordinal data. Significance is compared to baseline (Day 1) levels, and error bars represent 95% confidence intervals. Main effects of P-OM3: † † \( < 0.005 \), † ‡ \( < 0.05 \); main effects of aspirin: † † † \( < 0.005 \), † † ‡ < 0.05; n = 15.
**4. Discussion and conclusions**

The primary finding of this study was that treatment with omega-3 fatty acids interfered with collagen-mediated platelet activation. The reduction of PAC-1 binding, P-selectin exposure and annexin-V binding all indicate that platelets with high levels of DHA and EPA are less able to bind fibrinogen, secrete their granule contents, and generate thrombin on their surface. Globally diminished protein phosphorylation is consistent with an impaired signaling. The reduced reactivity of the platelets after P-OM3 treatment could, at least in part, account for reduced risks for atherothrombosis and its clinical sequelae, MI, and stroke.

The selection of collagen as the agonist in this study was based on the known role that collagen plays in early platelet arrest and activation at the sites of vessel injury [20]. Impairments in collagen-mediated platelet signaling have been associated with a significant reduction in thrombotic activity, and in some cases, excessive bleeding, highlighting the critical nature of collagen for the platelet function [21,22]. Indeed, collagen-mediated whole-blood aggregation was reduced with P-OM3 in an earlier study using the same protocol [14].

The impairment of collagen signaling was also evident by the reduction in protein phosphorylation after collagen signaling. The band at approximately 115 kDa could be the catalytic subunit of phospholipase C, an essential protein for collagen-induced platelet activation [29]. Since G VPI is localized in lipid rafts, DHA and EPA could disrupt collagen signaling complexes, as the raft integrity appears to be necessary for full collagen signaling [18,19]. However, disruptions of rafts do not appear to affect all downstream collagen signaling pathways [30], and the impairment of collagen signaling may be caused by the loss of secondary activation pathways, particularly since the granule secretion is also inhibited by the P-OM3 treatment. It is also possible that serine/threonine phosphorylation patterns could be altered and/or influence aspirin. P-OM3 reduced annexin-V binding (p = 0.0045), while aspirin treatment increased annexin-V binding (p = 0.0036). No interaction between P-OM3 and aspirin was observed in PAC-1 and annexin-V binding. For P-selectin exposure, P-OM3 and aspirin were expected to have even lower activity than with P-OM3 alone; however, we observed an interaction between the two drugs that ameliorated this effect. Statistical analysis with the platelet number as a covariate indicated that the change in platelet number after P-OM3 treatment did not influence the major effects observed in these assays.

Finally, we examined the effects of collagen on platelet phosphotyrosine induction using a phosphotyrosine-specific antibody in western blotting. We assessed only baseline and P-OM3 treatments, not aspirin, due to the absence of significant aspirin effects in the flow cytometric assays. As seen in Fig. 4a, most subjects showed a reduction in overall collagen-mediated tyrosine phosphorylation after the P-OM3 treatment when compared to baseline stimulation. When the density of the bands at ~50 and ~115 kDa (the most consistently observed bands in all subjects) were quantified and represented by an effect size, a significant reduction in band intensity after collagen stimulation compared to baseline (Day 1) was observed on Day 29 (Fig. 4b, p = 0.023), suggesting that intracellular signaling from collagen receptors is diminished after the P-OM3 treatment. We then blotted for a phosphorylated version of the major collagen pathway signaling protein, PLC/2, and found that levels of collagen-stimulated PLC/2 phosphorylation were also diminished after EPA and DHA treatment (Fig. 4c).

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**Fig. 3.** Flow cytometric assay of platelet responses to collagen stimulation before and after P-OM3 and aspirin treatment. Measurements show the level of fluorophore binding after collagen (13 μg/ml) treatment of PRP on each of the four visits of the study. Data were compiled on an Accuri C6 flow cytometer, which does not allow for the laser gain adjustment. The machine settings remain fixed between all assays, and data are reported untransformed as mean fluorescence. Significance is compared to baseline (Day 1) levels, error bars represent 95% confidence intervals. Main effects of P-OM3: * p < 0.005, ** p < 0.05; main effects of aspirin (Asp): † p < 0.005, ‡ p < 0.05; n = 14. Control stimulated fluorescence levels were not significantly different over the course of the study.
tyrosine phosphorylation, not just in response to EPA and DHA, but also in response to platelet washing conditions with prostacyclin addition. However, Ser/Thr phosphorylation induced by collagen signaling in platelets has not been as well characterized as tyrosine phosphorylation, thus making it difficult to quantify this potential interaction. Future studies will be needed to generate more platelet samples to examine the effect of P-OM3 on the collagen signaling network in more detail, and to identify which specific proteins and pathways are affected.

Treatment with P-OM3 did not affect the platelet function as measured by the clinical PFA-100 test. Since the test worked as expected with aspirinated platelets, this observation suggests that the PFA-100 test is either insensitive to the type of platelet activation inhibited by P-OM3 or requires a longer P-OM3 treatment time to show an effect, though the use of dual agonists in the cartridges (which would activate multiple pathways) could result in normal in vitro function even if collagen-mediated signaling was reduced. Since P-OM3 has been shown in prospective clinical trials and cohort studies to have vascular benefits, its mechanism of action (if indeed it is anti-thrombotic) might require the presence of omega-3 enriched platelets and arterial substrate (which the PFA-100 test cannot replicate). In addition, the PFA-100 test may not be reflective of clinically relevant anti-thrombosis, since it was recently reported to not correlate well to a primary endpoint of vessel blockage [31]. Given the compelling clinical evidence for a health benefit of omega-3 fatty acid supplementation and the other results from this study, some in vivo modulation of platelet function is more likely than not.

The fact that P-OM3 blunted collagen-induced platelet activation and aspirin did not suggest that these fatty acids operate via a cyclooxygenase-independent pathway. Indeed, previous studies have also observed cyclooxygenase-independent platelet activation when using the GPVI collagen receptor as an agonist [27,32]. While an occupation of cyclooxygenase is not likely the sole target of DHA and EPA in the platelets, we cannot rule out the possibility that metabolites of DHA and EPA can contribute to the inhibition. The production of thromboxane A3 from an EPA, which is less active than its cousin thromboxane A2 derived from an AA [33], may be playing a role, and resolvins have also been reported to have potent anti-platelet activities by themselves and could also represent a way for omega-3 fatty acids to act independently from aspirin, along with other oxylipins and epoxides [34].

P-OM3 treatment reduced platelet and erythrocyte cell count to a small but statistically significant extent. While the changes were not clinically significant, both of these effects could contribute to the reduced thrombotic risk. A recent study suggests a strong correlation between platelet counts and risk for the coronary heart disease [35], so even a slight reduction in platelet count could have a significant cardiovascular benefit. Since platelet deposition at sites of injury is known to create a thrombogenic surface [36], the reduced number of platelets may slow the progression of arterial occlusion. It should be noted that even pharmacologic doses of omega-3 fatty acids, such as those used here, do not increase the risk for clinically significant bleeding [37].

In conclusion, an increasing body of evidence continues to suggest EPA and DHA as promising therapeutics in combating cardiovascular disease. With their increased use, it will become imperative to better understand how these molecules influence cellular behavior. This study gives further evidence that platelet function can be favorably affected by DHA and EPA without adversely affecting normal hematology, and it suggests that at least part of the mechanism by which omega-3 fatty acids reduce risk for cardiovascular events is via effects on platelet metabolism.

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