Alpha-Linolenic Acid–Rich Wheat Germ Oil Decreases Oxidative Stress and CD40 Ligand in Patients With Mild Hypercholesterolemia

Cesare Alessandri, Pasquale Pignatelli, Lorenzo Loffredo, Luisa Lenti, Maria Del Ben, Roberto Carnevale, Alessandro Perrone, Domenico Ferro, Francesco Angelico, Francesco Violi

Epidemiological studies have underlined that a higher intake of α-linolenic acid (18:3n-3) is inversely associated with coronary and carotid atherosclerotic disease. Long-chain n-3 fatty acids have been suggested to delay the atherosclerotic process through an antiinflammatory activity and in turn to stabilize vulnerable plaque. Previous studies suggested, in particular, that n-3 fatty acids may exert an antiinflammatory action by reducing oxidative stress. As we have previously shown that oxidative stress is implicated in upregulating CD40 ligand (CD40L), a protein with inflammatory and prothrombotic property, we tested the hypothesis that 18:3n-3 may protect against atherosclerotic disease also via inhibiting oxidative stress–mediated CD40L expression. To investigate this issue, 32 patients with hypercholesterolemia were randomly allocated, in a double blind fashion, to 2 months supplementation with 2 vegetable oils (1 tbsp/d) containing low (maize oil, 0.63%) or high (wheat germ oil, 8.6%) percentage of 18:3n-3. Before and after treatment oxidative stress and CD40L, a protein that is implicated in the progression of atherosclerotic disease, were measured. Also, we analyzed in vitro whether 18:3n-3 fatty acids were able to influence platelet oxidative stress and CD40L expression (for expanded Methods, see supplementary data available online at http://atvb.ahajournals.org).

There were no differences in clinical and laboratory characteristics between the 2 groups; after supplementation with both vegetable oils, no change of serum lipid profile was observed (not shown).

Maize oil supplementation did not change either soluble CD40 Ligand (sCD40L) data or 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative stress (not shown). Conversely, a parallel (r = 0.534; P = 0.03) and significant decrease of 8-OHdG (from 1.06 ± 0.26 to 0.62 ± 0.20 ng/mL; P < 0.001) and sCD40L (from 223.5 ± 32.2 to 96.5 ± 7.1 pg/mL; P < 0.001) was seen in wheat germ oil-treated patients. Also, platelet CD40L significantly decreased (mean fluorescence from 45.4 ± 3.1 to 29.3 ± 3.1; −35.4%, P < 0.001) only in wheat germ oil-treated patients (Figure, panel A).

A significant increase of platelet content of linoleic acid was observed in patients given maize (n = 3, +14.28%, P < 0.005) or wheat germ oil (n = 3, +16%, P < 0.05). Conversely, a significant increase of platelet concentration of α-linolenic acid was observed only in patients given wheat germ oil (n = 3, +255%, P < 0.002 versus n = 3, +6%, P > 0.05 in patients given maize oil; Figure, panel B). Also a significant reduction in platelet arachidonic acid (n = 3, −16.5%, P < 0.05) was observed only in patients given wheat germ oil. Platelets from volunteers incubated with liposomes containing linoleic acid or α-linolenic acid showed a different behavior. Thus, linoleic acid demonstrated a nonsignificant decrease of CD40L platelet O$_2^-$, NADPH oxidase, and p38MAP kinase activation (data not shown); conversely platelets incubated with α-linolenic acid showed a marked reduction of platelet CD40L expression (Figure, panel C), O$_2^-$ production (Figure, panel D), NADPH oxidase (Figure, panel E), and p38MAP kinase activation (Figure, panel F).

This study showed that in patients with hypercholesterolemia, wheat germ oil supplementation was associated with parallel reduction of oxidative stress and platelet CD40L expression suggesting that n-3 fatty acids downregulated CD40L via an oxidative stress–mediated mechanism. In vitro experiments showing that platelet incubation with n-3 fatty acids elicited significant decrease of platelet O$_2^-$ could support such hypothesis. Moreover, platelets incubated with n-3 fatty acids showed reduced activation of NADPH oxidase and phosphorylation of p38 MAP kinase, which is a protein implicated in the activation of NADPH oxidase. The underlying mechanism cannot be fully elucidated at the moment. Accumulation of 18:3n-3 on platelet membrane could reduce the content of arachidonic acid and in turn lower NADPH oxidase activation. This hypothesis, which is consistent with previous data showing a key role for arachidonic acid in stimulating platelet NADPH oxidase–dependent O$_2^-$ generation, should be investigated in the future.

Even if we cannot exclude that the n-3 antioxidant effect could not only be the cause but also the consequence of downregulation of CD40L, our hypothesis is consistent with an interventional study showing that supplementation with ascorbic acid was associated with platelet CD40L downregulation.

In conclusion, we provide evidence that wheat germ oil is an important source of n-3 fatty acids, which may exert an
antiatherosclerotic effect via inhibition of oxidative stress–mediated CD40L upregulation.

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Disclosures
None.

References
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SUBJECTS AND METHODS

Clinical Study

Thirty-two consecutive patients (14 males and 18 females; mean age 60.3±6.1 years) with polygenic hypercholesterolemia, recruited from the same geographic area and following a similar typical Mediterranean diet, were randomly allocated, in a double-blind manner, to receive for two months a supplementation (1 tbsp/day) of maize oil or wheat germ oil.

<table>
<thead>
<tr>
<th>Fatty acids composition (%)</th>
<th>Maize oil</th>
<th>Germ oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>10%</td>
<td>20,14%</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>1,9%</td>
<td>0,48%</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>38,82%</td>
<td>12,18%</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>47,81%</td>
<td>58,30%</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>1%</td>
<td>8,60%</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>0,09%</td>
<td>0,07%</td>
</tr>
</tbody>
</table>

During the follow-up all patients kept following their regular Mediterranean diet. The randomization procedure was based on a random sequence. Sealed envelopes were used to conceal randomization.

All patients were attending our metabolic outpatient clinic and had a complete clinical and biochemical work up as part of routine clinical examination. Written consent was obtained from all subjects before the study, that was conform to the ethical guidelines of the 1975 Declaration of Helsinki. The ethics committee of our institution approved the study protocol.

None of the patients had clinical evidence of cardiovascular disease (as shown by clinical history, physical examination, or ECG) or diabetes mellitus and had not taken any lipid-lowering agent,
antiplatelet drug or antioxidants in the previous 30 days. Nine patients (5 in the maize oil group and 4 in the wheat germ oil group) were treated with anti-hypertensive drugs and showed blood pressure <140/90 during the follow-up.

Plasma lipids, sCD40L, 8-Hydroxy-2-deoxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress (a, b) and CD40L platelet expression were measured at baseline and after two months of treatment.

In a subgroup of patients (3 treated with maize oil and 3 with wheat germ oil) we also examined if vegetable oils influenced platelet content of polyunsaturated fatty acids (see below).

**Lipid Profile**

Fasting serum levels of total cholesterol and triglycerides were determined with enzyme-based methods. HDL cholesterol was measured after phosphotungstic acid/MgCl₂ precipitation of fresh plasma. LDL cholesterol was calculated according to the Friedewald formula.

**Analysis of sCD40L and 8-OHdG**

Blood samples mixed with 0.13 M Na citrate (ratio 9:1) were taken between 8 and 9 AM from patients who had fasted at least 12 hours; samples were immediately centrifuged at 2000 rpm for 20 minutes at 4°C, and the supernatant was collected and stored at -80°C until measurement. Plasma levels of sCD40L were measured with a commercial immunoassay (Quantikine CD40 ligand, R&D Systems). Intra-assay and inter-assay coefficients of variation were 7% and 9%, respectively. Serum levels of 8-OHdG, were analyzed using a competitive enzyme-linked immunosorbent assay (Bioxytech 8-OHdG-EIA, OXIS Health Products, Portland). Intra- and inter-assay coefficients of variation were 2.1% and 4.5%, respectively

**Flow Cytometric Analysis of CD40L Expression**

Blood samples were collected from patients between 8 and 9 AM without stasis from an antecubital vein with a 21-gauge needle after a 12-hour fast and mixed with 0.13 M Na citrate (ratio 9:1, vol/vol). Washed platelets were prepared as previously described (c).
CD40L on platelet membrane was analyzed with specific fluorescein isothiocyanate conjugated-labeled monoclonal antibodies (Mab). An irrelevant isotype-matched antibody (anti-IgG1) was used as a negative control. Mab (20 µL) was added to 200 µL of platelet suspension (2 x 10⁶/mL) previously fixed with 2% paraformaldehyde in PBS (0.1% bovine serum albumin) and incubated for 60 minutes at 4°C. The unbound Mab was removed by addition of 0.1% bovine serum albumin PBS and centrifugation at 500g for 3 minutes (twice). Fluorescence intensity was analyzed on an Epics XL-MCL Cytometer (Coulter Electronics) equipped with an argon laser at 488 nm. For every histogram, 50 000 platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity was reported as mean fluorescence of specific antibody minus mean fluorescence of control antibody.

**Analysis of platelet linoleic and α-linolenic acids**

Before and at the end of follow-up blood samples were collected from 6 patients (2 males and 1 female age 61±6 years were given maize oil and 2 males and 1 female age 60 ±5 years were given wheat germ oil), out of 32 included in the interventional trial. The samples were drawn between 8 and 9 AM without stasis from an antecubital vein with a 21-gauge needle after a 12-hour fast and mixed with 0.13 M Na citrate (ratio 9:1, vol/vol). Washed platelets were prepared as previously described (c).

Platelet lipids were extracted as described by Folch et al. (d) and dried into a clear screw-capped glass tube. The percentages of linoleic and α-linolenic acids in the platelet samples were determined as methyl esters after vigorous methanolysis with 0.5N anhydrous HCl at 100 °C for 18h. After cooling to room temperature, the solution was extracted four times with hexane. The hexane phases containing the fatty acids methyl esters were collected, dried, and injected (0.5µl in CH₂Cl₂) into a SPB 2380 fused silica capillary column programmed at 5°C/min from 140 to 170°C for gas chromatographic analysis.
Linoleic and α-linolenic acids were used as standards (Sigma) for qualitative and quantitative analyses (e).

**In vitro study**

In vitro studies were carried out to examine if incubation of platelets with liposomes containing linoleic or α-linolenic acid resulted in changes of $\text{O}_2^-$, CD40L, and NADPH oxidase and p38MAP kinase activity. Blood samples mixed with 0.13 M Na citrate (ratio 9:1, vol/vol) were obtained between 8 and 9 AM from three volunteers (3 males; mean age 60±4 years, total cholesterol 241±3.6 mg/dl, triglycerides 124±24.4 mg/dl) who had fasted for 12 hours and had provided their informed consent to participate in the study.

**Preparation of liposomes**

Liposomes were composed of dimyristoylphosphatidylcholine (LPC) and either linoleic (LPC/18:2n-6) or α-linolenic acid (LPC/18:3n-3). Using a rotary evaporator, the liposomes were dried by removing (all of the) chloroform at approximately 30°C under mild negative pressure. The dried liposomes were dispersed and suspended in aqueous medium. The swelling solution volume was always chosen such that the final phospholipid concentration in the aqueous dispersion was 10mM, linoleic acid was 20 mg/ml and α-linolenic acid 2 mg/ml. The amount of liposomes that was added to each sample was adjusted to approximately 50 nM of phospholipids, 25-100 ng/ml linoleic acid and 1.5-6 ng/ml α-linolenic acid.

Platelets (2 x $10^8$/mL: final concentration in tyrode buffer) were incubated at 37° C for 30 min. with liposomes in the form of LPC/18:2n-6 or LPC/18:3n-3. Platelet pellet obtained from the above procedure was resuspended in tyrode buffer and fixed by 1% paraformaldehyde in PBS containing 0.1% BSA/FFA and washed twice in PBS/BSA 0.1%. Fitc labeled anti-CD61 Mab (IL-Coulter) was used as positive control and a isotype-matched Mab was included in all assays. Analysis of collagen-induced platelet CD40L was performed as reported above (f).
Platelets incubated 30 min. at 37°C with liposomes were also treated to measure O$_2^-$, NADPH oxidase and p38 MAP kinase (see below).

**Platelet O$_2^-$ formation**

Platelet-produced O$_2^-$ was measured using lucigenin chemiluminescence. Platelets (2 x 10^8/mL: final concentration in tyrode buffer, treated or not with liposomes) from each individual (n=3) were analysed in both in basal conditions and after stimulation with collagen 6 µg/mL. Each sample was mixed with 5 µmol/L lucigenin, the chemiluminescence was measured after 3 min, and O$_2^-$ production was expressed as stimulation index (mean level of stimulated platelets divided by the mean of unstimulated platelets) (c). The chemiluminescence of lucigenin was detected with a Bio-Orbit 1251 luminometer. The chemical specificity of this light-yielding reaction for O$_2^-$ has been previously reported (c).

**Platelet NADPH oxidase activity**

Measurement of platelet NADPH oxidase activity was performed in platelet homogenates according to Pignatelli P. et al. (c). Washed platelets (treated or not with liposomes) (2 x 10^8/mL: final concentration in tyrode buffer) were centrifuged and the pellet suspended in homogenate buffer containing: 50 µM Tris/HCl (ph 7.4), 1.0 mM EDTA, 2.0 µM leupeptin and 2.0 µM pepsatin A, and then homogenized. Platelet homogenates were incubated 10 min 37°C with 25 mM NADPH. The assay solution contained 400 µl tyrode buffer and 0.5 µM lucigenin. After preincubation at 37°C for 3 min, the reaction was started by adding 100 µl of platelet homogenates in presence or absence of arachidonic acid 0.5 mM. The chemiluminescent signal was calculated as counts per minute (cpm) for an average of 10 min and corrected by protein concentration (cpm/mg); values were expressed as relative chemiluminescence units (g).
p38MAP kinase

Platelets (2x10^8/ml final concentration in tyrode buffer after treatment or not with liposomes as above described) were stimulated with collagen (6 µg/ml) 3 min at 37°C. Platelets were washed and resuspended in a 2X lysis buffer (5mM EDTA, 0.15M NaCl, 0.1M Tris pH 8.0, 1% Triton and Protease Inhibitor Cocktail).

Equal amounts of protein (30µg/lane) estimated by Bradford assay were solubilized in a 2X Laemmli Sample buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel.

Western Blot analysis was performed with monoclonal anti-p38 MAP KINASE (1µg/ml) incubated overnight at 4°C.

Immune complexes were detected by enhanced chemiluminescence. The rate of p38 MAP KINASE was analyzed by autoradiography. The developed spots were calculated by densitometric analysis on a NIHimage 1.62f analyser and the amount of phosphorylation was determined by dividing the areas of the phosphorylated spots of stimulated platelets by the area of control unstimulated platelets; the value was expressed as relative units.

Statistics

Assuming that treatment with n-3 rich vegetable oil would reduce sCD40L by 30%, we postulated that the sample size of the interventional trial should consist of at least 8 patients for each group (alpha=0.05 and 1-Beta=0.90) (f).

Comparisons between groups were carried out by one-way and repeated measures ANOVA and were replicated as appropriate with nonparametric tests such as the Wilcoxon and Kolmogorov-Smirnov (Z) test in case of non-homogeneous variances, as verified by Levene’s test. Categorical variables were tested by the χ²-test. The correlation analysis was carried out by Pearson’s test. Data
are presented as mean±SD. Statistical significance was defined at p<0.05. Statistical analysis was performed with SPSS 13.0 software for Windows.

REFERENCES


