Research article

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The synergistic effect of EPA and DHA with cyclooxygenase-1 inhibitors on platelet aggregation

Ourania Argyropoulou, Aikaterini N. Tsouka*, Despoina Pantazi*, Ioannis K. Koutsaliaris, Alexandros D. Tselepis

Atherothrombosis Research Centre, Department of Chemistry / Laboratory of Biochemistry, University of Ioannina, Ioannina, Greece
**Equal contribution*

ABSTRACT

Aim: Consumption of omega-3 fatty acids docosahexaenoic acid (DHA, 22:6ω-3) and eicosapentaenoic acid (EPA, 20:5ω-3) from a variety of foods and supplements could reduce cardiovascular events. The antiplatelet drugs aspirin (ASA) and triflusal, bind to platelet cyclooxygenase-1, thus preventing the biosynthesis of thromboxane A2 from arachidonic acid. ASA, triflusal, and their combinations with DHA or EPA were evaluated against *in vitro* platelet aggregation induced by the agonists AA, ADP, or TRAP-6.

Materials-Methods: Platelet Rich Plasma (PRP), isolated from blood of healthy volunteers was pre-incubated with acetylsalicylic acid, triflusal, EPA, DHA, or the drug-omega-3 combinations at various concentrations for 10min at 37ºC, prior to activation by AA, TRAP-6, and ADP (0.3mM, 10μM and 6μM, respectively). Platelet aggregation was determined by Light Transmittance Aggregometry.

Results: DHA significantly improves ASA and triflusal's inhibitory effect towards AA-induced platelet aggregation, while EPA enhanced only the antiplatelet activity of ASA. Moreover, only the combination of ASA with EPA exhibited enhanced inhibitory effect against platelet aggregation induced by ADP. Furthermore, the inhibition of ASA and triflusal on TRAP-6-induced platelet aggregation was enhanced when ASA or triflusal were combined with EPA.

Conclusions: Both DHA and EPA inhibit AA, ADP and TRAP-6-induced platelet aggregation in a dose dependent manner. The results of this study suggest a potentially beneficial use of EPA and DHA supplementation, in conjunction with the antiplatelet drugs ASA and triflusal.

KEY WORDS: *Aggregation, acetylsalicylic acid, aspirin, eicosapentaenoic acid, docosahexaenoic acid, triflusal*

Corresponding author:

Professor Alexandros D. Tselepis
Atherothrombosis Research Centre/Laboratory of Biochemistry, Department of Chemistry, University of Ioannina, Ioannina, GR-45110
Tel.: +30 26510 08365, Fax:+30 26510 08677
E-mail: atselep@uoi.gr

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) play a key role in reducing the risk of cardiovascular diseases (CVDs)[1]. PUFAs exhibit cardioprotective[2,3] and anti-inflammatory effects and have positive effects in neurological disorders[4,5]. More

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specifically, eicosapentaenoic acid (EPA, 20:5-3) and docosahexaenoic acid (DHA, 22:6-3), the most known omega-3 (ω-3) polyunsaturated fatty acids (n-3 PUFAs), are obtained from vegetables and their oils, from eggs, nuts and mainly from fatty fishes and their products, especially salmon, and tuna[6,7]. There is strong evidence from clinical studies that consumption of these fatty acids in pharmacological doses significantly reduce serum triglyceride levels and may exert a variety of anti-atherothrombotic effects[8]. Several clinical studies evaluated the effect of DHA and EPA through the years. The most recent studies, are the EVOLVE II (A Prospective Multicenter Trial to Assess the Safety and Effectiveness of the SYNERGY Everolimus-Eluting Platinum Chromium Coronary Stent System (SYNERGY Stent System) for the Treatment of Atherosclerotic Lesion(s)), the ASCEND (A Study of Cardiovascular Events iN Diabetes), the REDUCE-IT (Reduction of Cardiovascular Events with Icosapent Ethyl–Intervention Trial), the EVAPORATE (Effect of Icosapent Ethyl on Progression of Coronary Atherosclerosis in Patients With Elevated Triglycerides on Statin Therapy) and the STRENGTH (Outcomes Study to Assess STatin Residual Risk Reduction With EpaNova in HiGh CV Risk PatienTs With Hypertriglyceridemia) Clinical Trials. More specifically, the EVOLVE II study showed that 2g of PUFAs daily, reduce triglyceride and non-HDL cholesterol levels in patients with severe hypertriglyceridemia (SHTG) and benefit acute pancreatitis and CVD[9]. Patients with diabetes mellitus but without CVD showed no significant difference in serious vascular effects after ω-3 fatty acids administration, according to the ASCEND Study[10]. Moreover, the REDUCE-IT study showed that in patients with increased triglyceride levels receiving statins, the risk of CVD death was decreased in the group of patients treated with 2g of EPA twice daily as compared with the placebo group[11]. Furthermore, the EVAPORATE Study showed that the combination of EPA with statins slowed coronary plaque progression and regression over 18 months[12]. Finally, the STRENGTH Clinical trial showed that among patients at high CV risk, the addition of ω-3 fatty acids to usual background therapies did not reduce the composite endpoint of major adverse cardiac events[13].

Aspirin or acetylsalicylic acid (ASA), a COX-1 inhibitor, remains the primary therapeutic option for CVD prevention, including acute events[14–16]. ASA acts by blocking the production of thromboxane A2 (TXA2)[17,18]. Administration of ASA can lead to gastrointestinal toxicity[19,20]. Another antiplatelet drug very similar to ASA is triflusal. Triflusal inhibits irreversibly the COX-1 activity but to a lesser extent compared with ASA[21–23]. Due to these characteristics, triflusal exerts a comparable efficacy but with a safer profile compared to ASA[24,25]. Several clinical trials have designed to highlight triflusal as a promising alternative drug versus ASA[14–16,26].

The aim of our study was to investigate the possible synergistic effect of EPA or DHA on ASA- and triflusal-induce inhibition of platelet aggregation induced by arachidonic acid (AA), adenosine diphosphate (ADP) and thrombin receptor TRAP-6 *in vitro*. To examine the possible synergistic effect, fatty acids and antiplatelet drugs were used in concentrations that induce a minimum inhibition of platelet aggregation.

MATERIALS AND METHODS

Materials and Reagents

Docosahexaenoic acid (DHA) was purchased from Biosynth Carbosynth, Carbosynth Ltd United Kingdom and Biosynth AG Switzerland) and Eicosapentaenoic acid (EPA) was kindly provide by Libytec Pharmaceutical S.A. The antiplatelet drugs Acetylsalicylate lysine and Triflusal were obtained from Egicalm, Galinos Company, Greece and Galenica SA, Greece, respectively. Arachidonic Acid (A.A.) and Adenosine Diphosphate (ADP) were purchased from Sigma-Aldrich, St. Louis, MO, USA, and the Thrombin Receptor Activating Peptide-6 (TRAP-6) was purchased from Bachem, Bubendorf, Switzerland.

Platelet aggregation studies

The antiplatelet activity of the fatty acids and antiplatelet drugs was studied in platelet-rich plasma (PRP) by the Light transmittance aggregometry (LTA) assay, as we previously described[27–29]. Briefly, PRP was prepared from citrated blood samples withdrawn from peripheral venous blood of apparently healthy volunteers and the platelet number was adjusted to 250×10^6/mL with homologous platelet-poor plasma (PPP). The platelet aggregatory response to A.A. (300 μM), ADP (6 μM) or TRAP-6 (10 μM) was studied in aliquots of 0.5 ml PRP at 37 °C under continuous stirring at 1,200 rpm, in a Chronolog Lumi-Aggregometer (model 700 4-channel) equipped with the AggroLink software package. In some experiments, the PRP was pre-incubated for 10 min at 37°C with DHA or EPA, dissolved in Dimethyl sulfoxide (DMSO) or the antiplatelet drugs ASA or triflusal dissolved in sterile water or ethanol (EtOH), respectively, or their combination, before the initiation of the aggregation. In these experiments, the final concentration of EtOH and DMSO was 0.4 % (v/v). The inhibitory efficacy of PUFAs was expressed as IC50 values, in μM (the concentration that induces 50% inhibition of maximum platelet aggregation). In control experiments, PRP was incubated in the aggregometer with DHA or EPA for 5 min at 37 °C under continuous stirring to evaluate whether these PUFAs are platelet activators themselves. All aggregation assays were conducted within 3h after blood venipuncture.

Statistics

Values were normally distributed as was checked by the Shapiro-Wilk test. For statistical comparisons we used F test and post hoc analysis was performed using the Bonferroni adjustment to correct for multiple comparisons. A p-value of <0.05 was considered significant.

RESULTS

Effect of DHA and EPA on Platelet Aggregation induced by various agonists

The effect of DHA and EPA on platelet aggregation induced by AA, ADP and TRAP-6 is shown in Figure 1 (A, B, C, respectively). Both DHA and EPA significantly inhibited A.A.-induced platelet aggregation in a dose-dependent manner exhibiting IC50 values of 161.8 and 144.6 μM, respectively. The minimum significant inhibitory effect of 20.81±7.56% and 22.52±3.46% by DHA and EPA, respectively, was observed at the concentration of 125 μM. Platelet aggregation induced by ADP was inhibited by 20 % at the DHA concentration of 125 μM, an effect that was not significantly increased even at the concentration of 1000μM (28% inhibition). EPA at the concentration of 125 μM inhibited ADP-induced platelet aggregation by 20%, exhibiting a similar potency with DHA, however at concentrations of 500μM or higher, it inhibited platelet aggregation by 55%. Finally, both DHA and EPA were potent inhibitors of TRAP-6-induced platelet aggregation exhibiting IC50 values of 89.0 and 94.0 μM, respectively whereas a 20 % inhibition was observed at 75 μM of DHA or EPA (Fig.1).

Estimation of the aspirin and triflusal concentration that express a minimum antiplatelet effect

To define the synergistic effect of PUFAs with aspirin or triflusal, we estimated the concentration of these antiplatelet drugs that cause an up to 30% inhibition of platelet aggregation induced by the three agonists. ASA, at a concentration of 25 μM inhibited by 20% platelet aggregation induced by A.A., ADP, or TRAP-6 (Fig.2). Moreover, triflusal at a concentration of 400μM exhibited a 20% inhibition of platelet aggregation induced by A.A., whereas a 20% inhibition of platelet aggregation induced by ADP or TRAP-6 was observed at a triflusal concentration of 500μM (Fig. 2).

Effect of the combination of antiplatelet drugs with DHA or EPA on human platelet aggregation induced by A.A.

The combination of ASA (25 μM) with DHA or EPA inhibited platelet aggregation by more than 93%, p<0.0001 compared to the inhibition induced by ASA, DHA, or EPA alone (Fig. 2). In addition, the combination of triflusal with DHA induced an 87.6±10.78% inhibition of platelet aggregation, while the triflusal and EPA combination a 20.1±3.46% inhibition of platelet aggregation, p<0.0001 compared to triflusal, DHA, or EPA alone (Fig. 2).

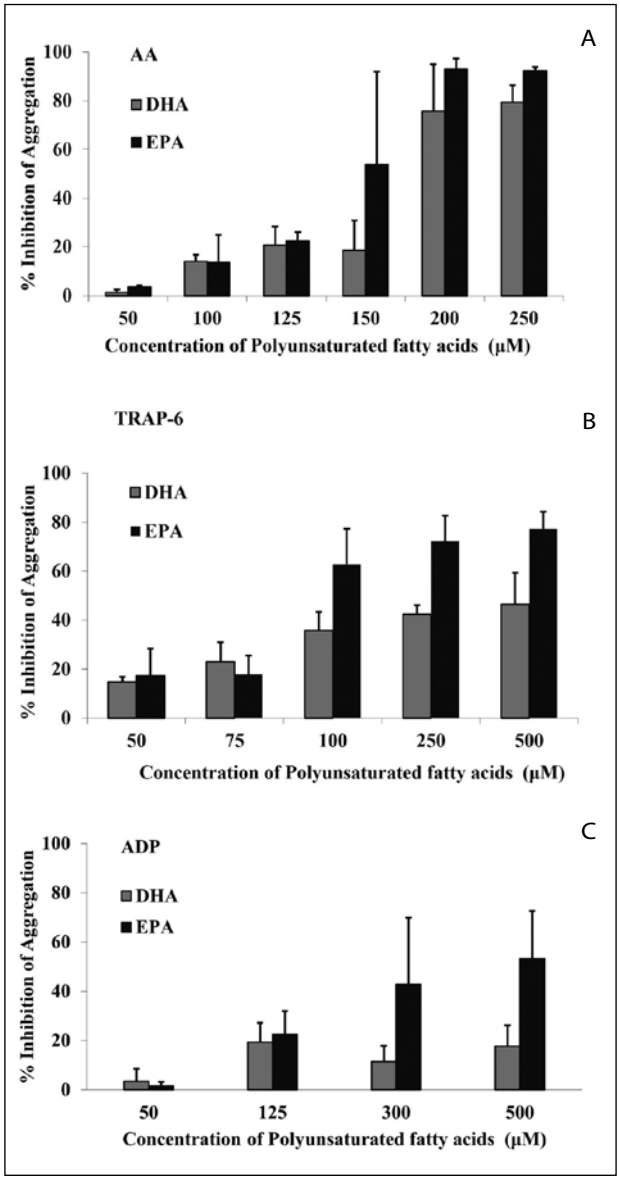

FIGURE 1. (A) Dose-dependent effect of EPA and DHA on platelet aggregation induced by AA (300μM). (B) Dose-dependent effect of EPA and DHA on platelet aggregation induced by ADP (6μM). (C) Dose-dependent effect of EPA and DHA on platelet aggregation induced by TRAP-6 (10μM).

Effect of the combination of antiplatelet drugs with DHA or EPA on human platelet aggregation induced by ADP.

The combination of ASA with DHA induced a 48.30±11.63 % inhibition of platelet aggregation induced by ADP, showing

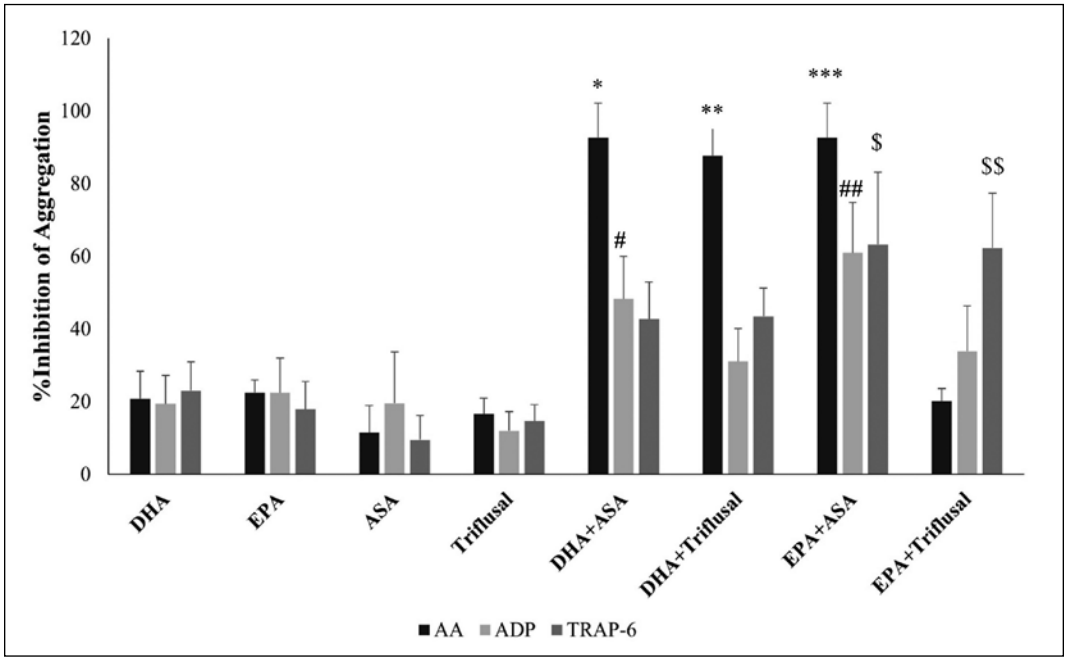

FIGURE 2. The effect of DHA (125μM) and EPA (125μM) in combination with Triflusal (400μM) or ASA (25μM) on platelet aggregation induced by AA, $^{*}p<0.001$ compared to DHA or ASA, $^{**}p<0.001$ compared to DHA or triflusal and $^{***}p<0.001$ compared to EPA or ASA. The effect of DHA (125μM) and EPA (125μM) in combination with Triflusal (500μM) and ASA (25μM) at platelet aggregation induced by ADP, $^{\#}p<0.01$ compared to DHA or ASA and $^{\#\#}p<0.001$ compared to EPA or ASA. The effect of DHA (75μM) and EPA (75μM) in combination with Triflusal (500μM) and ASA (25μM) at platelet aggregation induced by TRAP-6, $^{\$}p<0.008$ compared to ASA or EPA. Values represent the mean ± SD from at least 3 different experiments.

that this combination exhibits a cumulative effect against platelet aggregation induced by ADP. In addition, the combination of ASA with EPA caused an inhibitory effect of 60.93±13.48% against platelet aggregation induced by ADP, improving the inhibitory effect of ASA. Moreover, DHA and triflusal exhibited a 31.07±8.98% inhibition of platelet aggregation induced by ADP as well as EPA and triflusal inhibited by 33.89±12.47% the platelet aggregation induced by ADP, showing that both fatty acids did not improve the inhibitory effect of triflusal regarding the platelet aggregation induced by ADP (Fig. 2).

Effect of the combination of antiplatelet drugs with DHA or EPA on human platelet aggregation induced by TRAP-6

The combination of ASA with DHA induced a 42.74±10.21% inhibition of the platelet aggregation induced by TRAP-6 showing that this combination exhibits a cumulative effect against platelet aggregation induced by TRAP-6. In addition, the combination of ASA with EPA induced a 63.21±19.89% inhibition of platelet aggregation induced by TRAP-6. Furthermore, the combination of triflusal with DHA exhibited a 43.42±7.80% inhibitory effect towards platelet aggregation induced by TRAP-6 showing a cumulative effect, whereas triflusal with EPA exhibited an inhibitory effect of 62.24±15.17% towards platelet aggregation induced by TRAP-6 showing a synergistic effect. (Fig. 2).

DISCUSSION

Both EPA and DHA show antiplatelet activity against A.A., ADP and TRAP-6-induced platelet aggregation, while EPA is the more potent of the two. The results of this study show a potentially beneficial use of antiplatelet drugs ASA and triflusal in conjunction with EPA and DHA supplementation. A number of *in vitro* and *in vivo* studies in mouse models support the idea that omega-3 fatty acids can enhance the activity of other platelet inhibitors.[30,31]

The combinations of EPA and DHA with ASA yield an enhanced inhibitory activity against platelet activation through the A.A. pathway, that indicate a possible synergistic effect. Long chain omega-3 fatty acids antagonize A.A. as the substrate to produce several eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes.[32–35] Inhibition of TxA2 synthesis results in TxA3 production, that exhibits weak pro-aggregatory properties.[35–37]

Furthermore, the combination of EPA and ASA yields an enhanced antiplatelet effect against ADP induced platelet activation. E-resolvins (RvE1), the specialized pro-resolving mediators (SPMs) that occur after the metabolism of EPA, selectively engage with ADP activated platelets, indicating a possible mechanism of antiplatelet activity.[38,39] Similarly, the DHA and triflusal combination

resulted in an enhanced inhibitory effect toward platelet activation induced by ADP. Furthermore, EPA potentiates the inhibitory effect of ASA and triflusal toward TRAP-6 dependent platelet aggregation.

The underlying mechanisms regarding the pleiotropic activity and the possible beneficial effects of EPA and DHA are currently under investigation by our laboratory team. According to our knowledge, this is the first *in vitro* study of a possible triple antiplatelet effect of ASA and triflusal with DHA or EPA.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ΠΕΡΙΛΗΨΗ

Η συνεργατική επίδραση του EPA και του DHA με αναστολείς της κυκλοοξυγενάσης-1 στη συσσώρευση των αιμοπεταλίων

Ουρανία Αργυροπούλου, Αικατερίνη Ν. Τσούκα*, Δέσποινα Πανταζή*, Ιωάννης Κ. Κουτσαλιάρης, Αλέξανδρος Δ. Τσελέπης

Ερευνητικό Κέντρο Αθηροθρόμβωσης/Εργαστήριο Βιοχημείας, Τμήμα Χημείας, Πανεπιστήμιο Ιωαννίνων, Ιωάννινα, Ελλάδα
**Ίση συνεισφορά*

Σκοπός: Η κατανάλωση των ωμέγα-3 λιπαρών οξέων εικοσιδυοεξαενοϊκό οξύ (DHA, 22:6ω-3) και εικοσιπενταενοϊκό οξύ (EPA, 20:5ω-3), από μια ποικιλία τροφίμων και συμπληρωμάτων διατροφής θα μπορούσε να μειώσει τα καρδιαγγειακά συμβάντα. Τα αντιαιμοπεταλιακά φάρμακα ασπιρίνη (ASA) και τριφλουζάλη, δεσμεύονται στην κυκλοοξυγενάση-1 των αιμοπεταλίων, εμποδίζοντας τη βιοσύνθεση του θρομβοξανίου Α2 από αραχιδονικό οξύ. Η ASA, η τριφλουζάλη και οι συνδυασμοί τους με DHA ή EPA μελετήθηκαν στην επαγόμενη από Α.Α, ADP ή TRAP-6 αιμοπεταλιακή συσσώρευση.

Υλικά-Μέθοδοι: Πλάσμα πλούσιο σε αιμοπετάλια απομονώθηκε από αίμα υγιών εθελοντών και προεπωάστηκε με ακετυλοσαλικυλικό οξύ, τριφλουζάλη, DHA, EPA ή τους συνδυασμούς φαρμάκων-ωμέγα 3 οξέων σε διάφορες συγκεντρώσεις για 10min, 37°C, πριν την ενεργοποίηση με Α.Α., TRAP-6 ή ADP (0.3mM, 10μM and 6μM, αντίστοιχα). Η αιμοπεταλιακή συσσώρευση προσδιορίστηκε με συσσωρευομετρία οπτικής διαπερατότητας.

Αποτελέσματα: Το DHA βελτιώνει σημαντικά την ανασταλτική δράση της ASA και της τριφλουζάλης έναντι της προκαλούμενης από Α.Α. αιμοπεταλιακής συσσώρευσης, ενώ το EPA ενίσχυσε μόνο την αντιαιμοπεταλιακή δράση της ASA. Επιπλέον, μόνο ο συνδυασμός της ASA με EPA παρουσίασε ενισχυμένη ανασταλτική δράση έναντι της αιμοπεταλιακής συσσώρευσης που επάγει το ADP. Επιπρόσθετα, η αναστολή της ASA και της τριφλουζάλης στην επαγόμενη από TRAP-6 αιμοπεταλιακή συσσώρευση ενισχύθηκε όταν η ASA και η τριφλουζάλη συνδυάστηκαν με EPA.

Συμπεράσματα: Αμφότερα τα DHA και EPA αναστέλλουν δοσοεξαρτώμενα την επαγόμενη από Α.Α., ADP και TRAP-6 αιμοπεταλιακή ενεργοποίηση. Τα αποτελέσματα αυτής της μελέτης υποδεικνύουν μια πιθανή ευεργετική χρήση των EPA και DHA, σε συνδυασμό με τα αντιαιμοπεταλιακά φάρμακα ASA και τριφλουζάλη.

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: *Ακετυλοσαλικυλική λυσίνη, ασπιρίνη, εικοσιδυοεξαενοϊκό οξύ, εικοσιπενταενοϊκό οξύ, συσσώρευση, τριφλουζάλη*

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