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Arachidonic Acid Enhances the Tissue Factor Expression of Mononuclear Cells by the Cyclo-Oxygenase-1 Pathway: Beneficial Effect of n-3 Fatty Acids

Yves Cadroy, Dominique Dupouy, and Bernard Boneu

Monocytes express tissue factor (TF) upon stimulation by inflammatory agents. Dietary administration of fish oil rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) results in an impairment of TF expression by monocytes. EPA and DHA are metabolized differently from arachidonic acid (AA), the major fatty acid present in cell membranes. We examined the effects of AA on the TF expression of isolated human PBMC, and we determined whether EPA and DHA modulated this phenomenon differently. Nonstimulated PBMC had a low TF-dependent procoagulant activity. When PBMC were incubated with increasing concentrations of AA, the TF-dependent procoagulant activity increased in a dose-dependent manner to 190% at 7.5 μM. Indomethacin, a cyclo-oxygenase inhibitor, totally abolished the stimulating effect of AA, whereas specific pharmacologic inhibitors of cyclo-oxygenase-2 or of 5-lipoxygenase had no inhibitory effect. A thromboxane (TX)A2/endoperoxides receptor antagonist and a methacin, a cyclo-oxygenase inhibitor, totally abolished the stimulating effect of AA, whereas specific pharmacologic inhibitors of cyclo-oxygenase-1 metabolites; the combined action of PGG2 and TXA2, which potentiated it, was greater than that of PGE2, which inhibited it. Dietary n-3 fatty acids exert part of their beneficial effect by modulating this procoagulant activity differently from AA.


Arachidonic acid (AA; 20:4, n-6) is a polyunsaturated fatty acid present in cell membranes that modulates diverse physiologic and pathologic responses. These effects are primarily mediated by its metabolic products, eicosanoids. Eicosanoids include notably the cyclo-oxygenase products, i.e., PG and TX, and the lipoxygenase products, i.e., leukotrienes and hydroxyeicosatetraenoic acids (HETEs). Their effects, which depend on the nature of compounds, principally concern inflammation and thromboregulation (1). Eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3) are other polyunsaturated fatty acids contained in fish oil. EPA is metabolized into eicosanoids that are different from the corresponding compounds derived from AA and are considered to develop reduced biologic activities (2–5). DHA is not metabolized by cyclo-oxygenase or lipoxygenase enzymes (6). Experimental (7, 8), epidemiologic, and interventional (9, 10) studies suggest that consumption of fish oil, which causes a progressive decrease in the AA content of tissue phospholipids and an increase in the tissue level of n-3 fatty acids, exerts beneficial effects on thrombosis and atherosclerosis. The molecular mechanisms by which dietary n-3 fatty acids exert their beneficial effects are complex and have not been totally clarified. Some of these effects have been attributed to the activity of n-3 fatty acids on monocytes (4, 8, 11–13).

Monocytes are centrally involved in numerous pathophysiologic processes, such as thrombosis, atherosclerosis, wound repair, and inflammation. These properties are partly related to their ability to express various procoagulant activities (14–17). The procoagulant activities of monocytes are mediated to a large extent by cell surface-associated tissue factor (TF). TF is the cellular receptor and cofactor for plasma factor VIII(a), which initiates the coagulation protease cascade leading ultimately to the generation of thrombin and fibrin (18). Dietary administration of fish oil results in an impairment of TF expression by monocytes (8, 12, 13). The molecular mechanisms underlying TF activity reduction after n-3 fatty acids intake are unknown. However, since monocytes are important generators of eicosanoids and possess both cyclo-oxygenase and lipoxygenase enzymes, changes in the monocyte capacity to generate eicosanoids by these pathways have been suspected to influence monocyte TF activity. Thus, several recent studies have indicated that eicosanoids may modulate the procoagulant properties of monocytes. For example, prostacyclin analogues and PGE1 cause a decrease in cytokine-induced TF activity by monocytes (19, 20), whereas, conversely, platelet 12-HETE enhances PBMC procoagulant activity (21).

To clarify the mechanisms of the beneficial effect of dietary fish oil on the procoagulant activity of monocytes, we investigated in vitro the effects of AA, EPA, and DHA on TF expression by isolated PBMC. We show for the first time that AA, but not EPA or DHA, enhanced the TF-dependent procoagulant activity of isolated PBMC. The roles of the cyclo-oxygenase and lipoxygenase pathways were also examined. We describe a novel role for the cyclo-oxygenase-1 pathway in modulating the TF expression of AA-stimulated PBMC that appeared to be balanced by the opposite effects of...
arachidonic acid and mononuclear cell tissue factor

Materials and Methods

Cell isolation and cell cultures

PBMC were isolated using a modification of the method described by Balter et al. to efficiently deplete platelet from the cell preparation (22). Whole blood was obtained with a 19-gauge needle from healthy volunteers who had not taken aspirin or other nonsteroidal anti-inflammatory drugs in the 7 days preceding the donation. Blood was anticoagulated with trisodium citrate (0.129 M; Becton Dickinson, Meylan, France) and centrifuged at 280 × g for 15 min at 4°C. Platelet-rich plasma was removed. The sedimented cells were diluted to twice the original blood volume with PBS (pH 7.4; Seromed, Biochrom, Berlin, Germany), layered onto Hypaque PLUS (Pharmacia Biotech, Uppsala, Sweden), and centrifuged at 400 × g for 35 min at 4°C. The resulting PBMC were washed in 5 mM EDTA-PBS four to six times to remove remaining platelets. The resulting mononuclear fraction contained less than two platelets per leukocyte. Non-specific α-naphthyl-acetate esterase staining indicated that the mononuclear fraction contained more than 90% monocytes. Cells (500 μl) were incubated at 2.5 × 10^6 PBMC/ml in medium 199 (ATGC Biotechnologie, Noisy-le-Grand, France) containing 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in stoppered, sterile, pyrogen-free tubes.

Sodium arachidonate, eicosapentaenoate, and docosahexaenoate (Sigma, Saint Quentin Fallavier, France), stored under argon at −80°C, or carbocyclic TXA2 (CTXA2; Sigma), TXB2 (Cayman, Ann Arbor, MI), PGG2, E2, D2, and F2α (Calbiochem, La Jolla, CA) were dissolved in medium 199 just before use and added to the cell suspension at the concentration indicated that they did not affect the cell morphology. Cells were incubated for 4 h in medium 199 and carefully scraped with a rubber policeman just before measurement of their procoagulant activity.

The cells were identified by their typical morphology. Cells were incubated for 4 h in medium 199 and carefully scraped with a rubber policeman just before measurement of their procoagulant activity.

Cell viability, as assessed by the measurement of lactate dehydrogenase release in the supernatant of cultured cells and by trypan blue exclusion, was >90%. All reagents used for cell isolation and culture were prepared according to the method of Jaffe et al. (23). The culture medium was composed of RPMI 1640 and medium 199 (ATGC Biotechnologie, Noisy-le-Grand, France) supplemented with 20% human pooled serum (Institut Pasteur, Paris, France). The cells were identified by their typical morphology. Cells were incubated for 4 h in medium 199 and carefully scraped with a rubber policeman just before measurement of their procoagulant activity.

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Measurement of procoagulant activity

Procoagulant activity was measured on intact cells using a one-step plasma recalciﬁcation time assay performed on a coagulometer (KC4, Amelung, Lemgo, Germany). The PBMC were placed on ice for 10 to 20 min to remove any adherent monocytes. A 20-μl sample of PBMC or scrape-harvested endothelial cells was added to 90 μl of citrated normal human plasma without platelets. One hundred microliters of 25 mM ω-Carboxy Clofibrate (ω-CCF) was added to initiate the reaction. Coagulation times were converted into procoagulant activity units (AU) using reference curves determined with a standard human blood TF preparation containing 10^6 AU/ml (Thromborel S, Behring, Marburg, Germany); the logarithm of the procoagulant activity was related to the logarithm of the coagulation time. The procoagulant activity of mononuclear cells was characterized by incubating the cells with a mixture of two mouse anti-human TF mAbs (10 μg/ml; American Diagnostica, Greenwich, CT) for 30 min at 37°C.

TF Ag was measured on cell lysates by commercially available ELISA (Imubind Tissue Factor, American Diagnostica). Cell lysates were prepared by lysing PBMC in PBS containing 1% Triton X-100. 1 mM EDTA (Merck, Chelles, France), 16 mM octyl PD glucopyranoside (Boehringer Mannheim, Meylan, France), 10 μM pepstatin A, 10 μM leupeptin, 0.1 mM PMSF (Sigma), and 100 kallkine inhibitor units/ml aprotinin (Sanofi-Chovay, Paris, France). The cell lysates were then frozen and thawed three times. They were stored at −80°C until assayed. TXB2 and PGE2 produced by cultured PBMC were measured in the supernatant by enzyme immunoassays (Cayman).

Statistical analysis

All results represent the mean ± 1 SEM of 4 to 10 separate experiments. Depending on the data, statistical analysis was performed using Student’s t test for paired variables or ANOVA followed by a Neuman-Keuls test when p > 0.05. Differences were considered significant at p ≤ 0.05.

Results

AA enhanced the procoagulant activity and TF synthesis of mononuclear cells

Nonstimulated PBMC had a very low procoagulant activity (138 ± 17 AU/10^6 cells; n = 7). Considering that the procoagulant activity of the cell suspension was solely due to monocytes, which represented 28% of the PBMC suspension, the procoagulant activity expressed by monocytes (493 ± 61 AU/10^6 monocytes) was comparable to that expressed by cultured resting endothelial cells (498 ± 110 AU/10^6 endothelial cells; n = 9), indicating that monocytes were not significantly stimulated by the procedure of isolation. The procoagulant activity was TF dependent, since it was inhibited >90% by preincubating the cells with anti-TF mAbs. When PBMC cells were incubated with AA (5 μM) for 4 and 20 h, respectively, their procoagulant activity increased, but this increase was less marked with 4-h incubation (i.e., by 132% at 4 h vs 335% at 20 h; n = 1). Therefore, all additional experiments were performed with 20-h incubation. In these conditions, AA increased the procoagulant activity in a dose-dependent manner (Table I) to a mean of 190% at 7.5 μM (n = 10), which corresponds to a mean reduction in clotting times of 40 s (from 240 to 200 s). Higher concentrations resulted in cell lysis. Similarly, AA significantly enhanced the cell content of TF Ag (Table II). By comparison, LPS (100 ng/ml) increased the procoagulant activity of PBMC by 294% (406 ± 50 AU/10^6 cells; n = 7).

The effect of AA was mediated by the cyclo-oxygenase-1 pathway

Preincubation of PBMC with indomethacin (10 μM), a cyclo-oxynase-1 and -2 inhibitor, which blocked TXA2 formation by 96 ± 1%, totally abolished the stimulating effect of AA, whereas specific pharmacologic inhibitors of cyclo-oxygenase-2 (NS398) or 5-lipoxygenase (L655,238) had no inhibitory effect (Table I). Importantly, the stimulating effect of AA was not due to a low contamination by endotoxins, since the procoagulant activity induced by LPS remained unchanged in the presence of indomethacin (563 ± 123 vs 570 ± 88 AU/10^6 cells with and without indomethacin, respectively; n = 8). Therefore, these results indicate that AA enhanced the procoagulant activity of PBMC by the cyclo-oxygenase-1 pathway.

PGG2 and CTXA2 enhanced the procoagulant activity and TF synthesis of PBMC

The major metabolites of AA produced by the cyclo-oxygenase-1 pathway are represented by the endoperoxides PGG2/PGH2 and further metabolized into TXA2, PGE2, PGD2, and PGFα. To determine those that are responsible for the enhancing effect of AA on the procoagulant activity of PBMC, we tested the effect of

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purified PG (Table III). PGG₃ and CTXₐ₂, a TXₐ₂ agonist, enhanced, in a dose-dependent manner, the procoagulant activity of PBMC and TF Ag (from 0.32 ± 0.12 to 1.15 ± 0.33 ng/10⁶ cells at 2 μM PGG₃; p < 0.05; n = 8). In contrast, PGE₂ significantly decreased the procoagulant activity of the cells. TXB₂, PGD₂, and PGF₉α had no significant effect.

To further test the hypothesis that the formation of endoperoxides and TXₐ₂ was involved in the potentiating effect of AA on the procoagulant activity of PBMC, we studied the effect of furegrelate, a TX synthase inhibitor, which blocked TXₐ₂ formation by 94 ± 1%, and that of SQ29,548, a TXₐ₂/endoperoxides receptor antagonist. Both molecules inhibited the potentiating effect of AA on the procoagulant activity of PBMC (Table I). As expected, the procoagulant effect of CTXₐ₂ was not inhibited by furegrelate (266 ± 51 vs 142 ± 30 AU/10⁶ cells with and without CTXₐ₂; 1 μM; p < 0.01; n = 4).

The potentiating effect of AA on the procoagulant activity of PBMC and the respective amounts of TXB₂, the stable metabolite of TXₐ₂, and PGE₂ released by PBMC varied among the different blood donors from whom the cells were isolated. The effect of AA on the procoagulant activity of cells was not directly related to the amount of TXB₂ or PGE₂ released (p > 0.20), but was significantly and positively related to the ratio of TXB₂/PGE₂ formed by cells in presence of AA (r = 0.61; p < 0.001; Fig. 1). This correlation remained significant when the outlying data with the highest procoagulant activity (937% of the baseline procoagulant activity) were deleted (r = 0.44; p < 0.01). Therefore, the variability of response of blood donors to AA appeared to be related to the respective amounts of endoperoxides/TXₐ₂ and PGE₂ produced by PBMC: there was a balance between these eicosanoids, which showed opposite effects on the procoagulant activity of PBMC.

To confirm that the procoagulant activity of PBMC was determined in part by a balance between TXₐ₂ and PGE₂, PBMC were incubated with PGE₂ (0.5 μM) and increasing concentrations of CTXₐ₂ (0 to 1 μM). As found previously (Table III), the addition of PGE₂ to PBMC decreased their baseline procoagulant activity by 54% (Fig. 2). When the molar ratio CTXₐ₂/PGE₂ was increased from 0 to 2, the procoagulant activity of PBMC increased in a ratio-dependent manner. However, although the increase in procoagulant activity produced by the addition of CTXₐ₂ to PGE₂-treated cells was statistically significant (p < 0.01), the procoagulant activity of PGE₂-treated cells remained significantly lower (p < 0.05) than that of cells incubated with CTXₐ₂ but without PGE₂ (p < 0.05).

**EPA and DHA did not enhance the procoagulant activity and TF synthesis of mononuclear cells**

The n-3 fatty acids EPA and DHA are metabolized differently from AA by cyclo-oxygenase and lipoxygenase enzymes. For example, when these fatty acids were incubated with PBMC, EPA, unlike AA, did not increase the basal production of TXB₂, and DHA inhibited it (Table II). In these conditions, EPA and DHA did not enhance the procoagulant activity (Fig. 3) and TF Ag level (Table II) of PBMC. At 5 and 7.5 μM, the procoagulant activity was significantly lower with EPA and DHA (p = 0.01) than with AA. Therefore, these results confirm that the effect of AA was mostly mediated by the production of endoperoxides and TXₐ₂.

**Discussion**

We have shown in the present study that the procoagulant activity of isolated PBMC was enhanced by AA. This increase in procoagulant activity was due to an increased generation of TF and not to an increased surface expression of procoagulant activity (Table II). The effect of AA was mediated through the production of cyclo-oxygenase-1 metabolites, especially endoperoxides and TXₐ₂. These findings differ from those of a previous study in which AA enhanced the procoagulant activity of PBMC when they were...
The procoagulant activity with 5 mM AA was expressed as a percentage of the procoagulant activity of resting PBMC. The role of this platelet lipoxygenase product was negligible in our study, since there were very few platelets, an important source of 12-HETE, and since monocytes produce only very low amounts of 12-HETE, especially in the absence of LPS. The enhancing effect of AA observed in our study was mediated by cyclo-oxygenase-1. It was inhibited by indomethacin, a cyclo-oxygenase-1 and -2 inhibitor (29), but it remained unchanged with a selective inhibitor of cyclo-oxygenase-2 (NS398; Table I). The latter result is not surprising, since the cyclo-oxygenase-2 pathway is only functional when monocytes are exposed to inflammatory stimuli, which were absent in our study (24, 25).

Our experiments were performed with exogenous AA. However, we do consider the role of cyclo-oxygenase-1 pathway in AA-induced TF expression to be pathophysiologically relevant. Indeed, this polyunsaturated fatty acid was effective at concentrations as low as 5 \( \mu \text{M} \). The plasma concentrations of polyunsaturated fatty acids have been reported to vary between 0.2 and 2 mM (31), and the percentage of AA in plasma free fatty acid can reach 9% (32). Interestingly, and in contrast to AA, endotoxin-stimulated TF expression of PBMC was not inhibited by inhibitors of the cyclo-oxygenase or lipoxygenase pathway. Thus, endogenous AA, present in the cell membrane, and/or the cyclo-oxygenase or lipoxygenase pathways did not appear to be involved in endotoxin-induced TF expression.

In the presence of AA, monocytes release endoperoxides (PGG\(_2\), TXA\(_2\), and PGH\(_2\)) through the cyclo-oxygenase-1 pathway that are further metabolized into two major metabolites, TXA\(_2\) and PGE\(_2\) (22, 24, 25). Purified PGG\(_2\) and CTXA\(_2\), a TXA\(_2\) agonist, enhanced the TF expression of PBMC (Table II). The role of this platelet lipoxygenase product was negligible in our study, since there were very few platelets, an important source of 12-HETE, and since monocytes produce only very low amounts of 12-HETE, especially in the absence of LPS (26).

### Table III. Effect of purified prostaglandins on the procoagulant activity of mononuclear cells

<table>
<thead>
<tr>
<th>PG (( \mu \text{M} ))</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD(_2) (AU/10(^6) cells, ( n = 8 ))</td>
<td>108 ± 10</td>
<td>ND</td>
<td>114 ± 32</td>
<td>146 ± 33**</td>
<td>192 ± 40**</td>
</tr>
<tr>
<td>CTXA(_2) (AU/10(^6) cells, ( n = 4 ))</td>
<td>130 ± 33</td>
<td>174 ± 62</td>
<td>211 ± 28</td>
<td>272 ± 28*</td>
<td>ND</td>
</tr>
<tr>
<td>TXB(_2) (AU/10(^6) cells, ( n = 5 ))</td>
<td>122 ± 30</td>
<td>ND</td>
<td>120 ± 30</td>
<td>116 ± 18</td>
<td>114 ± 16</td>
</tr>
<tr>
<td>PGE(_2) (AU/10(^6) cells, ( n = 9 ))</td>
<td>108 ± 10</td>
<td>80 ± 9</td>
<td>75 ± 4**</td>
<td>99 ± 8</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>PGE(_2) (AU/10(^6) cells, ( n = 5 ))</td>
<td>154 ± 14</td>
<td>163 ± 30</td>
<td>160 ± 24</td>
<td>158 ± 20</td>
<td>182 ± 28</td>
</tr>
<tr>
<td>PGG(_2) (AU/10(^6) cells, ( n = 5 ))</td>
<td>149 ± 31</td>
<td>164 ± 40</td>
<td>139 ± 26</td>
<td>160 ± 46</td>
<td>108 ± 22</td>
</tr>
</tbody>
</table>

\* PBMC (2.5 \( \times \) 10\(^6\) cells/mL) were incubated for 20 h with increasing concentrations of PGG\(_2\), CTXA\(_2\), TXB\(_2\), PGE\(_2\), PGD\(_2\), and PGG\(_2\) (AU/10\(^6\) cells). Statistical comparisons of the procoagulant activity of mononuclear cells incubated in presence of PG vs that without PG are represented by asterisks, where * indicates \( p < 0.05 \) and ** indicates \( p < 0.01 \). Data are the mean ± 1 SEM of \( n \) experiments.

### Figure 1

Relationship between the procoagulant activity and the molar ratio of TXB\(_2\)/PGE\(_2\) produced by PBMC isolated from different blood donors and incubated with AA. PBMC (2.5 \( \times \) 10\(^6\) cells/mL) isolated from different blood donors were incubated without or with AA (5 \( \mu \text{M} \)) for 20 h. The procoagulant activity with 5 \( \mu \text{M} AA \) was expressed as a percentage of the baseline procoagulant activity obtained in the absence of AA. TXB\(_2\) and PGE\(_2\) were measured in the supernatant of PBMC (\( n = 35 \)).

### Figure 2

Effects of increasing concentrations of CTXA\(_2\) on the procoagulant activity of PBMC treated or not with PGE\(_2\) (\( n = 5 \)). PBMC (2.5 \( \times \) 10\(^6\) cells/mL) were incubated for 20 h with PGE\(_2\) and CTXA\(_2\) (AU/10\(^6\) cells). Results are expressed as a percentage of the procoagulant activity of resting PBMC incubated for 20 h without PGE\(_2\) or CTXA\(_2\). Statistical comparisons of the procoagulant activity of PBMC incubated with purified PGE\(_2\) and CTXA\(_2\) vs that obtained without PGs are represented by asterisks (* indicates \( p < 0.05 \); ** indicates \( p < 0.01 \)).
inducing a rise in the concentration of ionized calcium in the cell cytoplasm (1). Our study adds one more mechanism by which TXA₂ may be prothrombotic. Other mechanisms include its capacity to cause platelet aggregation and vasoconstriction, to augment monocyte and neutrophil adhesiveness (1, 35–38), and to promote diapedesis (3). Finally, our results also suggest that part of the antithrombotic effect of aspirin, which irreversibly inactivates cyclo-oxygenase and inhibits the production of endoperoxides/TXA₂, may be mediated by a diminution of the procoagulant activity of PBMC.

References


