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Parallel decrease of tissue factor surface exposure and increase of tissue factor microparticle release by the n-3 fatty acid docosahexaenoate in endothelial cells

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Summary

Tissue factor (TF) is expressed on the endothelium in response to inflammatory mediators, giving endothelial cells a pro-thrombotic phenotype. Since fish-derived n-3 fatty acids (FA) have been associated with reduced incidence of myocardial infarction, we investigated the endothelial effects of the most abundant n-3 FA, docosahexaenoate (DHA), on TF expression. Human umbilical vein endothelial cells were pre-incubated with DHA (or stearate and arachidonate as controls) for 48–72 hours, and then stimulated with bacterial lipopolysaccharide (LPS) or tumor necrosis factor-α. Pre-incubation of endothelial cells with DHA (but not stearate or arachidonate) concentration-dependently reduced surface protein exposure, independent of TF mRNA or total protein expression regulation. Conversely, DHA treatment in conjunction with activating stimuli, induced the release of endothelial TF-exposing microparticles from endothelial cells, quantitatively accounting for the decreased TF cell surface exposure. In conclusion, DHA treatment, with a time-course consistent with its incorporation in membrane phospholipids, increases the release of TF-exposing microparticles from endothelial cells, accounting for decreased endothelial cell TF surface exposure, thus potentially modifying the overall endothelial control of microparticle-related effects.

Keywords

Endothelial cells, microparticles, omega-3 fatty acids, n-3 fatty acids, procoagulant activity, tissue factor

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Introduction

Tissue Factor (TF) is an integral membrane protein considered to be the trigger of the extrinsic coagulation pathway and the initiator of thrombogenesis *in vivo* (1). Once in contact with the blood, TF forms a high-affinity complex with factors VII/VIIa, activating factors IX and X, thus leading to thrombin generation, fibrin deposition, and platelet activation. Many pathophysiological stimuli, such as LPS and inflammatory cytokines, are capable of inducing the transcription, surface expression and activity of TF in endothelial cells and monocytes (1–3). Intravascular TF antigen has now been reported to exist in multiple forms, specifically as a soluble protein and as a membrane protein in granulocytes and in platelet α-granules (4), as well as in circulating microparticles (MP) (5). TF can be released from the cell surface by shedding of TF-exposing MP as an inactive form in healthy individuals (6), and as an activated form in some pathological conditions associated with the activation of coagulation (7, 8).

Large prospective studies have found a significant inverse association between the intake of long-chain n-3 polyunsatured fatty acids (FA) (including α-linolenic, 18:3 n-3), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3), and the risk of fatal and non-fatal coronary heart disease and sudden cardiac death (9–11). The mechanisms through which n-3 FA exert their benefits are likely multiple, including effects on atherogenesis, thrombogenesis and arrhythmogenesis (12). However, the effects of n-3 FA on most hemostatic variables investigated, including plasma activity of clotting factors and fibrinolytic mediators (13–16), are quite controversial. Overall, studies performed so far on the effects of n-3 FA on TF

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procoagulant activity have shown both enhancing (17) and inhibitory effects (18). Since we previously reported on the inhibition of pro-atherogenic mediators, such as endothelial leukocyte adhesion molecules and inflammatory cytokines, by n-3 FA (19, 20), here we tested the effects of the most abundant n-3 FA, DHA, on TF-induced expression and activity in human cultured vascular endothelial cells.

Methods

Cell cultures and experimental design

Human umbilical vein endothelial cells (HUVEC) were isolated and grown as described (21). HUVEC monolayers were pre-incubated for 0–72 hours (h) with the n-3 FA docosahexaenoate (DHA) (22:6, n-3) or, in control experiments, with eicosapentanoic acid (EPA) (22:5, n-3 FA), arachidonic acid (20:4, n-6 FA) or the saturated FA stearate (18:0). FA (>99% pure, as sodium salts (Sigma, St. Louis, MO, USA) were dissolved in a 100% ethanol stock under nitrogen to ensure minimal oxidation, and maintained at –80° C until used. Most experiments were performed with 48–72 h of treatment with FA, a time necessary for their maximum incorporation into cell membranes, paralleled by maximum biological activity on several biological end points previously explored (19). Monolayers were then stimulated with *Escherichia Coli* lipopolysaccharide (LPS, 10–1,000 ng/ml, Sigma) or TNF-α (20 ng/ml, Valter Occhiena, Torino, Italy). All reagents used for cell isolation and culture were assayed to exclude endotoxin contamination using a *Limulus* amebocyte lysate assay (Sigma).

Experiments with enzyme inhibitors of FA metabolism and measurement of lipid peroxidation

In specific experiments testing the possible involvement of FA metabolites generated through metabolizing enzymes, we inhibited cyclooxygenases, 5-, 12- and 15-lipoxygenase and the cytocrome P450 epoxygenase, by pharmacological and molecular approaches (siRNA), with a strategy similar to the one recently adopted by us to probe mechanisms of DHA-mediated effects on cyclooxygenase-2 (COX-2) (22). We used acetyl-salicylic acid (aspirin, from Sigma), at 100 μM as an inhibitor of cyclooxygenases, the compound SKF-525A (proadifen, from Calbiochem, San Diego, CA, USA), at 50 μM, as an inhibitor of cytocrome P450 epoxygenase, the compound MK886 (Calbiochem), at 100 nM, as a selective inhibitor of 5-lipoxygenase, the compound 5,8,11-eicosatriynoic acid (ETI, Alexis, Lausen, Switzerland) at 10 μM as a combined inhibitor of 5- and 12-lipoxygenases, and nordihydroguaiaretic acid (NDGA, from Sigma), at 30 μM, as a combined inhibitor of 5-, 12- and 15-lipoxygenases. Effective concentrations of such compounds were derived from their reported IC50 and our previous experience (22). In such experiments, endothelial cells were treated with DHA at 50 μM for 48 h, after which, in the same medium, each inhibitor was added for 30 min before adding LPS or TNF-α for further 12 h. Additionally, targeted silencing of 15-lipoxygenase-1 was obtained with three different siRNA sequences obtained from Ambion (Austin, TX, USA; identification numbers 2306, 2309, 2486). Briefly, HUVEC were detached and seeded in the appropriate (6– or 96-well) multi-well plates, already containing 36 pM of a mixture of the three different siRNA against 15-LO-1 or 36 pM negative control siRNA (Ambion), having no homology to known sequences from mice, rats or humans, using the siPort NeoFX reagent (Ambion) and following manufacturer's instructions. After adhesion to the well, EC were treated with DHA 50 μM for 48 h, with daily additions of the same siRNA species. Afterwards, EC were challenged with LPS or TNF-α for 12 h and then tested for TF exposure and MP release as detailed below.

Membrane lipid peroxidation in DHA-treated HUVEC with or without the radical scavenging antioxidant (+)-α-tocopherol acid succinate (vitamin E) was determined by measuring thiobarbituric acid (TBA) reactivity of malondialdehyde (MDA), an end-product of lipid peroxidation, as previously described (23). Absorbance at 532 nm was converted to pmol MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane. Lipid peroxidation was expressed as pmol MDA per mg of total protein.

Controls of toxicity and apoptosis assay

The effects of FA treatments on cell survival were monitored by assessing total cellular proteins by the amido-black assay (24). Cell viability was evaluated by assessing the surface expression of the endothelial-specific constitutive and non-inducible E1/1 antigen (25), by an enzyme immunoassay (EIA) and by a tetrazolium-based colorimetric assay, measuring the ability of viable cells to reduce the tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan (Roche Diagnostics, Monza, Italy). DNA laddering, to evaluate apoptosis, was carried out by the TACS™ kit (R&D Systems, Milan, Italy).

The intensity of bands between 1,000 bp and 200 bp was quantified by densitometry and normalized by comparison with positive controls (DHA/staurosporine=densitometric ratio).

Measurement of TF total procoagulant activity in HUVEC

HUVEC seeded in 12-well plates were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed for 15 minutes (min) at 37° C with 300 mM of 0.1 M n-octyl-β-D-glycopyranoside in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, pH 7.5, 5 mM $CaCl_2$, 1 mg/ml bovine serum albumin [BSA]). TF activity was measured as its ability to promote the activation of factor X (150 nM) to factor Xa in the presence of factor VIIa (2 nM) and $CaCl_2$ (5 mM). The reaction (5 min at 37°C) was stopped by adding ethylenediaminotetra-acetic acid (EDTA, 5 mM final concentration). A chromogenic substrate for factor Xa (S-2765, Chromogenix, Instrumentation Laboratory, Milan, Italy) was then added (0.1 mM final concentration), and the absorbance at 405 nm was measured. TF activity was expressed in reference to a calibration curve constructed with different dilutions of a standard thromboplastin (Thromborel, S, Dade Behring, Liederbach, Germany), and results were expressed as standard thromboplastin arbitrary units (AU). To prove the TF-dependence of factor Xa generation, experiments were performed pre-incubating (30 min at room temperature) the sample with a specific anti-TF neutralizing antibody (No. 4509, American Diagnostica Inc, Stamford, CT, USA).

Detection of TF surface protein by EIA and flow cytometric analysis

Assay of TF cell surface protein was performed by EIA using a mouse anti-human antibody against TF (No. 4504; American Diagnostica), as described (19).

For the flow cytometric analysis, HUVEC (2.5 x 10^6) were harvested, washed and subsequently resuspended in 200 µl of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, 0.1% BSA). TF surface protein was detected by anti-human TF-fluoresceine isothiocyanate(FITC)-conjugated immunoglobulin(Ig)G (No. 4508J, 1 µg/ml, from American Diagnostica) for 20 min. Cell suspensions were analyzed with a Beckton Dickinson FACScan flow cytometer (San Jose, CA, USA), and results plotted as intensity of fluorescence (in arbitrary units, on a logarithmic scale, on the abscissa) versus cell number (on the ordinate). At least 10,000 events were counted.

Detection of TF total protein by Western blotting

TF protein was determined in cell lysates from HUVEC treated with DHA for 72 h and LPS for further 4 h, Western analysis was performed with 40 µg of total proteins, and TF detected by an anti-TF monoclonal antibody (1:500, No. 4509, American Diagnostica), as described (26).

Analysis of TF mRNA

Total cellular RNA was isolated with a guanidinium thiocyanate-phenol-chloroform extraction method using RNAzol™ B (Life Technologies, Milan, Italy), as described (27), and RNA (100 ng) was reverse-transcribed and amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) (Ready-To-Go RT-PCR Beads, Amersham). Primers used for TF mRNA amplification were as follows: sense primer: 5'-ACTACTGTTTCA-GTGTCAAGCAGTGATTC-3'; antisense primer: 5'-ATT-CAGTGGGGAGTTCTCCTTCCAGCTCTG-3'; sense primer for glyceraldehyde phosphate-dehydrogenase(GAPDH): 5'-GGTGAAGGTCGGAGTCAACGGA-3'; antisense primer: 5'GAGGGATCTCGCTCCTGGAAGA-3' (MWG Biotech, Florence, Italy). PCR was performed with 25 cycles (GeneAmp PCR System 9700, Perkin Elmer, Wellesley, MA, USA) under the following conditions: 5 min at 95°C and 1 min at 94°C for filament denaturation; 1 min at 60°C for annealing; 1 min at 72°C for elongation; and 5 min at 72°C for final extension. PCR products were visualized on 4% agarose gels stained with ethidium bromide, and photographed.

Detection of endothelium-derived TF microparticles (MP) and measurement of TF-dependent procoagulant activity

HUVEC (2 x 10^6 cells) were incubated with 25–50 µM DHA for 72 h and then stimulated with 1 µg/ml LPS or 20 ng/ml TNF-α for 6 h in serum-free medium. Cell supernatants, containing MP, were collected and cleared from cell fragments by centrifugation at 4,300 x *g* for 5 min. The supernatant was further ultracentrifuged at 100,000 x *g* for 1 h at 10°C, and MP were then resuspended in 100 µl of annexin-V buffer (7). Ten-µl aliquots of each MP-containing sample were incubated with annexin-V-FITC (Bender MedSystems, Vienna, Austria) or anti-TF FITC (No. 4508J, American Diagnostica) for 20 min in the dark. Labeled MP were then diluted in 350 µl of HEPES, and analyzed for a total of 2,000 events by flow cytometry at a high flow rate (60 µl/min) setting, with a logarithmic scale used both for light scatter and fluorescence. MP were identified by their size on a forward scatter/side scatter (FSC/SSC) cytogram, on which the MP gate was defined by 0.8-µm polystyrene latex beads (Sigma) (7). Only events included in this gate were further analyzed for fluorescence associated with anti-TF FITC. Fluorescence thresholds for annexin-V-FITC and anti-TF antibody binding were set based on the binding of annexin V in an inappropriate buffer (with EDTA 50 mM) and on the binding of an isotype-matched control (IgG1) antibody, respectively. Corrected data (MP number) for each sample were then obtained as number of events (N) recorded above this threshold. Mean fluorescence intensity (MFI) values of TF-positive MP population were reported as arbitrary units (AU).

MP obtained by ultracentrifugation were resuspended in HEPES buffer and TF activity was then measured as described above for HUVEC.

Data analysis

Results are all expressed as mean ± SD. Multiple comparisons were performed by one-way analysis of variance (ANOVA), followed by testing of individual contrasts by the Fisher's protected least significant difference test after checking for normality of distributions. Simple comparisons were performed by the unpaired Student's t-test. Comparisons of flow cytometry distributions were carried out by the Kolgomorov-Smirnov testing, using the Becton-Dickinson *Cell Quest* software. The level of significance in all tests was set at $p<0.05$.

Results

Effect of DHA on TF total cell-associated procoagulant activity

LPS and TNF-α both caused a marked increase in TF total cell-associated procoagulant activity (PCA) in a concentration- and time-dependent manner, as previously demonstrated (28, 29). Total TF activity was ~13-fold and ~15-fold greater compared with medium alone when measured 4 h after the addition of LPS 1 µg/ml (3,765 ± 123 AU/10^6 cells vs. 300 ± 27 AU/10^6 cells) and TNF-α at 20 ng/ml (4,290 ± 92 AU/10^6 cells vs. 286 ± 33 AU/10^6 cells), respectively. The inhibition of TF activity with the anti-TF IgG indicated that this procoagulant activity is indeed attributable to TF. HUVEC were incubated for 48 or 72 h with increasing concentrations of DHA (0, 10, 25, 50 µM), conditions that reflect a significant and highly reproducible inhibition of stimulated vascular cell adhesion molecule(VCAM)-1 surface expression (19), followed by treatment with LPS (0.01–10 µg/ml) for 4 h. DHA had no significant effect on baseline or LPS-induced TF activity at 48 h and 72 h (Fig. 1), or on TNF-α-induced TF activity (not shown).

Effect of DHA on TF surface protein

LPS induction of TF surface protein, as assessed by EIA, was time- and concentration-dependent, with peak expression occurring at 6 h after treatment with LPS 1 µg/ml (198 ± 5 OD mU vs. medium alone 15 ± 4 OD mU). When HUVEC were also stimu-

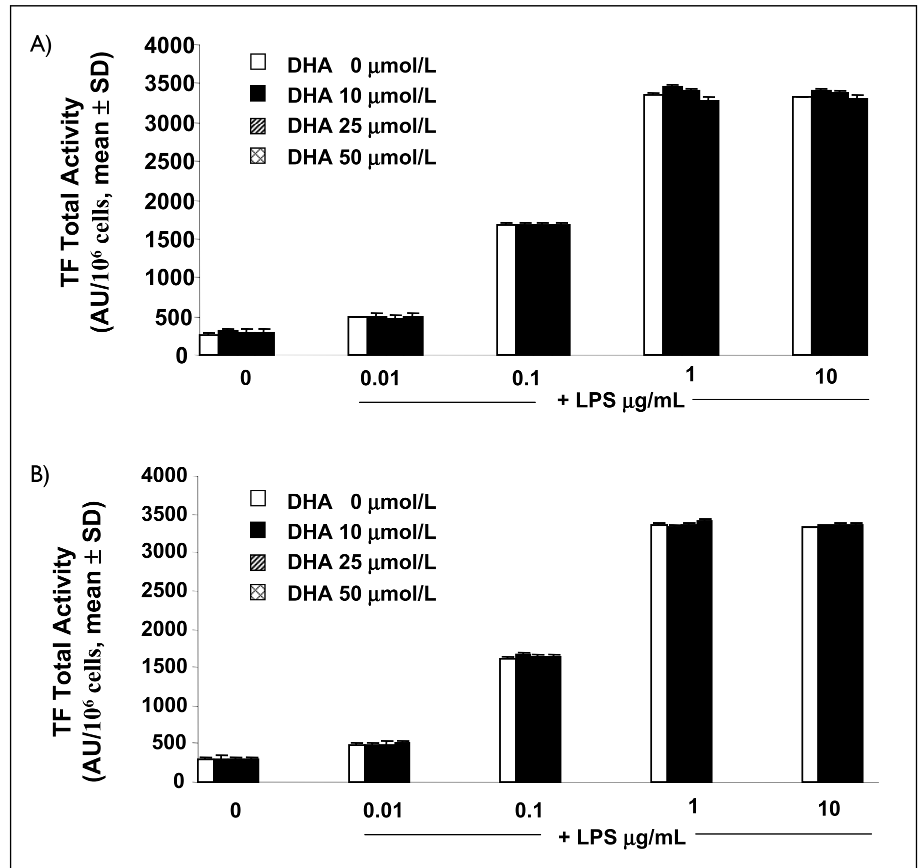

Figure 1: Effect of DHA on LPS-induced TF cellular activity in HUVEC. HUVEC were incubated with increasing concentrations of DHA (0, 10, 25 and 50 µM) for 48 h (A) and 72 h (B). At the end of this incubation, HUVEC were exposed to LPS 0.01–10 µg/ml for an additional 4 h, still in the presence of DHA. Data shown here are mean ± SD of five separate experiments, each performed in duplicate. No significant differences were seen with any of DHA concentration used on LPS-induced TF procoagulant activity.

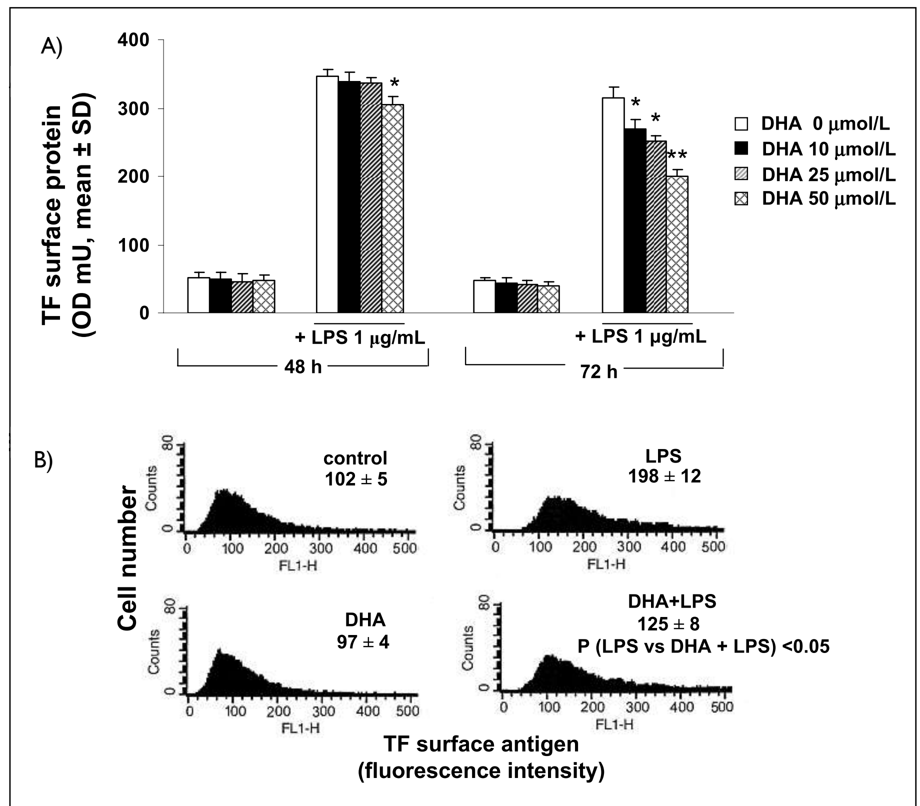

Figure 2: Effect of DHA on stimulated TF surface protein in HUVEC. A) HUVEC were incubated with DHA (0, 10, 25, 50 µM) for 48 h and 72 h, after which LPS 1 µg/ml was added for a further 5 h, still in the presence of the FA under investigation, to induce the surface exposure of TF protein. Surface protein was here detected by EIA. Representative DHA concentration-response curves of LPS-stimulated TF surface protein exposure with 48 h (left panel) and 72 h DHA pre-incubation (right panel) are shown. Results are mean ± SD of at least four replicates (*p<0.05 and **p<0.01 compared with stimulated condition). B) Flow-cytometry distributions of TF surface protein protein (fluorescence intensity, on the abscissa vs. cell number, on the ordinate) in HUVEC treated with 25 µM DHA for 72 h and then stimulated with LPS for an additional 4 h, still in the presence of DHA. Insets indicate median values ± SEM of fluorescence intensity units (proportional to the amount of antigen expressed) from one experiment representative of a series of three.

Table 1: Effects of DHA treatment for up to 72 h on endothelial total protein content, expression of E1/1 antigen, cell viability and apoptosis. DHA = docosaehexaenoic acid; Ag = antigen; MTT = tetrazolium salt (see text); OD = optical density. Values are mean ± SD.

	Control	DHA 25 µM	DHA 50 µM
Total protein (OD mU)			
0 h	256 ± 10	260 ± 14	249 ± 15
48 h	437 ± 10	440 ± 14	435 ± 26
72 h	445 ± 20	450 ± 15	425 ± 18
E1/1 Ag (OD mU)			
0 h	870 ± 55	910 ± 40	890 ± 20
48 h	1390 ± 21	1425 ± 40	1370 ± 38
72	1400 ± 30	1385 ± 25	1340 ± 40
MTT assay			
0 h	100%	100%	100%
48–72 h	> 95%	> 95%	> 95%
DNA laddering (densitometric ratio)			
0 h	0.1 ± 0.03	0.2 ± 0.07	0.2 ± 0.05
48–72h	0.1 ± 0.07	0.1 ± 0.09	0.2 ± 0.06

lated with increasing concentrations of TNF-α for 6 h, TF surface protein was concentration-dependently related to TNF-α stimulation, with a peak observed at 20 ng/ml (340 ± 27 OD mU vs. medium alone, 25 ± 11 OD mU). DHA, at all concentrations used, had no effects with pre-incubation time < 48 h (not shown). With 48 h pre-incubation, DHA inhibited LPS-stimulated TF surface protein only at 50 µM (Fig. 2A), while pre-incubation for as long as 72 h caused a clearer concentration-dependent inhibition of LPS-stimulated TF surface protein. This concentration-dependent inhibition of TF surface protein was independent of the intensity of the stimulation, since achieved, to a similar degree, when cells were stimulated with LPS 0.1 µg/ml (by 11% at DHA 10 µM, by 19% at DHA 25 µM, by 25% at DHA 50 µM). Similar results were obtained when HUVEC were stimulated with 20 ng/ml TNF-α after 48 h of pre-treatment with 50 µM DHA (318 ± 20 OD mU vs. TNF-α alone: 365 ± 15 OD mU) and 72 h of 10–25–50 µM DHA (314 ± 17 OD mU, 296 ± 14 OD mU, 233 ± 25 OD mU, respectively, vs. TNF-α alone: 365 ± 15 OD mU). This concentration-dependent inhibition of TF surface protein was also achieved when cells were stimulated with LPS 0.1 µg/ml (by 11% at DHA 10 µM, 19% at DHA 25 µM, by 25% at DHA 50 µM) and TNF-α 20 ng/ml (by 12% at DHA 10 µM, by 19% at DHA 25 µM, by 31% at DHA 50 µM).

Flow-cytometric analysis of HUVEC treated with DHA 25 µM for 72 h also showed decreased LPS-stimulated cell surface exposure of TF, confirming EIA data (Fig. 2B). Since DHA produced the largest inhibition of TF surface protein with 72 h at 25–50 µM, further experiments focused on this long incubation time. DHA treatment in all conditions did not produce detectable toxic effects, nor apoptosis, as evaluated by DNA laddering (Table 1).

Effect of saturated, monounsaturated and polyunsaturated fatty acids on TF surface protein and procoagulant activity

We explored whether the results just described are specific for DHA or also obtainable with other FA. Under same experimental conditions associated with marked effects of DHA, neither

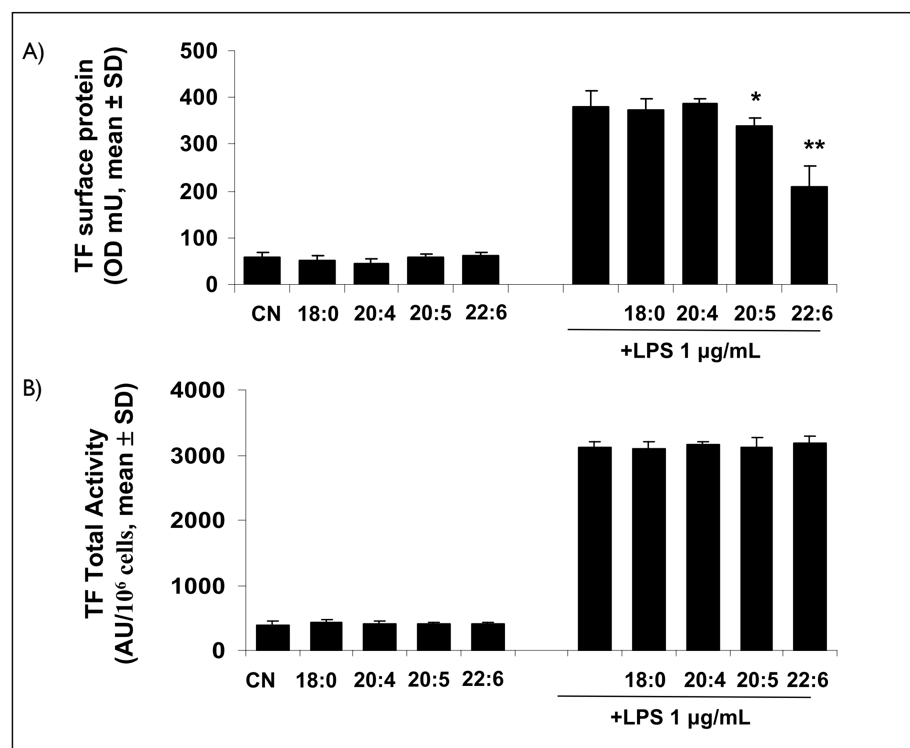

Figure 3: Effect of different FA on TF surface protein and procogulant activity in HUVEC. HUVEC were incubated with stearic acid (18:0), arachidonic acid (20:4 n-6), EPA (20:5 n-3), or DHA (22:6 n-6) for 72 h at 50 µM, or with medium alone (control, CN), after which LPS 1 µg/ml was added for a further 5 h, still in the presence of the FA. At the end of this incubation, TF surface protein and procoagulant activity were assessed by EIA and a coagulation assay, respectively. Data shown are mean ± SD of four separate experiments, each performed in duplicate (*p<0.05 and **p<0.01 compared with stimulated condition without the addition of FA). EPA produced a significant, albeit modest, inhibition of TF surface protein. DHA inhibited TF surface protein more potently. TF total procoagulant activity was not affected in any experimental conditions.

stearic acid nor n-6 polyunsaturated FA (AA) here inhibited LPS-induced endothelial TF surface protein (Fig. 3A) and procogulant activity. Figure 3 shows the effects of various classes of FA on TF surface protein (Fig. 3A) and procoagulant activity (Fig. 3B) in the same experimental conditions in which we had obtained maximal inhibition with DHA. Here the omega-n-3 FA EPA also attenuated TF surface protein, but not procogulant activity, while the maximum reduction of TF was achieved with DHA, suggesting a class effect for n-3 FA, but also that the presence of the extra double bond in DHA, compared with EPA, accounts for its greater activity. On the basis of these results, all further experiments were performed with DHA.

Effect of DHA on TF total protein and mRNA

To further understand the mechanisms of decreased TF surface protein after DHA incubation, we tested whether DHA treatment affected TF total protein and gene expression. HUVEC were pretreated with DHA 25 μM and 50 μM for 72 h, and then stimulated with LPS for 90 min and 4 h to induce full-blown TF mRNA and protein expression, respectively. Western analyses, however, revealed no significant inhibition of TF total protein in our experimental conditions (Fig. 4A). RNA prepared from untreated control cells or from cells treated with 1 μg/ml LPS with or without 50 μM DHA for 72 h was analyzed by RT-PCR. Baseline levels of TF were barely detectable in the negative control. LPS treatment of HUVEC caused a strong increase in TF mRNA, but this RNA level was not reduced by DHA treatment (Fig. 4B).

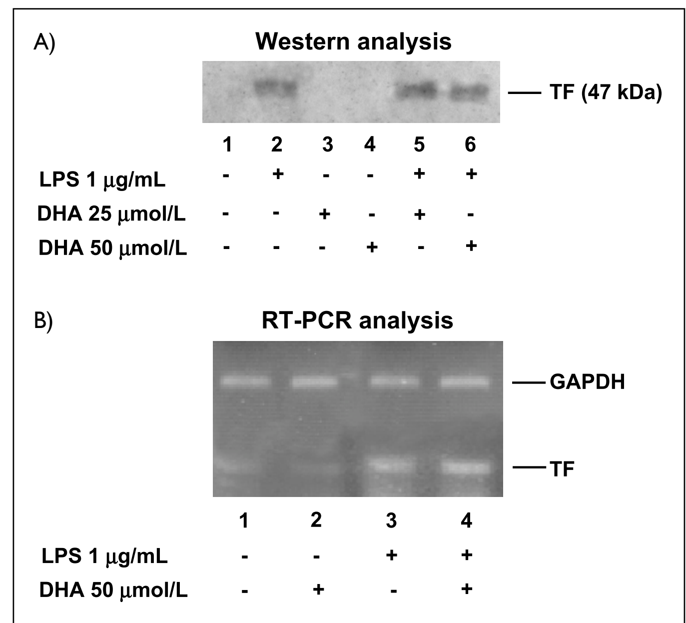

Figure 4: DHA does not modify TF total protein and mRNA steady state levels in HUVEC. A) Western analysis of TF expression in HUVEC incubated with 25–50 μM DHA for 72 h before the addition of 1 μg/ml LPS for further 4 h, still in the presence of DHA. Extracts of total cellular proteins were subjected to immunoblotting using an anti-TF antibody, as detailed in Methods. B) TF mRNA steady state levels, assessed by RT-PCR after pre-incubation of HUVEC with 50 μM DHA for 72 h, followed by the exposure to 1 μg/ml LPS or buffer for further 90 min, still in the presence of DHA. RT-PCR analyses were performed with 100 ng total RNA per sample. GAPDH mRNA levels were assessed as an internal control for the equality of amplification. The blots shown here are representative of three Western and three RT-PCR analyses performed.

Effect of DHA on endothelial TF-exposing MP release and TF-associated MP procoagulant activity

Because TF is also associated with circulating MP, and because these may mediate, after their local release, systemic effects of TF, we investigated whether DHA induces MP formation in HUVEC. If this were to occur, it might explain the above-reported data on reduced TF surface exposure prompted by incubation with DHA.

We used 1 μg/ml LPS and 20 ng/ml TNF-α for 6 h to induce endothelial MP release (Fig. 5). We evaluated the total number of MP release by annexin-V-FITC labeling. DHA (25–50 μM) for 72 h did not increase MP release significantly (events: 59 ± 25, at 50 μM, vs. control: 24 ± 10). Total MP number was increased by about 5- and 6-fold after TNF-α (20 ng/ml) and LPS (1 μg/ml) stimulation, respectively, compared with control (data not shown). DHA treatment (25–50 μM) increased MP release in a concentration-dependent manner when the cells were stimulated with TNF-α (events: 275 ± 60 and 347 ± 71, respectively, vs. TNF-α alone: 170 ± 12, $p<0.05$). or LPS (301 ± 41 and 378 ± 51, respectively, vs. LPS alone 204 ± 19, $p<0.05$).

LPS and TNF-α, at the above-reported concentrations, increased also the shedding of TF-exposing MP in cell supernatants, by ~3- and ~5-fold respectively, compared with unstimulated cells (where TF is not exposed) (Fig. 5A). DHA (25–50 μM for 72 h) did not influence the number of TF-exposing MP in unstimulated conditions, but increased TF-exposing MP release in a concentration-dependent manner when the cells were stimulated with LPS (events: 45 ± 5 and 54 ± 8, respectively, vs. control: 12 ± 10, $p<0.001$) and TNF-α (events: 51 ± 6 and 67 ± 8, respectively, vs. control: 9 ± 11, $p<0.001$). This concentration-dependent increase by DHA in TF-exposing MP was also achieved when the cells were stimulated with a lower concentration of LPS (0.1 μg/ml). MFI values, a reflection of the relative abundance of TF per microparticle, increased after stimulation with LPS or TNF-α (44 ± 5 AU and 52 ± 3 AU, respectively), compared with control (10 ± 5), but did not apparently change when HUVEC were pretreated with DHA (DHA 25 μM + LPS: 47 ± 2 AU; DHA 50 μM + LPS: 51 ± 6 AU; DHA 25 μM + TNF-α: 56 ± 2 AU, DHA 50 μM + TNF-α: 57 ± 3 AU) (Fig. 5B), suggesting that DHA treatment did not alter the intensity of TF exposure per microparticle, but only altered their total number.

In parallel experiments, neither stearic nor AA influenced MP release, with or without LPS or TNF-α (data not shown). Stimulation with LPS and TNF-α led to an ~4-fold and ~5-fold rise in MP-associated TF activity (5.9 ± 0.3 AU and 8.3 ± 0.5 AU, respectively) at 6 h after stimulation, compared with control conditions (1.6 ± 0.5 AU, $P<0.001$, Fig. 5C). As shown in Figure 5C, however, MP-associated TF activity was not enhanced by pretreatment with DHA before stimulation with LPS or TNF-α (DHA 25 μM + LPS: 6.2 ± 0.8 AU; DHA 50 μM + LPS: 6.1 ± 0.61 AU; LPS: 5.9 ± 0.3 AU, p=N.S. at ANOVA; DHA 25 μM + TNF-α: 8.4 ± 0.8 AU; DHA 50 μM + TNF-α: 9.1 ± 0.65 AU; TNF-α: 8.3 ± 0.5 AU, p=NS at ANOVA), indicating that DHA did not influence MP-associated TF procoagulant activity.

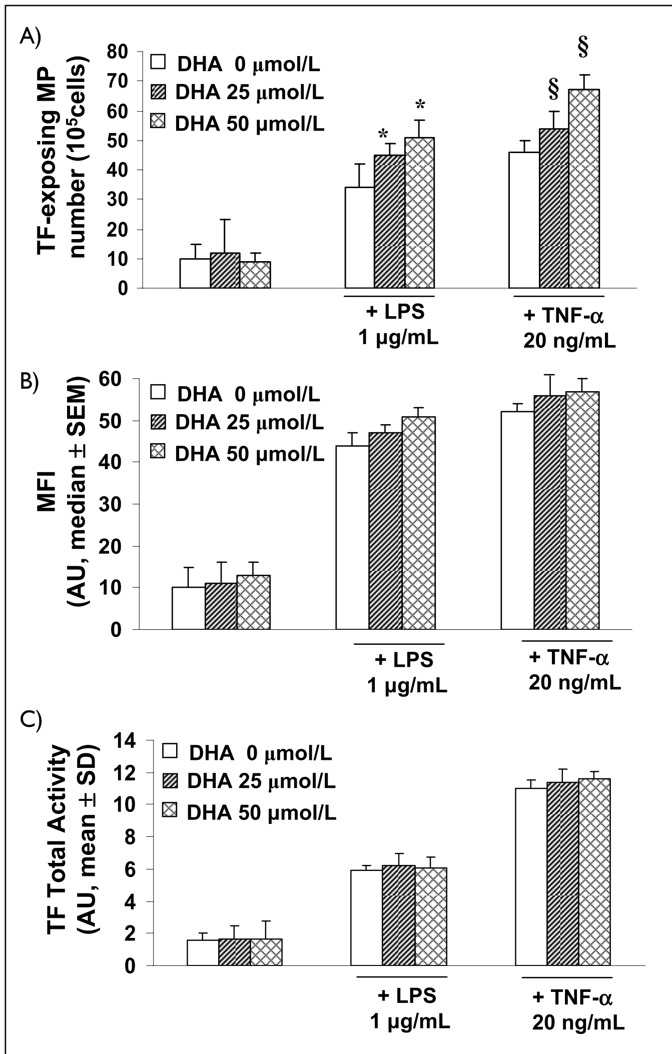

Figure 5: DHA increases number of TF-exposing MP, but not their procoagulant activity. MP released after pre-treatment with 25–50 µM DHA for 72 h, followed by stimulation with 1 µg/ml LPS and 20 ng/ml TNF-α for 6 h. A) MP number is indicated as the number of fluorescence events (N), after subtraction for autofluorescence, as described in *Methods*. Results are mean ± SD of at least three replicates (*p<0.05 DHA 25–50 µM + LPS vs. LPS; §p<0.05 DHA 25–50 µM + TNF-α vs. TNF-α). B) Mean fluorescence intensity (MFI) of MP positive for TF. Results, expressed as arbitrary units (AU), are median values ± SEM of at least three determinations. Differences of LPS or TNF-α stimulation vs. unstimulated control are all statistically significant at p<0.001. Within each LPS or TNF-α stimulation setting, differences in the presence vs. the absence of DHA are non-significant. C) TF-dependent procoagulant activity of MP shed from HUVEC. The results are expressed as arbitrary units. Differences of LPS or TNF-α stimulation vs. unstimulated control are all statistically significant at p<0.001. Within each LPS or TNF-α stimulation setting, differences in the presence vs. the absence of DHA are non-significant.

Involvement of DHA enzymatic metabolization or of peroxidation in the effects of DHA on TF surface exposure and MP release

Polyunsaturated fatty acids are known to be highly susceptible to metabolization and peroxidation, and – theoretically – a number of enzymatic, through one or more metabolizing enzymes, or spontaneous metabolic products might account for the observed effects of DHA on TF surface exposure and MP shedding. To test for effects mediated by metabolites of DHA, we inhibited the main pathways of FA metabolization with the use of pharmacological or molecular tools. As shown in Table 2, the inhibition of cyclooxygenases and cytocrome P450 epoxygenase did not affect DHA action on TF exposure and MP release. Moreover, the inhibition of all lipoxygenase activities by NDGA and specific inhibition of 5- and 12-lipoxygenase by MK-886 and ETI also did not reverse DHA inhibitory effects on TF exposure and MP release.

Finally, to test whether spontaneous peroxidation products of PUFA might play a role in the observed effects, levels of MDA were measured in HUVEC after exposure to DHA. In our experimental conditions, DHA did not affect the oxidative status of membrane FA. In fact, after 72 h of incubation with various DHA concentrations in the absence or presence of vitamin E (10 µM), MDA formation did not increase in HUVEC in comparison with untreated cells (at 10 µM DHA: 58 ± 3 pM; at 25 µM DHA: 65 ± 7 pM; at 50 µM DHA: 61 ± 3 vs. untreated cells 57 ± 5 pM; n=3). Such low, baseline levels of MDA were not affected in any condition by the radical scavenging antioxidant vitamin E (not shown).

Such a set of experiments therefore pointed out to a membrane effect of DHA in our system, without the involvement of any downstream metabolite or peroxidation product.

Discussion

Since the effects of n-3 FA on coagulation are controversial, it was of relevance to investigate the effects of these natural substances on TF expression and activity by human endothelial cells, on the surface of which TF may appear in conditions of endothelial activation. The major interest of our report is therefore in offering a detailed investigation of the biological effects of the most abundant n-3 FA, DHA, on such phenomena. This is, to the best of our knowledge, the first detailed examination of the effects of DHA on TF expression and activity in endothelial cells. Firstly, our results show that treatment of endothelial cells with DHA, in conditions including long pre-incubation times previously reported to be associated with reduced expression of VCAM-1, intercellular adhesion molecule-1 (ICAM-1) and E-selectin (19, 30), or COX-2 (22), reduces TF surface protein induced by LPS and TNF-α, but not its gene expression. In short-time experiments (<48 h pre-incubation time), TF surface protein was essentially not affected by DHA, but a significant reduction was detected in cells treated with DHA in the micromolar range (0–50 µM) for longer time periods (72 h). These results indicate that only the chronic exposure to DHA, consistent with its optimal incorporation in membrane phospholipids (as previously documented by us in the same experimental conditions [19, 20]), modulates TF surface protein in endothelial cells. The effects shown here occur within a concentration range compatible with nutritional or pharmacological interventions *in vivo*. Our concentrations of DHA, in the order of 10 µM, are in the order of magnitude of the plasma concentrations detectable after a moderate in-vivo supplementation with n-3 FA of approximately 2–5 g/day (31, 32), in accordance to other reports of n-3

Table 2: Effect of pharmacological and molecular inhibition of cyclooxygenase(s), lipoxygenases and p450 epoxygenase on DHA-mediated effects of tissue factor (TF) surface protein and microparticle (MP) release. HUVEC were treated with DHA at 50 μM for 48 h, after which, in the same medium, each inhibitor was added for 30 min before adding TNF-α for 5 h and 6 h to detect TF surface protein and MP release, respectively. In the experiments using siRNA, detached endothelial cells were seeded in the appropriate culture plates containing tranfection complexes. After cell adhesion, DHA was added for 48 and then the monolayer was stimulated as above. All comparisons of conditions with and without DHA are significant at p<0.01. All comparisons of conditions with and without the pharmacological inhibitors or siRNA are non-significant. MP: microparticles; DHA= docosaehexaenoic acid; ETI = 5,8,11-Eicosatriynoic acid (10 μM); MK886 (100 nM); SKF-525A = Proadifen (50 μM); NDGA = nordihydroguaiaretic acid (30 μM).

Treatment	Enzyme inhibited	TF surface protein (OD, mU)	MP release (number of events)
No treatment		31 ± 17	19 ± 11
TNF-α		362 ± 31	198 ± 21
TNF-α + DHA		268 ± 20	333 ± 42
TNF-α + aspirin	Cyclooxygenases	379 ± 25	201 ± 17
TNF-α + aspirin + DHA	Cyclooxygenases	268 ± 15	341 ± 16
TNF-α + SKF-525A	Cytocrome P450 epoxygenase	368 ± 18	211 ± 22
TNF-α + SKF-525A + DHA	Cytocrome P450 epoxygenase	263 ± 30	342 ± 15
TNF-α + MK886	5-lipoxygenase	382 ± 18	188 ± 20
TNF-α + MK886 + DHA	5-lipoxygenase	282 ± 17	339 ± 23
TNF-α + ETI	5- and 12-lipoxygenases	347 ± 20	197 ± 10
TNF-α + ETI + DHA	5- and 12-lipoxygenases	271 ± 12	325 ± 33
TNF-α + NDGA	5-, 12- and 15-lipoxygenases	361 ± 20	205 ± 18
TNF-α + NDGA + DHA	5-, 12- and 15-lipoxygenases	280 ± 23	318 ± 23
TNF-α + 15-LOX siRNA	15-lipoxygenase	389 ± 11	183 ± 21
TNF-α + 15-LOX siRNA + DHA	15-lipoxygenase	272 ± 31	323 ± 19

FA effects (33–35). Neither the saturated FA stearate, nor the n-6 polyunsaturated FA AA had any effects on TF surface protein, indicating the specificity of action of DHA on this endpoint. Moreover, DHA, which has a longer chain length and a greater degree of unsaturation than EPA, consistently exhibited greater potency than EPA on TF surface protein and procoagulant activity, in agreement with our own previous findings on other endpoints (19, 30).

Other previous reports have shown that n-3 FA inhibit TF activity in monocytic cell lines (36), or in monocytes isolated from hypertriglyceridemic patients treated for several weeks with n-3 FA (18). However, the mechanisms for these effects also in these other cell types are poorly characterized. Contrary to our original reasoning, we consistently found no modulatory activity of DHA on TF gene expression, with no directional changes observed in TF mRNA and total TF protein and activity. Such results indicate that DHA regulates TF surface exposure on HUVEC, independent of TF mRNA regulation and gene expression. We deliberately use, in this context, the wording "TF exposure", according to Lindmark (37), in referring to changes in surface protein not accompanied by changes in gene expression. This occurred with DHA concentrations and incubation times identical to those associated with inhibition of adhesion molecules and COX-2 (19, 22). Post-transcriptional mechanisms, likely dealing with post-translational modifications in the antigenic recognition of the protein, had therefore to be involved.

Previous studies in a variety of cell types have found that a portion (up to 30%) of TF antigen can be segregated in discrete intracellular pools after stimulation with agonists (38). In particular, it has been observed that, both in smooth muscle cells – expressing TF constitutively (39) – and in endothelial cells – where TF is induced by vascular endothelial growth factor (VEGF), LPS and TNF-α – there are "patches" on the plasma membrane containing TF pools (26), providing a rich source of procoagulant activity under conditions associated with endothelial damage or activation. Since endothelial activation is also associated with MP release, able to mediate vascular responses to pro-apoptotic, pro-inflammatory or pro-thrombotic stimuli (7, 40–42), and since MP release might explain the reduction of cell surface-associated TF exposure by treatment with DHA, we tested the hypothesis that DHA affects MP release. Our results indeed indicate that DHA, in the same conditions associated with decreased TF surface exposure, can promote the release of TF-exposing MP in co-stimulation with TNF-α or LPS, without inducing cellular toxicity or apoptosis, as demonstrated by the assay of several cytotoxicity markers and of DNA fragmentation (laddering). The two phenomena – reduced TF cell surface exposure and increased TF MP release – are likely related, since quantitatively of the same order of magnitude and sharing similar concentration- and time-dependence.

The action of DHA on TF exposure may theoretically be an effect of the FA *per se* or of some products of its metabolization or peroxidation. DHA is now indeed recognized to be precursor of novel anti-inflammatory lipid mediators such as 7S,8,17R-trihydroxydocosahexaenoic acid and 10,17S-docosatriene, obtained from acetylated COX-2 and/or lipoxygenase activities, respectively, in several cell types and tissues (43), which might – theoretically – mediate downstream effects. We have recently found that DHA-derived metabolites are indeed involved in the regulation of cyclooxygenase (COX)-2 gene ex-

pression (22). The effect here described appears, however, to be different, since it does not involve any change in TF gene expression (our results on mRNA levels) and does not appear to be changed to any substantial extent by treatment with any of several inhibitors of the known enzymes able to metabolize DHA into bioactive lipid mediators. Such effect also does not appear to be associated with an increase in MDA levels (as an index of lipid peroxidation), or to be affected by the antioxidant vitamin E. These sets of results therefore suggest that effects of DHA confined to the plasma membrane, including changes in membrane stability and fluidity, influencing the activity of receptors and the translocation of phosphatidylserine in the outer membrane leaflet, possibly also influencing cytosolic calcium concentration (44) – phenomena all involved in the formation of MP (7, 45) – are probably involved. Exact molecular mechanisms in such a direction deserve further investigations.

Despite increased TF-exposing MP by DHA in stimulated conditions, we observed no enhanced TF-dependent procoagulant activity. This suggests that TF on the surface of MP, the shedding of which appears enhanced by DHA, does not affect coagulation. Anti-thrombotic actions of n-3 FA are generally attributed to reduced platelet function through reduced production of AA-derived prostanoids, or decreased fibrogen levels. No significant influence of such FA has been so far observed on several coagulation variables investigated, including the activity of coagulation factors, or fibrinolytic activity (13–16, 46). Overall, the in-vitro studies performed by us indicate that, once incorporated in cell membrane phospholipids, DHA reduces TF surface exposure (but not activity) in endothelial cells concomitant, probably, to a facilitation in the release of TF MP. These two phenomena appear to be quantitatively symmetrical, with a probably overall neutral effect on TF-mediated blood coagulation *in vivo*. Such phenomena may still, however, be involved in non-coagulative aspects of TF activity, such as transcellular signalling or angiogenesis, the biological significance of which remain uncertain at the moment. In general, on the one hand MP have a pathologic potential, since they can trigger and disseminate a pro-inflammatory and pro-thrombotic response (45); on the other hand, MP can serve the function of "sensors" and "effectors" for the maintenance of homeostasis in multicellular organisms, and thus contribute to tissue development, angiogenesis, wound healing, and – more generally – tissue remodeling (47). By modulating TF MP release, n-3 FA might thus regulate diverse non-coagulative functions associated with MP shedding and TF exposure.

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References

1. Steffel J, Luscher TF, Tanner FC. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. Circulation 2006; 113: 722–731.

2. Szotowski B, Antoniak S, Poller W, et al. Procoagulant soluble tissue factor is released from endothelial cells in response to inflammatory cytokines. Circ Res 2005; 96: 1233–1239.

3. Camerer E, Kolsto AB, Prydz H. Cell biology of tissue factor, the principal initiator of blood coagulation. Thromb Res 1996; 81: 1–41.

4. Muller I, Klocke A, Alex M, et al. Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets. FASEB J 2003; 17: 476–478.

5. Rauch U, Nemerson Y. Tissue factor, the blood, and the arterial wall. Trends Cardiovasc Med 2000; 10: 139–143.

6. Berckmans RJ, Neiuwland R, Boing AN, et al. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost 2001; 85: 639–646.

7. Combes V, Simon AC, Grau GE, et al. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. J Clin Invest 1999; 104: 93–102.

8. Mallat Z, Benamer H, Hugel B, et al. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation 2000; 101: 841–843.

9. GISSI Prevenzione Group. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet 1999; 354: 447–455.

10. Albert CM, Campos H, Stampfer MJ, et al. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. N Engl J Med 2002; 346: 1113–1118.

11. de Lorgeril M, Salen P, Defaye P, et al. Dietary prevention of sudden cardiac death. Eur Heart J 2002; 23: 277–285.

12. Das UN. Beneficial effect(s) of n-3 fatty acids in cardiovascular diseases: but, why and how? Prostaglandins Leukot Essent Fatty Acids 2000; 63: 351–362.

13. Agren JJ, Vaisanen S, Hanninen O, et al. Hemostatic factors and platelet aggregation after a fish-enriched diet or fish oil or docosahexaenoic acid supplementation. Prostaglandins Leukot Essent Fatty Acids 1997; 57: 419–421.

14. Conquer JA, Cheryk LA, Chan E, et al. Effect of supplementation with dietary seal oil on selected cardiovascular risk factors and hemostatic variables in healthy male subjects. Thromb Res 1999; 96: 239–250.

15. Schmidt EB, Arnesen H, Christensen JH, et al. Marine n-3 polyunsaturated fatty acids and coronary heart disease: Part II. clinical trials and recommendations. Thromb Res 2005; 115: 257–262.

16. Schmidt EB, Arnesen H, De Caterina R, et al. Marine n-3 polyunsaturated fatty acids and coronary heart disease. Part I. Background, epidemiology, animal data, effects on risk factors and safety. Thromb Res 2005; 115: 163–170.

17. Muller AD, van Houwelingen AC, van Dam-Mieras MC, et al. Effect of a moderate fish intake on haemostatic parameters in healthy males. Thromb Haemost 1989; 61: 468–473.

18. Tremoli E, Eligini S, Colli S, et al. n-3 fatty acid ethyl ester administration to healthy subjects and to hypertriglyceridemic patients reduces tissue factor activity in adherent monocytes. Arterioscler Thromb 1994; 14: 1600–1608.

19. De Caterina R, Cybulsky MI, Clinton SK, et al. The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells. Arterioscler Thromb 1994; 14: 1829–1836.

20. De Caterina R, Liao JK, Libby P. Fatty acid modulation of endothelial activation. Am J Clin Nutr 2000; 71: 213S-23S.

21. Massaro M, Basta G, Lazzerini G, et al. Quenching of intracellular ROS generation as a mechanism for oleate-induced reduction of endothelial activation and early atherogenesis. Thromb Haemost 2002; 88: 335–344.

22. Massaro M, Habib A, Lubrano L, et al. The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKCepsilon inhibition. Proc Natl Acad Sci USA 2006; 103: 15184–15189.

23. Jain SK. Hyperglycemia can cause membrane lipid peroxidation and osmotic fragility in human red blood cells. J Biol Chem 1989; 264: 21340–21345.

24. De Caterina R, Tanaka H, Nakagawa T, et al. The direct effect of injectable cyclosporine and its vehicle, cremophor, on endothelial vascular cell adhesion molecule-1 expression. Ricinoleic acid inhibits coronary artery endothelial activation. Transplantation 1995; 60: 270–275.

25. Pober JS, Gimbrone MA, Jr., Lapierre LA, et al. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. J Immunol 1986; 137: 1893–1896.

26. Camera M, Giesen PL, Fallon J, et al. Cooperation between VEGF and TNF-alpha is necessary for exposure of active tissue factor on the surface of human endothelial cells. Arterioscler Thromb Vasc Biol 1999; 19: 531–537.

27. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156–159.

28. Colucci M, Balconi G, Lorenzet R, et al. Cultured human endothelial cells generate tissue factor in response to endotoxin. J Clin Invest 1983; 71: 1893–1896.

29. Bevilacqua MP, Pober JS, Majeau GR, et al. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc Natl Acad Sci USA 1986; 83: 4533–4537.
30. De Caterina R, Bernini W, Carluccio MA, et al. Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. J Lipid Res 1998; 39: 1062–1070.
31. Thies F, Miles EA, Nebe-von-Caron G, et al. Influence of dietary supplementation with long-chain n-3 or n-6 polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. Lipids 2001; 36: 1183–1193.
32. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet 1989; 2: 757–761.
33. Maziere C, Conte MA, Degonville J, et al. Cellular enrichment with polyunsaturated fatty acids induces an oxidative stress and activates the transcription factors AP1 and NFkappaB. Biochem Biophys Res Commun 1999; 265: 116–122.
34. Diep QN, Touyz RM, Schiffrin EL. Docosahexaenoic acid, a peroxisome proliferator-activated receptor-alpha ligand, induces apoptosis in vascular smooth muscle cells by stimulation of p38 mitogen-activated protein kinase. Hypertension 2000; 36: 851–855.
35. Kim HJ, Vosseler CA, Weber PC, et al. Docosahexaenoic acid induces apoptosis in proliferating human endothelial cells. J Cell Physiol 2005; 204: 881–888.
36. Chu AJ, Walton MA, Prasad JK, et al. Blockade by polyunsaturated n-3 fatty acids of endotoxin-induced monocytic tissue factor activation is mediated by the depressed receptor expression in THP-1 cells. J Surg Res 1999; 87: 217–224.
37. Lindmark E, Tenno T, Siegbahn A. Role of platelet P-selectin and CD40 ligand in the induction of monocytic tissue factor expression. Arterioscler Thromb Vasc Biol 2000; 20: 2322–2328.
38. Schecter AD, Giesen PL, Taby O, et al. Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation. J Clin Invest 1997; 100: 2276–2285.
39. Mulder AB, Smit JW, Bom VJ, et al. Association of smooth muscle cell tissue factor with caveolae. Blood 1996; 88: 1306–1313.
40. Kagawa H, Komiyama Y, Nakamura S, et al. Expression of functional tissue factor on small vesicles of lipopolysaccharide-stimulated human vascular endothelial cells. Thromb Res 1998; 91: 297–304.
41. Abid Hussein MN, Meesters EW, Osmanovic N, et al. Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo. J Thromb Haemost 2003; 1: 2434–2443.
42. Martinez MC, Tesse A, Zobairi F, et al. Shed membrane microparticles from circulating and vascular cells in regulating vascular function. Am J Physiol Heart Circ Physiol 2005; 288: H1004–1009.
43. Serhan CN, Arita M, Hong S, et al. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. Lipids 2004; 39: 1125–1132.
44. Zamaria N. Alteration of polyunsaturated fatty acid status and metabolism in health and disease. Reprod Nutr Dev 2004; 44: 273–282.
45. VanWijk MJ, VanBavel E, Sturk A, et al. Microparticles in cardiovascular diseases. Cardiovasc Res 2003; 59: 277–287.
46. Grundt H, Nilsen DW, Hetland O, et al. Atherothrombogenic risk modulation by n-3 fatty acids was not associated with changes in homocysteine in subjects with combined hyperlipidaemia. Thromb Haemost 1999; 81: 561–565.
47. Freyssinet JM. Cellular microparticles: what are they bad or good for? J Thromb Haemost 2003; 1: 1655–1662.