Review

Docosahexaenoic acid (DHA) and human platelet reactivity

Douglas C. Gaudette and Bruce J. Holub

Department of Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

Introduction

Epidemiological evidence suggests an inverse relationship between the consumption of marine foods rich in omega-3 fatty acids and the incidence of cardiovascular disease.[1-4] Numerous studies have shown a dampening of platelet function following the ingestion of fish or fish oils (for review see References 5,6). Despite the fact that fish/fish oils contain both eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids (EPA and DHA), relatively few studies have attempted to discriminate between the effects of these two fatty acids on platelet reactivity. This article will review the evidence to date using human platelets which indicates that DHA, in addition to the more well-known effects of EPA, may contribute to the dampened platelet reactivity observed following fish/fish oil feeding. The metabolism and possible mechanisms of action of this fatty acid in human platelets will also be discussed. The structure for DHA is given in *Figure 1* (shorthand version).

DHA and platelet aggregation

Various approaches have been used to study the effect of DHA on platelet aggregation: short-term incubation with exogenous DHA; in vitro enrichment of platelet phospholipids by longer incubations with DHA; and ex vivo platelet aggregation following oral administration of purified DHA.

Short-term incubations with DHA

Rao et al.[7] were the first to report inhibition of platelet aggregation by exogenous DHA. In platelet rich plasma (PRP), 150 μM DHA completely inhibited platelet aggregation induced by 450 μM arachidonic acid (20:4n-6, AA), as well as the second wave of aggregation induced by epinephrine and adenosine diphosphate (ADP). Using similar conditions, Srivastava[8] also reported a dose-dependent inhibition of collagen-, epinephrine-, and AA-induced aggregation by exogenous DHA. In washed platelet suspensions, DHA has been shown to inhibit platelet aggregation induced by the endoperoxide analogue (U46619),[9,10] AA,[9] and to a limited extent by low dose thrombin (0.05 U/ml).[9] DHA was without effect when a higher dose of thrombin (0.25 U/ml) was used.[9] In the studies using washed platelet suspensions,[9,10] DHA was considerably more potent than EPA at inhibiting platelet reactivity.

In contrast to these results, DHA was reported to be much less potent than either EPA or dihomo-γ-linolenic acid (20:3n-6) (DGLA) at inhibiting ADP (3.75 μM) induced aggregation in PRP.[11] A potential problem with this study was that free fatty acids (FFA), in toluene, were added to aggregometer cuvettes, and the toluene evaporated under nitrogen prior to the addition of PRP. Since the PRP was not subsequently analyzed for FFA content, it is impossible to determine the actual concentration of DHA to which the platelets were exposed. An interesting observation of this study, however, was that the concentration of DHA required for inhibition of mitogen release from stimulated platelets was considerably lower than that required for inhibition of aggregation. Platelet mitogens are believed to play a major role in the progression of atherosclerotic disease.[12]

Recently O'Keefe and colleagues[13] have determined that a *trans* isomer of DHA (22:6Δ19†) was equally effective as the all-*cis* form at inhibiting AA-induced aggregation. Although trans-19 bond DHA (22:6Δ19†) has been indentified in the liver lipids of rats fed heated linseed oil,[14] and *trans* linolenic acid (a potential precursor of 22:6Δ19†) has been found in oils used commercially,[15] it is not presently known whether this isomer of DHA exists in human tissues or plasma at significant levels.

Although the above results have demonstrated that FFA-DHA is indeed antiaggregatory, a potential problem exists as to the physiological relevance of these findings. Since DHA is poorly released from platelet phospholipids upon agonist stimulation[16,17] (as will be

Address reprint requests to Dr. Bruce J. Holub at the Department of Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada, N1G 2W1.
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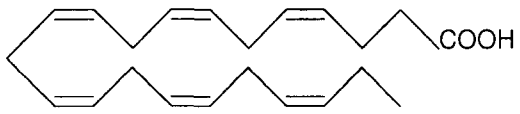

DHA, Docosahexaenoic Acid, 22:6n-3

Figure 1 Structure (simplified) of docosahexaenoic acid (22:6n-3)

discussed shortly), we reasoned that plasma may be an important source of free DHA to which platelets are exposed. To test the hypothesis that plasma DHA may contribute to the dampened platelet aggregation observed following fish/fish oil feeding, we designed an in vitro system which attempted to simulate the in vivo situation in regard to plasma FFA-DHA.[18] Using this protocol, 20 μM DHA, a concentration potentially attainable in vivo,[19,20] significantly inhibited platelet aggregation induced by a sub-maximal dose of collagen. EPA at the same concentration, which is higher than would be expected in plasma FFA in vivo, was much less potent. Interestingly, 20 μM AA and DGLA, which have been shown to stimulate[21] or inhibit[11] platelet aggregation, respectively, under different incubation conditions, had no effect on collagen induced aggregation in this study.

Platelet aggregation following in vitro incorporation of DHA

With short-term incubation (as in the above section), little esterification of fatty acid into platelet phospholipids occur.[22] In order to study the effect of phospholipid esterified with DHA on platelet aggregation in vitro, a longer period of incubation (e.g., 2 hrs) followed by washing of the platelet suspension is required. Using this approach, Croset and Lagarde[17] reported that the dosage of thrombin, collagen, and U46619 required for 50% aggregation was significantly higher when platelets were pre-enriched with DHA. In all cases, the effect of DHA enrichment was greater than the corresponding effect of EPA. DHA was also more effective alone than when platelets were simultaneously enriched with EPA.

The in vitro approach has the advantage of speed and prevents the potential problem of retroconversion of DHA to EPA as occurs in vivo.[23,24] It is uncertain, however, whether DHA is esterified to the same pool of platelet phospholipids during in vitro incubation as compared to when it is orally administered.

Platelet aggregation following oral administration of purified DHA

Very few studies, as compared to the large number of fish/fish oil feeding trials,[6] have examined platelet aggregation following oral administration of purified DHA. Von Schacky and Weber[23] reported a significant inhibition of both collagen (0.50 μg/ml) and ADP (1 μM) induced aggregation following the ingestion of 6 g of DHA for 6 days. At the same dosage, EPA was less effective. Hirai and colleagues,[24,25] on the other hand, have found DHA to be less effective than EPA at dampening platelet function when consumed in the same amount (3.6 g for 24 days). While EPA intake significantly inhibited collagen- (0.50 and 1.0 μg/ml), ADP- (2.0 and 3.0 μM), and epinephrine- (0.5 and 1.0 μg/ml) induced aggregation, the effect of DHA ingestion was only significant in the case of collagen.[24] Similar results were obtained by this group when hyperlipidemic (type IIa and IIb) subjects were studied.[25] Although the reason for this discrepancy is not known, it could be due to differences in the populations studied (Western vs Japanese), the duration, or dosage of DHA used. In both studies, platelet aggregation was performed in PRP. Since plasma from fasting Japanese subjects has been reported to contain approximately 3 times the level of DHA in the non-esterified fraction[20] than corresponding samples from Western subjects,[16] a less dramatic change in this pool may have occurred following DHA supplementation.

Metabolism and mechanisms of DHA action

Incorporation and release of DHA from platelet phospholipids. In vitro incorporation of DHA into platelet phospholipids has been extensively studied by Lagarde and colleagues and summarized recently.[26] Incubation of washed platelets with albumin-bound DHA resulted in the incorporation of approximately 10 nmol/10^9 platelets, the majority of which (85%) was found in the phospholipid fraction.[17] Simultaneous incubation of DHA with EPA significantly reduced the amount of DHA incorporated (5 nmol/10^9 platelets). This observation may explain the reduced efficacy of DHA + EPA, as compared to DHA alone, on the inhibition of platelet aggregation mentioned above.[24] Under similar conditions, linoleic acid (18:2n-6, LA) and AA, at physiologic molar ratios (fatty acid/albumin), also reduced the incorporation of DHA.[27] The percentage of DHA incorporated into the phospholipid fraction was not altered to a significant extent by LA or AA.[27] Among individual phospholipids, the bulk of DHA was observed in phosphatidylcholine (PC) (64%).[17] Simultaneous incubation with EPA, LA, or AA shifted the balance of DHA incorporation; proportionately less being found in PC, but more in phosphatidylethanolamine (PE) (13.5, 22.8, 22.2, and 26.2% for DHA alone or in the presence of EPA, LA, or AA, respectively).[17,27]

In contrast to the aforementioned findings, Fischer et al.[16] have observed a slightly greater in vitro incorporation of DHA into PE than into PC (45.2 and 37.0%, respectively). This discrepancy may be due to the use of PRP as the incubation media in the latter study. In agreement with Fisher et al., oral administration of DHA results in approximately equal incorporation into PC and PE.[24] Although labeled DHA is incorporated into phosphatidylinositol (PI) in vitro (about 7% of phospholipid DHA)[16,17] this has not been observed in vivo following purified DHA ingestion.[23,24] A small amount of DHA incorporation into PI has been observed following fish oil administration.[28] Although

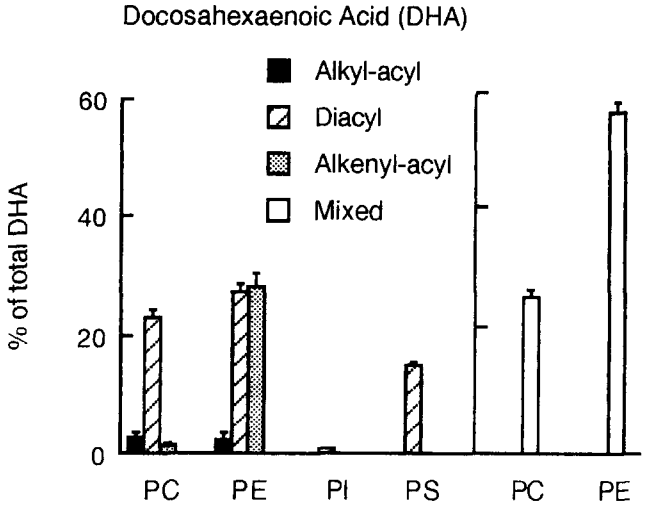

Figure 2 The percentage contributions of the individual subclasses (alkyl-acyl, diacyl, alkenyl-acyl) of the various platelet phospholipids to the total mass of DHA esterified to platelet phospholipid. The subjects consumed a fish oil concentrate (MaxEPA) providing 3.6 g EPA plus 2.4 g DHA per day for 3 weeks.[31] Values are means ± SE (n = 6).

this may indicate significant differences between in vivo and in vitro incorporation of DHA, it could also reflect the fact that the in vitro data are based on levels of radioactivity. As pointed out by Weiner and Sprecher,[29] changes in radioactivity in platelet phospholipids do not reflect corresponding changes in mass, particularly in the case of PI which has a much shorter half life.

Recently, it has been shown that alkenylacyl PE (plasmalogen) is disproportionately enriched in both DHA and EPA following fish oil feeding.[30] In such subjects, the alkenylacyl PE, diacyl PE, and diacyl PC represented 28.1, 27.5, and 23.0%, respectively, of the total mass of DHA-containing phospholipid[31] as seen in *Figure 2*. At the present time, it is not known if such a distribution also occurs when DHA alone is ingested.

In addition to increased levels of DHA, increased levels of EPA[23,24] and docosapentaenoic acid (22:5n-3)[24] have also been reported in platelet PC and PE following oral DHA administration. This reflects the capacity of human tissues to retroconvert DHA to EPA. In contrast to fish oil[5] or EPA administration,[24] DHA ingestion does not appear to decrease the content of AA in platelet phospholipids.[23,24] This is of interest since reduced substrate (AA) for platelet cyclooxygenase has been suggested as a contributing mechanism to explain the reduced thromboxane A_2 (TxA_2) formation following fish oil feeding.[28]

Incorporation of DHA into phospholipids could lead to dampened platelet reactivity in at least two ways: agonist induced release of DHA from platelet phospholipids leading to interference of AA metabolism and/or production of DHA metabolites (see below); alteration of platelet membrane properties and hence enzyme activities due to DHA incorporation itself. Since, in contrast to AA and EPA, there appears to be no significant release of DHA following agonist stimulation,[16,17] the latter seems to be the more likely possibility. Recently, Lagarde et al.[32] have reported that although DHA is not released per se, agonist stimulation of platelets enriched with DHA in vitro results in a substantial transacylation from PC into PE. The significance of this observation in terms of platelet reactivity is not known presently.

Decreased TxA_2 formation. Decreased formation of thromboxane A_2 (TxA_2) has been suggested as a major mechanism in the reduced platelet reactivity following fish oil supplementation of the diet.[33,34] DHA in free fatty acid form has been demonstrated to be a potent competitive inhibitor of AA metabolism by sheep vesicular gland prostaglandin synthetase.[35] In platelets exposed to [^{14}C]-AA, exogenous DHA has been reported to inhibit TxA_2 formation in a dose-dependent manner.[7,8] Under more physiological conditions in terms of plasma non-esterified DHA, we have observed a decreased formation of 12-hydroxy-heptadecatrienoic acid (HHT), an indicator of TxA_2 formation in collagen stimulated platelets.[18] Interestingly, doubling the concentration of albumin bound DHA (40 μM), which produced a greater inhibition of platelet aggregation, had less effect on HHT formation. This suggested that at the higher concentration of DHA used, mechanisms in addition to inhibition of TxA_2 formation must be operating. One possibility which requires further investigation was the observation of a small but significant increase in the level of [^{3}H]-PGD_2 in the presence of 40 μM DHA. PGD_2, through activation of adenylate cyclase, is a known inhibitor of platelet aggregation.[36]

In vitro incorporation of DHA into platelet phospholipids has also been shown to reduce TxA_2 generation.[37] Since DHA was not released to any appreciable extent upon agonist stimulation, it was concluded that altered membrane properties, as a result of DHA incorporation, reduced the release or cyclooxygenation of AA. Although it has subsequently been concluded by these same authors[26] that phospholipase mediated release of AA was probably not affected by DHA incorporation into platelet phospholipids, this hypothesis has not been directly tested to date.

In contrast to the in vitro studies, oral administration of DHA has not been shown to reduce TxA_2 formation.[23,24] It should be noted, however, that in both of these studies, TxA_2 formation was not assessed under conditions in which decreased platelet aggregation was observed. Assessment of TxA_2 in the serum of clotted whole blood[23] or in washed platelet suspensions exposed to high concentrations of thrombin (1 U) or collagen (50 μg/ml)[24] may not have been sensitive enough to detect small changes in TxA_2 formation, which may have occurred when platelets were exposed to low doses of ADP or collagen. Further research is therefore required to determine if a decreased formation of TxA_2 contributes to the dampened platelet reactivity observed following purified DHA ingestion.

Formation of 11- and 14-HDHE. Aveldano and Sprecher[38] were the first to characterize the major platelet lipoxygenase products of DHA as 11- and 14-hydroxydocosahexaenoic acid (11- and 14-HDHE). The formation of these products from exogenous DHA has been confirmed by others[16,37] and has been shown to be somewhat enhanced by simultaneous incubation with AA.[37] In vitro 14-HDHE has been shown to be a potent inhibitor of U46619-induced platelet aggregation ($IC_{50}=0.45$ μM).[39] This inhibition was attributed to antagonism at the platelet TxA_2/endoperoxide (TX/EP) receptor although binding studies with [^{3}H]-U46619 were not conducted.

Although 14-HDHE formation may explain partly the decreased platelet aggregation observed in the presence of high levels of exogenous DHA in vitro, it is uncertain whether this mechanism is operative under more physiological conditions. Only trace amounts of hydroxylated DHA metabolites have been observed following stimulation of in vitro DHA-enriched platelets.[16,37] Similar results were obtained following oral administration of DHA[24] or cod liver oil.[16] Unfortunately, these latter analyses were performed in washed platelet suspensions, thereby neglecting the potential contribution of plasma albumin-bound DHA. Quantification of the total amount of 14-HDHE produced from both endogenous (platelet) and exogenous (plasma) sources of DHA, following DHA or fish oil feeding, would be very useful in determining the physiological relevance of this metabolite on platelet function.

Antagonism of TX/EP receptor. In addition to its hydroxylated metabolite 14-HDHE, DHA itself has been suggested to act as a TX/EP receptor antagonist.[9,10] In washed platelet suspensions, the IC_{50} value for inhibition of U46619-induced aggregation was very similar to the IC_{50} value for inhibition of specific binding of [^{3}H]-U46619 (2.2 ± 0.6 μM and 1.5 ± 0.22 μM, respectively).[10] To put these results into perspective, however, it should be noted that as little as 0.5 μM AA will cause substantial platelet aggregation under similar experimental conditions.[40] As well, although plasma levels of DHA may be considerably higher than those used in this study, it is predominantly bound to albumin.[41] Further research is necessary to determine if this is a physiologically relevant mechanism of DHA action.

Recently, Lagarde and colleagues[26] have reported that in vitro enrichment of platelet phospholipids with DHA can also decrease [^{3}H]-U46619 binding to platelet TX/EP receptors. It was suggested that this effect was due to a functional alteration of the TX/EP receptor site as a result of DHA incorporation into membrane phospholipids. It would be of interest to determine if this same effect occurs following oral administration of DHA.

DHA and platelet signal transduction. Apart from studies on eicosanoid formation and TX/EP receptor function, little attention has been paid to the effect of DHA on other aspects of platelet signal transduction. This may prove to be a fruitful area of research given that the presently postulated mechanisms of DHA action may not totally explain the effect of this fatty acid on platelet function.

We have studied the effect of exogenous albumin-bound DHA on phosphoinositide metabolism following collagen stimulation. In [^{3}H]-glycerol prelabelled platelets, we observed a moderate decrease in [^{3}H]-phosphatidic acid formation, an indicator of phospholipase C activity, in the presence of 20 μM albumin-bound DHA.[18] This effect, however, was also observed when other fatty acids which did not significantly affect platelet aggregation were tested.

In subsequent studies using [^{3}H]-inositol prelabelled platelets, collagen-induced formation of [^{3}H]-phosphatidylinositol 4-monophosphate (PIP) and [^{3}H]-phosphatidylinositol 4,5-bisphosphate (PIP_2) were significantly inhibited in the presence of exogenous albumin-bound DHA.[42] At the same concentration, EPA was without effect on this parameter. DHA incubation did not inhibit U46619 and phorbol ester-induced increases in [^{3}H]-PIP and [^{3}H]-PIP_2. These findings suggested that the effect of DHA likely occurs prior to protein kinase C activation and TX/EP receptor occupancy. Further research is required to determine the exact mechanism of this effect of DHA as well as to what extent reduced TxA_2 formation could be responsible. Further research is also required to determine the biochemical and functional significance of collagen-induced increases in [^{3}H]PIP and [^{3}H]PIP_2, although, in addition to supplying substrate for second messenger generation (i.e., inositol trisphosphate and diacylglycerol), PIP and PIP_2 have been suggested to play a role in the regulation of calcium mobilization[43] and actin polymerization,[44] independent of their hydrolysis by phospholipase C.

Conclusions and possible research directions

DHA appears to contribute significantly to the inhibition of platelet aggregation which has been observed following fish oil feeding. At the present time, major gaps exist in our understanding of how dietary DHA affects platelet functioning. Some of the critical questions which could be addressed in the future include: following oral ingestion of DHA, to what extent does exogenous (plasma) as opposed to endogenous (platelet) DHA contribute to the reduced platelet aggregation? Can the plasma non-esterified pool of DHA be metabolized into a physiologically relevant concentration of 14-HDHE? If so, does 14-HDHE have other sites of action besides the TX/EP receptor? Are non-platelet derived metabolites (other cells, hepatic) of DHA of importance? Does in vivo DHA incorporation into platelet phospholipids affect [^{3}H]-U46619 binding to TX/EP receptor sites? To what extent does dietary DHA reduce TxA_2 formation in platelets stimulated with various agonists? If dietary DHA reduces TxA_2 synthesis, to what extent do endogenous (platelet) and exogenous (plasma) pools of DHA contribute to this

effect? If TxA_2 formation in stimulated platelets is decreased following DHA ingestion, to what extent can this explain the decreased platelet aggregation? Does dietary DHA or its metabolites affect other aspects of platelet signal transduction? Answers to these and other questions hopefully will lead to a greater understanding of the mechanisms involved in the dampened platelet reactivity observed following DHA ingestion.

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