Exogenous modification of platelet membranes with the omega-3 fatty acids EPA and DHA reduces platelet procoagulant activity and thrombus formation

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Larson MK, Tormoen GW, Weaver LJ, Luepke KJ, Patel IA, Hjelmen CE, Ensz NM, McComas LS, McCarty OJ. Exogenous modification of platelet membranes with the omega-3 fatty acids EPA and DHA reduces platelet procoagulant activity and thrombus formation. Am J Physiol Cell Physiol 304: C273–C279, 2013. First published November 21, 2012; doi:10.1152/ajpcell.00174.2012.—Several studies have implicated the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in inhibition of normal platelet function, suggesting a role for platelets in EPA- and DHA-mediated cardioprotection. However, it is unclear whether the cardioprotective mechanisms arise from alterations to platelet-platelet, platelet-matrix, or platelet-coagulation factor interactions. Our previous studies led us to hypothesize that EPA and DHA alter the ability of platelets to catalyze the generation of thrombin. We tested this hypothesis by exogenously modifying platelet membranes with EPA and DHA, which resulted in compositional changes analogous to increased dietary EPA and DHA intake. Platelets treated with EPA and DHA showed reductions in the rate of thrombin generation and exposure of platelet phosphatidylserine. In addition, treatment of platelets with EPA and DHA decreased thrombus formation and altered the processing of thrombin precursor proteins. Furthermore, treatment of whole blood with EPA and DHA resulted in increased occlusion time and a sharply reduced accumulation of fibrin under flow conditions. These results demonstrate that EPA and DHA inhibit, but do not eliminate, the ability of platelets to catalyze thrombin generation in vitro. The ability of EPA and DHA to reduce the procoagulant function of platelets provides a possible mechanism behind the cardioprotective phenotype in individuals consuming high levels of EPA and DHA. EPA, DHA, and their metabolites. While these findings suggest that platelet inhibition may be a contributing factor to n-3 FA-induced cardioprotection, it is not clear how these alterations affect platelet function under physiological conditions within the circulation. Our findings suggest that platelet aggregation in a basic clinical measurement is not impeded after 4 wk of high-dose EPA and DHA intake (17), suggesting that the cardioprotective mechanisms of EPA and DHA are not due to reduced platelet-platelet or platelet-collagen interactions. The focus of this study was to test the hypothesis that EPA and DHA inhibit the procoagulant phenotype of platelets. Vanschoonbeek and colleagues (30) observed a prolonged thrombin generation time in plasma in subjects on a high EPA and DHA regimen. They attributed this increased time to the concurrent reduction in factor V and fibrinogen levels in these subjects. However, another potential role for an n-3 FA-mediated effect on coagulation is the interface between platelets and the coagulation cascade. Platelets are a key cellular component in thrombin generation (3, 23). Acidic phospholipids on the platelet surface can form Ca2+-dependent complexes of factor Xa (derived from plasma), factor Va (derived from plasma and/or secreted from platelets), and prothrombin (24, 29). This complex assembly allows platelets to serve as a surface for the catalysis of thrombin generation at sites of vascular injury (9). In addition to an effect of EPA and DHA treatment on the levels of soluble coagulation factors, we hypothesized that n-3 FAs could also inhibit platelet-mediated thrombin generation. Therefore, we investigated the role of EPA and DHA in the assembly of coagulation enzyme complexes on the surfaces of activated platelets. Specifically, we aimed to determine if the procoagulant function of platelets was inhibited by the presence of increased levels of EPA and DHA in the platelet plasma membrane.

DECADES OF EPIDEMIOLOGICAL case-control, and pharmacological studies have established a clear cardiovascular benefit from high doses of the omega-3 fatty acids (n-3 FAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (5, 26). While several potential mechanisms underlying the EPA- and DHA-mediated reduction of myocardial infarction and stroke have been proposed and investigated, recent studies have focused on n-3 FA-mediated alterations to platelets, the primary cellular component of blood coagulation and a frequent target of therapeutic intervention. These studies have shown that basic platelet functions, including refractory signal transduction (10, 12, 17), reduced aggregation (4, 7, 8, 16), lowered platelet counts (17), reduced adhesion (19), lowered sensitivity to collagen (33), and reduced platelet-monocyte adhesion (6), can be significantly altered in the presence of high levels of EPA, DHA, and their metabolites. While these findings suggest that platelet inhibition may be a contributing factor to n-3 FA-induced cardioprotection, it is not clear how these alterations affect platelet function under physiological conditions within the circulation. Our findings suggest that platelet aggregation in a basic clinical measurement is not impeded after 4 wk of high-dose EPA and DHA intake (17), suggesting that the cardioprotective mechanisms of EPA and DHA are not due to reduced platelet-platelet or platelet-collagen interactions. The focus of this study was to test the hypothesis that EPA and DHA inhibit the procoagulant phenotype of platelets. Vanschoonbeek and colleagues (30) observed a prolonged thrombin generation time in plasma in subjects on a high EPA and DHA regimen. They attributed this increased time to the concurrent reduction in factor V and fibrinogen levels in these subjects. However, another potential role for an n-3 FA-mediated effect on coagulation is the interface between platelets and the coagulation cascade. Platelets are a key cellular component in thrombin generation (3, 23). Acidic phospholipids on the platelet surface can form Ca2+-dependent complexes of factor Xa (derived from plasma), factor Va (derived from plasma and/or secreted from platelets), and prothrombin (24, 29). This complex assembly allows platelets to serve as a surface for the catalysis of thrombin generation at sites of vascular injury (9). In addition to an effect of EPA and DHA treatment on the levels of soluble coagulation factors, we hypothesized that n-3 FAs could also inhibit platelet-mediated thrombin generation. Therefore, we investigated the role of EPA and DHA in the assembly of coagulation enzyme complexes on the surfaces of activated platelets. Specifically, we aimed to determine if the procoagulant function of platelets was inhibited by the presence of increased levels of EPA and DHA in the platelet plasma membrane.

MATERIALS AND METHODS

Human subjects and statistics. All procedures were approved by Institutional Review Boards at Augustana College and Oregon Health and Science University and followed institutional guidelines. All subjects (male and female) gave informed consent. Healthy human donors not knowingly taking medication were recruited for the study. All subjects served as their own controls, with blood and plasma split into two fractions. All statistics were performed using Student’s t-test, paired for each donor; significance was set at P < 0.05.

In vitro platelet DHA and EPA modification. For whole blood assays, blood was drawn 1:10 into 3.2% sodium citrate, 150 μM DHA and EPA (NuChek Prep, Elysian, MN) or vehicle control (1% fatty acid-free BSA in HEPES-Tyrode buffer) was added, and the samples were incubated for 4 h at 37°C. DHA and EPA were prepared under argon to prevent oxidation. For washed platelet assays, whole blood was drawn 1:10 into 3.2% sodium citrate, and acid citrate dextrose
was added. Blood was spun at 200 g for 20 min, and plasma was treated with 750 μM DHA and EPA or vehicle control and incubated as described above. Plasma was then spun at 1,000 g for 10 min in the presence of 50 ng/ml prostaglandin I2. Platelets were resuspended at desired concentrations in HEPES-Tyrode buffer (Fig. 1). For analysis of fatty acid content, platelets were subjected to centrifugation, methylated, and analyzed via gas chromatography, as previously described (16). In selected experiments, platelet samples were run on TLC plates and compared with phospholipid standards. TLC bands were collected and analyzed for fatty acid content via gas chromatography.

**Flow cytometry.** Platelets were diluted to 5 × 10^7 platelets/ml and added to microtubes containing 5 μl of FITC-annexin V (BD Biosciences, Franklin Lakes, NJ) in the presence of 11 mM Ca^{2+}, along with varying concentrations of collagen in equal volumes (Chronolog, Havertown, PA) or 100 ng/ml convulxin (Centerchem, Norwalk, CT). After 10 min of incubation, samples were diluted in 500 μl of 0.2% paraformaldehyde and analyzed on a flow cytometer (Accuri C6, BD Biosciences); 2 × 10^4 platelets were counted for each sample.

**Lipid raft staining.** Glass coverslips were coated with 50 μg/ml collagen and subsequently blocked with 5 mg/ml denatured BSA. Washed platelets were treated with vehicle or EPA/DHA, layered on collagen-coated coverslips at 4°C for 30 min at room temperature. Platelets were then washed and mounted on glass slides and imaged with a confocal microscope (BX51 Fluoview, Olympus, Melville, NY).

Western blotting. Blotting for thrombin precursor proteins was performed largely as described by Wood et al. (32). Briefly, platelets were resuspended at 3 × 10^10 platelets/ml in HEPES-Tyrode buffer and then added to reaction tubes containing 11 mM Ca^{2+}, 0.2 μg/ml human prothrombin (Haematologic Technologies, Essex Junction, VT), 10 μg/ml collagen, 1 mM RGDS peptide (Tocris Bioscience, Ellisville, MO), and 5 nM factor Xa (Haematologic Technologies). Platelets were incubated with samples removed at varying time points and added to an equal volume of 2× sample buffer. After separation of proteins on reducing 10% SDS-polyacrylamide gels, Western blotting was carried out using an anti-human prothrombin monoclonal antibody that recognizes prothrombin, prethrombin-1, fragment 2, and meizothrombin (Haematologic Technologies) via its epitope in fragment 2.

**Thrombin generation assay.** For thrombin generation assay, 15 μl of Spectrozyme TH (American Diagnostica, Stamford, CT) were added. Blood was spun at 200 g for 20 min, and plasma was treated with 750 μM DHA and EPA or vehicle control and incubated as described above. Plasma was then spun at 1,000 g for 10 min in the presence of 50 ng/ml prostaglandin I2. Platelets were resuspended at desired concentrations in HEPES-Tyrode buffer (Fig. 1). For analysis of fatty acid content, platelets were subjected to centrifugation, methylated, and analyzed via gas chromatography, as previously described (16). In selected experiments, platelet samples were run on TLC plates and compared with phospholipid standards. TLC bands were collected and analyzed for fatty acid content via gas chromatography.

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**Thrombin generation assay.** For thrombin generation assay, 15 μl of Spectrozyme TH (American Diagnostica, Stamford, CT) were combined with 1.5 μl of factor Xa (500 nM) and 1.75 μl of prothrombin (122 μM) in individual wells of a transparent 96-well plate. Next, 146 μl of 16 mM CaCl2 and 100 μl of vehicle- or EPA/DHA-treated platelets were added to the well; immediately thereafter, absorbance at 405 nm was recorded by spectrophotometer (Tecan, San Jose, CA) every 60 s for 1 h. After each experiment, absorbance was plotted vs. time for each well, and the maximum slope of each plot was measured to yield enzyme activity.

**Flow adhesion and fibrin quantification.** Whole blood collected into 3.2% sodium citrate was incubated for 4 h at 37°C with 150 μM EPA/DHA or vehicle. Whole blood was recalculated with 7.5 mM CaCl2 and 3.75 mM MgCl2 immediately prior to perfusion through fibrillar collagen-coated capillary tubes at a wall shear rate of 250 s⁻¹. Thrombus formation was visually recorded using differential interference contrast microscopy, and the degree of fibrin formation was analyzed by Western blotting for the fibrin degradation product D-dimer following clot lysis with plasmin (2 mM), as previously described (31).

**Oclusion assay.** Occlusion assays were performed as previously described (2). Briefly, treated whole blood was recalculated by the addition of Ca^{2+} and Mg^{2+} and then driven by a constant-pressure gradient through collagen-coated capillaries at an initial shear rate of 285 s⁻¹, with the time required for flow to cease recorded as the occlusion time.

**RESULTS**

Initial experiments were designed to characterize the effect of EPA and DHA on platelet function. Platelet-rich plasma from the same donor was treated for 4 h with vehicle or a combination of EPA and DHA to facilitate the incorporation of EPA and DHA into the platelet membrane (Fig. 1). Analysis of platelet fatty acid composition via gas chromatography demonstrated that the incubation of platelets with EPA and DHA in vitro increased EPA levels fivefold and DHA levels by 50% (Fig. 1B) and incorporation of EPA and DHA increased in a concentration-dependent manner (Fig. 1C). Analysis of lipid fractions via TLC showed that the alterations in lipid content were due solely to increases in EPA and DHA in platelet phospholipids, rather than in free fatty acids, which were not found in measurable quantities (data not shown). While each subject showed variability in the degree of change, EPA and DHA content increased in all subjects (n = 19), regardless of their vehicle-control levels (Fig. 1).

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Fig. 1. Alteration of platelet lipid content via exogenous addition of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A: methodology for treating platelet-rich plasma (PRP) with EPA and DHA. Each subject served as his/her own control, with half of the PRP being treated with fatty acids and the other half treated with the BSA-Tyrode vehicle. B: platelet membrane composition of EPA and DHA, expressed as percentage of total platelet fatty acids in vehicle-treated washed platelets and EPA/DHA-treated (FA) washed platelets. Data are shown as box-and-whisker plots; all subjects (n = 19) showed a significant increase in EPA and DHA after fatty acid incubation. C: platelets were incubated with vehicle or 10, 100, or 750 μM EPA and DHA for 4 h, and platelet composition of EPA and DHA as percentage of total platelet fatty acids was measured. Values are means ± SE.
Next, we aimed to determine whether platelet levels of EPA and DHA affected the activation of prothrombin. Washed platelets incubated with EPA and DHA showed a significant (~40%) reduction in the rate of prothrombin activation compared with platelets incubated with vehicle (Fig. 2A). Activation of prothrombin on the surface of activated platelets is dependent on the exposure of acidic phospholipids [i.e., phosphatidylserine (PS)] on the outer leaflet of the platelet membrane. We hypothesized that the reduction in prothrombin activation rates may be due to a reduction in PS exposure for platelets incubated with EPA and DHA. PS exposure was measured following labeling of collagen-activated platelets with FITC-annexin V. Our results show that FITC-annexin V labeling of platelets stimulated with 2.5 or 25 μg/ml collagen was reduced by 40% and 60%, respectively, by pretreatment of platelets with EPA and DHA (Fig. 2B). Moreover, we observed a concomitant reduction in the level of annexin V labeling of convulxin-stimulated platelets in response to increasing doses of EPA and DHA (Fig. 2C).

Platelet PS exposure is regulated by a series of receptor-mediated signaling events (1). Since platelet collagen receptors are found concentrated in lipid raft microdomains (20), we tested if increased EPA and DHA incorporation into platelet membranes disrupted lipid raft microdomains. Our results show that treatment of platelets with EPA and DHA reduced the degree of labeling by the raft-specific probe FITC-CTB (Fig. 3A). Quantification of the fluorescent signal of a large population of FITC-CTB-labeled platelets showed that the average FITC-CTB binding was significantly reduced after EPA and DHA treatment, suggesting an n-3 FA-mediated reduction in raft assembly (Fig. 3B).

We next designed experiments to determine whether treatment of platelets with EPA and DHA affected thrombus formation. Thrombi were formed by addition of Ca²⁺, prothrombin, and factor Xa to washed platelets in microplate wells. After only 15 min of platelet incubation with EPA and DHA, there was little difference in the extent or morphology of thrombus formation compared with vehicle-treated platelets (Fig. 4, top). In contrast, treatment of platelets with EPA and DHA for 4 h resulted in dispersed, small thrombi compared with the large intact thrombus formed by vehicle-treated platelets (Fig. 4, bottom).

The delayed rate of thrombin generation in EPA- and DHA-treated platelets may be explained by a disruption of normal prothrombin proteolytic conversion. EPA- and DHA-treated platelets were incubated with prothrombin, factor Xa, RGDS peptide (to prevent aggregation), and collagen and allowed to catalyze thrombin generation for varying lengths of time. Lysates were blotted with an antibody that recognizes a prothrombin epitope located in fragment 2. Our results showed an increased degree of fragment 2 (Fig. 5) at earlier time points in EPA- and DHA-treated platelets than in vehicle controls, suggesting that treatment of platelets with EPA/DHA affects kinetics of prothrombin cleavage.

We next designed experiments to characterize the ability of EPA and DHA to affect thrombus formation in whole blood.
Citrated whole blood was recalcified with Mg\(^{2+}\) and Ca\(^{2+}\) and then driven through a collagen-coated capillary tube by a constant-pressure gradient. The time to occlusion was measured. The time for an occlusive thrombus to form was nearly doubled following treatment of whole blood with EPA and DHA (Fig. 6). Despite the increased occlusion time, all n-3 FA-treated blood samples did occlude within 1 h (Fig. 6).

Experiments were designed to characterize platelet deposition and fibrin formation following perfusion of whole blood over collagen under constant shear. While no major differences were seen in the degree of platelet deposition or aggregation, the degree of fibrin formation was dramatically reduced in the EPA- and DHA-treated samples (Fig. 7A). To characterize the degree of fibrin formation under shear, rinsed capillary tubes were digested with plasmin, and the solubilized lysates were blotted for D-dimer. Our results show that treatment of whole blood with EPA and DHA dramatically reduced the level of fibrin relative to vehicle, as determined by Western blotting (Fig. 7B).

**DISCUSSION**

This study is the first to provide evidence that EPA and DHA regulate the procoagulant phenotype of platelets. Our results demonstrating that EPA and DHA reduced the ability of platelets to catalyze thrombin generation and form occlusive thrombi under shear complement previous work demonstrating that thrombin generation in platelet-rich plasma was reduced after fatty acid treatment (30) and that platelet PS exposure was reduced in subjects taking high doses of EPA and DHA (17). The ability of EPA and DHA to decrease the rate of platelet-mediated thrombin generation may represent another mechanism contributing to some of the cardioprotective effects of EPA and DHA. While other mechanisms certainly play a significant role in the cardioprotective effects of n-3 FAs [e.g., antiarrhythmic effects (18), improvements in myocardial efficiency (25, 27), and reductions in blood pressure (11)], the ability of n-3 FAs to reduce, but not abrogate, platelet-mediated thrombin generation supports the notion that n-3 FAs prevent occlusive thrombus formation in diseased blood vessels while minimally affecting hemostasis. Our ex vivo results presented in this study mirror the reduced thrombosis, with minimal bleeding side effects in populations taking high doses EPA and DHA (13, 22).

The in vitro incorporation of EPA and DHA into platelet membranes described here provides an experimental platform for the analysis of the physiological effects of EPA and DHA on platelet function. The change in lipid composition following incubation of platelets with EPA and DHA is comparable to that in our clinical studies with healthy subjects taking high doses of EPA and DHA over 4 wk (17). In those studies, EPA levels were increased sixfold (compared with 5-fold in the in vitro incorporation described here; Fig. 1B), and DHA levels were increased by ~75% (compared with 50% incorporation of DHA described here). While changes in the EPA and DHA compositions between the clinical studies and in vitro incorporation are similar, it is possible that the differences could affect the ability of n-3 FAs to alter thrombin generation in vivo, although we would hypothesize that the increased incorporation of EPA and DHA into platelet membranes reported for clinical samples might increase the antithrombotic effects of n-3 FAs. The in vitro incorporation of EPA and DHA into platelets has the potential drawback of an unknown degree of cellular metabolism. As such, the potential contribution of EPA- and DHA-derived metabolites in vitro, which have been shown to alter platelet function (4, 7), was not assessed in the current study. However, we found that the degree of in vitro incorporation of EPA and DHA in platelet membranes was maximized after 1 h of incubation (data not shown), suggesting that EPA and DHA may be incorporated long before any substantial metabolism has occurred.
Another potential drawback of the in vitro model system presented here is that arachidonate levels were not significantly altered by EPA and DHA in the current study (22.35% of total platelet fatty acid content in vehicle-treated platelets vs. 21.91% in EPA/DHA-treated platelets, $P = 0.57$), while arachidonate levels were significantly reduced in the in vivo studies (16, 17). While all these factors suggest that in vitro incorporation of EPA and DHA into platelet membranes is not a perfect recapitulation of in vivo incorporation, the fact that the procoagulant phenotype of platelets is significantly inhibited in vitro in the results presented here suggests that EPA and DHA are capable of eliciting an antiplatelet effect. Characterization of the ability of n-3 FAs to regulate the procoagulant phenotype in vivo warrants further study.

The 4-h incubation of EPA and DHA with platelets was utilized on the basis of research demonstrating that platelets can dramatically alter their plasma membranes in vitro within 4–6 h (28). However, this lengthy incubation time may affect platelet function. For instance, we found slightly increased levels of annexin V binding in platelets after 4 h in the absence of exogenous stimulation (data not shown). We chose the 4-h incubation time based on the fact that this time point yielded changes in EPA and DHA content similar to those observed in vivo. Similarly, the concentration of fatty acids used in this study was chosen because of its ability to provide a level of EPA and DHA membrane incorporation analogous to that observed in our previous clinical study (17), along with an inhibition of collagen-mediated annexin V binding comparable to that observed in our previous study (17). However, it is difficult to compare the in vitro concentration used in the present study with the concentration of EPA and DHA in plasma in subjects with increased dietary supplementation of n-3 FAs. The normal total plasma fatty acid concentration for all fatty acids is 450 μM in plasma, with a substantially smaller fraction of that total being EPA and DHA (21). However, the pharmacodynamics of EPA and DHA in plasma may allow for longer incorporation times in vivo than those observed in the in vitro method described here, which may affect the procoagulant phenotype of platelets in subjects consuming n-3 FAs.

Our previous work suggested that EPA and DHA inhibited platelet signaling (17); however, EPA and DHA only minimally affected platelet-mediated closure time, as measured by the platelet function analyzer (17). Therefore, it seems unlikely that the increased n-3 FA content in the platelet membrane was substantially altering the ability of platelets to adhere at sites of vascular injury or to mediate platelet aggregation. This trend is also observed in the present study, where we report that treatment of whole blood with EPA and DHA did not affect the degree of platelet adhesion or aggregation on collagen under physiologically relevant shear flow conditions (Fig. 7). Together, these studies suggest that antithrombotic properties of n-3 FAs may be due to the ability of EPA and DHA to inhibit the procoagulant function of platelets and, in particular, the inhibition of platelet-mediated thrombin generation. Growing evidence suggests that platelet membrane-coagulation factor binding events may be limiting in coagulation enzyme generation (14), and our research supports a role for EPA/DHA in inhibiting the platelet membrane’s ability to facilitate proco-
agulant enzyme complex formation and enzyme generation. We propose a model by which EPA and DHA incorporate into the platelet plasma membrane, disrupting lipid raft distribution and the assembly of prothrombinase complexes, leading to decreased rates of thrombin generation and thrombus formation.

The antithrombotic properties of n-3 FAs reported here suggest that this effect is due to the antiplatelet effects of EPA and DHA. We found that treatment of platelet-poor plasma with EPA and DHA did not affect the prothrombin or activated partial thromboplastin time (data not shown), suggesting that the effect of EPA and DHA is cell-mediated. However, in whole blood assays, the contribution of cellular populations other than platelets cannot be ruled out. For instance, EPA and DHA intake can alter red blood cell lipid profiles (16), and this alteration could influence coagulability of blood, perhaps by altering erythrocyte viscosity. Moreover, perhaps n-3 FAs incorporate into the membrane of leukocytes, which have been shown to support the binding of coagulation factors (15). Future studies will focus on characterizing the ability of n-3 FAs to regulate the function of red blood cells and leukocytes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


