Flaxseed Oil Supplementation Increases Plasma F$_1$-Phytoprostanes in Healthy Men$^{1,2}$

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Abstract
Supplementation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been reported to reduce lipid peroxidation products formed from arachidonic acid (F$_2$-isoprostanes) in healthy humans, as well as in those under oxidative stress. $\alpha$-Linolenic acid (ALA) is a precursor to EPA and DHA; however, its conversion in humans is thought to be inefficient. ALA can also undergo free radical oxidation, forming compounds known as F$_1$-phytoprostanes, which are found in all plants and are in high concentrations in plant pollens. In this study, we examined the effect of ALA supplementation on plasma and urine F$_1$-phytoprostane and F$_2$-isoprostane concentrations in men. Thirty-six nonsmoking men, aged 20–65 y, were recruited from the general population and randomly allocated to consume 9 g/d of either flaxseed oil (62% ALA, 5.4 g/d) or olive oil (placebo) for 4 wk in a parallel design. At baseline and after 4 wk of supplementation, blood samples and a 24-h urine sample were collected for measurement of plasma and urinary F$_1$-phytoprostanes and F$_2$-isoprostanes and plasma fatty acids. Compared with the olive oil group, plasma phospholipid ALA was greater ($P = 0.0001$), as were F$_1$-phytoprostanes in plasma ($P = 0.049$) and urine ($P = 0.06$) in the flaxseed oil group after 4 wk supplementation. Flaxseed oil did not affect plasma or urinary F$_2$-isoprostanes. The greater plasma F$_1$-phytoprostane concentration in the flaxseed oil group most likely resulted from the increased plasma concentration of the ALA substrate and/or the F$_1$-phytoprostane content of the flaxseed oil. Future studies are needed to determine the physiological importance of increased plasma and urine F$_1$-phytoprostanes and their relevance to heart disease prevention.

Introduction
There is considerable evidence that the long chain (n-3) fatty acids eicosapentaenoic acid (EPA)$^6$ and docosahexaenoic acid (DHA) derived from fish are beneficial in the prevention of cardiovascular disease (CVD) (1). These long-chain fatty acids appear to act via multiple mechanisms that include improvements in lipid profile, blood pressure and heart rate, platelet and immune function, and reducing oxidative stress (2). The role of the plant-derived (n-3) fatty acid, $\alpha$-linolenic acid (ALA), in reducing CVD risk is less clear (3,4). This may be partly because conversion of ALA to the longer chain fatty acids EPA and DHA in humans is relatively inefficient (5).

Oxidative stress resulting in lipid peroxidation is thought to be an important contributor to the atherosclerosis process. Linoleic acid is an abundant PUFA in vivo and its free radical oxidation gives rise to free and esterified hydroperoxyoctadecanoic acids. Free radical attack on arachidonic acid (AA) results in the formation of a group of metabolites called F$_2$-isoprostanes that are produced in situ esterified to phospholipids via the formation of bicyclic endoperoxide intermediates and released in free form by phospholipases. The levels of hydroperoxyoctadecanoic acids and F$_2$-isoprostanes have been shown to be elevated in animal models of oxidative stress, and in humans with conditions associated with increased oxidative stress (6). The F$_2$-isoprostanes are considered to be reliable markers of in vivo lipid peroxidation (7,8). In vitro studies have shown that the long-chain (n-3) fatty acids EPA and DHA can also undergo oxidative modification to forming bicyclic endoperoxide intermediates and F$_3$-isoprostanes and F$_4$-isoprostanes (neuroprostanes), respectively (9,10). In randomized controlled trials in individuals with type 2 diabetes or hyperlipidemia, we showed that EPA and DHA reduced urinary F$_2$-isoprostanes (11–14). We also showed that F$_2$-isoprostanes were reduced in cord blood from neonates whose mothers were supplemented with fish oil during pregnancy (15). In addition, the F$_2$-isoprostane, 8-isoprostane, was significantly reduced after fish oil consumption in normal healthy individuals, suggesting benefits even when individuals are not under overt oxidative stress (16). The
effect of ALA on oxidative stress and specifically F2-isoprostanes has not been assessed. Like the long-chain (n-3) fatty acids, ALA can undergo free radical oxidation to an analogous group of compounds known as F1-phytoprostanes (17). These compounds have been identified in all plants and are found in high concentrations in plant pollens (17). Although little is known of the biological effects of phytoprostanes, they have been identified in urine and are found esterified to lipids in plasma in healthy men given vegetable oil (18). There is some evidence that they play a role in regulation of immune function (19). The purpose of this study was to evaluate the effect of supplementation with ALA on F2-isoprostanes and F1-phytoprostanes. Men were chosen for the study because of the gender differences in conversion of ALA to the long-chain (n-3) fatty acids, in particular EPA (20).

Methods

Participants. Thirty-six men aged between 20 and 65 y were recruited by advertisement from the general population. They were excluded if they had a history of major chronic disease, were smokers, took medication or dietary (n-3) fatty acid supplements, or drank >30 g ethanol/d, equivalent to ~0.3 L/d of wine. All individuals gave informed written consent to participate in the study that was approved by the Human Ethics Committee of the University of Western Australia. The trial was registered with the Australian clinical trials registry no. ACTRN01260600243516.

Study design. After a 2-wk screening period during which blood pressure, plasma lipids, and glucose and liver enzymes were monitored, baseline samples were collected and the men were randomized to take either 9 g/d of flaxseed oil (5.4 g ALA/d) or olive oil, each taken as 1-g capsules for a period of 4 wk in a parallel design. This dose of ALA was chosen because it was similar to that used in a study examining the effects of flaxseed oil on the AA metabolite thromboxane B2 (21). The flaxseed oil capsules contained 620 mg of ALA, 150 mg linoleic acid, and 130 mg oleic acid were obtained from By Nature Natural Health Products. Olive oil capsules contained 67% oleic acid and were obtained from Cardinal Health Australia. The men were asked not to alter their usual food intake and alcohol consumption and to avoid any antioxidant supplements and over-the-counter medication. A questionnaire was administered at the beginning and end of the intervention to assess dietary changes. At baseline and at the end of the 4-wk intervention, blood samples were taken from fasting participants and a 24-h urine sample was collected. Urine F2-isoprostanes and F1-phytoprostanes were measured as were plasma phospholipid fatty acids and serum total and HDL cholesterol. A venous blood sample for measurement of plasma F2-isoprostanes and F1-phytoprostanes was collected into cold tubes containing EDTA and reduced glutathione and centrifuged immediately at 1000 × g for 10 min at 4°C. The plasma was protected from oxidation by the addition of butylated hydroxytoluene at a final concentration of 0.18 mmol/L (40 mg/L) of plasma and stored at ~8°C until analysis. Urine for the same measurements was divided into aliquots and stored at ~8°C until analysis.

Plasma and urinary F1-phytoprostanes. F1-phytoprostanes in plasma (0.25 mL) were hydrolyzed under nitrogen with 1 mol/L KOH in methanol for 40 min at 40°C, utilizing d4-15-F2t-isoprostane (Cayman Chemicals) as an internal standard. This method enables measurement of total F1-phytoprostanes derived from all lipid classes. The sample was acidified to pH 4.6, prior to solid phase chromatography on a prewashed anion exchange column (25 mm × 0.2 mm × 0.33 μm) with helium as the carrier gas. The mass spectrometer was operated in electron capture negative ionization mode using methane as the ionizing gas. F1-phytoprostanes were detected by SIM monitoring mass/charge (m/z) 543 and m/z 573 for d4-15-F1-phytoprostane. Total F1-phytoprostanes were measured in the sample by integrating the peaks corresponding to the authentic F1-phytoprostanes standard (a mixture of all isomers) provided by Imbusch and Mueller (17) and El Fangour et al. (22). Urinary F1-phytoprostanes were measured in the same manner but without alkaline hydrolysis.

Plasma phospholipid fatty acids and serum lipids. Plasma fatty acids were measured in the phospholipid fraction as previously described (24). The phospholipid fatty acid pool was chosen, because it represents the major lipid class from which F1-phytoprostanes are derived (25). Serum triglycerides, total cholesterol, and HDL cholesterol were measured on a COBAS MIRA analyzer (Roche Diagnostics) in the PathWest Laboratories at Royal Perth Hospital.

Statistical power. We estimated that with 18 individuals per group, we would have 80% power to detect a 25% difference in plasma and urinary F1-phytoprostanes and F1-phytoprostanes at a significance level of P = 0.05.

Results

At baseline, the 2 groups were similar with respect to age, BMI, blood pressure, and serum triglycerides and total and HDL cholesterol (Table 1). The men were slightly overweight, but their serum triglycerides were <1.7 mmol/L, total cholesterol was <5.5 mmol/L, and blood pressure was <140/90 mm Hg. After the 4-wk intervention, the groups did not differ in body weight, serum triglycerides, total or HDL cholesterol, or blood pressure (Table 1).

Plasma phospholipid fatty acids. (n-3) Fatty acids were significantly increased following flaxseed oil, but not olive oil,
supplementation. ALA [18:3 (n-3)], EPA [20:5 (n-3)], and 22:5 (n-6) were all elevated (P < 0.01), and the (n-6) fatty acids 20:3 (n-6) and 22:3 (n-6), were reduced (P < 0.01) in the flaxseed oil group (Table 2). However, DHA [22:6 (n-3)] was not significantly altered after supplementation with flaxseed oil (Table 2). Relative to the olive oil group, AA [20:4 (n-6)], the substrate for F2-isoprostanes, was not significantly altered by flaxseed oil supplementation and no significant change in linoleic acid [18:2 (n-6)] was observed with flaxseed oil (Table 2).

### Table 1: Characteristics of men at baseline and after 4 wk of supplementation with 9 g/d olive or flaxseed oil

<table>
<thead>
<tr>
<th></th>
<th>Olive oil</th>
<th>Flaxseed oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Baseline</td>
<td>51 ± 2.0</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>Baseline</td>
<td>25.6 ± 0.7</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>Baseline</td>
<td>81.0 ± 2.9</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>Baseline</td>
<td>81.3 ± 3.0</td>
</tr>
<tr>
<td>Serum total cholesterol, mmol/L</td>
<td>Baseline</td>
<td>1.06 ± 0.09</td>
</tr>
<tr>
<td>Serum HDL-cholesterol, mmol/L</td>
<td>Baseline</td>
<td>5.04 ± 0.18</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>Baseline</td>
<td>119 ± 3.5</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>Baseline</td>
<td>69 ± 1.7</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 18. Groups did not differ at baseline or after 4 wk, adjusted for baseline value.

### Table 2: Plasma phospholipid fatty acid composition at baseline and after 4 wk of supplementation with 9 g/d olive or flaxseed oil

<table>
<thead>
<tr>
<th>Fatty acid, g/100 g total fatty acids</th>
<th>Olive oil</th>
<th>4 wk</th>
<th>Flaxseed oil</th>
<th>4 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.43 ± 0.03</td>
<td>0.39 ± 0.02</td>
<td>0.43 ± 0.02</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>16:0</td>
<td>0.28 ± 0.29</td>
<td>0.27 ± 0.31</td>
<td>0.28 ± 0.27</td>
<td>0.27 ± 0.30</td>
</tr>
<tr>
<td>18:1(n-7)</td>
<td>0.88 ± 0.06</td>
<td>0.83 ± 0.06</td>
<td>0.88 ± 0.05</td>
<td>0.85 ± 0.06</td>
</tr>
<tr>
<td>18:0</td>
<td>13.83 ± 0.24</td>
<td>13.83 ± 0.22</td>
<td>13.89 ± 0.24</td>
<td>14.21 ± 0.23</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>12.37 ± 0.28</td>
<td>12.45 ± 0.34</td>
<td>11.95 ± 0.37</td>
<td>11.66 ± 0.27</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>20.85 ± 0.81</td>
<td>21.33 ± 0.75</td>
<td>20.5 ± 0.58</td>
<td>20.20 ± 0.53</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.23 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.71 ± 0.05*</td>
</tr>
<tr>
<td>18:4(n-3)</td>
<td>0.53 ± 0.03</td>
<td>0.56 ± 0.02</td>
<td>0.50 ± 0.02</td>
<td>0.56 ± 0.20</td>
</tr>
<tr>
<td>20:3(n-6)</td>
<td>3.21 ± 0.19</td>
<td>3.23 ± 0.18</td>
<td>3.18 ± 0.16</td>
<td>2.83 ± 0.15*</td>
</tr>
<tr>
<td>20:4(n-6)</td>
<td>10.19 ± 0.39</td>
<td>10.03 ± 0.41</td>
<td>10.05 ± 0.36</td>
<td>9.58 ± 0.38</td>
</tr>
<tr>
<td>20:5(n-3)</td>
<td>1.20 ± 0.06</td>
<td>1.06 ± 0.08</td>
<td>1.40 ± 0.96</td>
<td>2.05 ± 0.12*</td>
</tr>
<tr>
<td>22:3(n-6)</td>
<td>0.39 ± 0.03</td>
<td>0.37 ± 0.02</td>
<td>0.34 ± 0.02</td>
<td>0.30 ± 0.01*</td>
</tr>
<tr>
<td>22:4(n-6)</td>
<td>1.23 ± 0.05</td>
<td>1.24 ± 0.03</td>
<td>1.17 ± 0.03</td>
<td>1.27 ± 0.04</td>
</tr>
<tr>
<td>22:5(n-3)</td>
<td>1.21 ± 0.05</td>
<td>1.19 ± 0.05</td>
<td>1.22 ± 0.04</td>
<td>1.46 ± 0.05*</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>3.65 ± 0.17</td>
<td>3.53 ± 0.12</td>
<td>4.55 ± 0.32</td>
<td>4.29 ± 0.29</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 18. Groups did not differ at baseline. *Different from olive oil at 4 wk, adjusted for baseline values, P < 0.01.
F2-isoprostanes, a peak corresponding to the retention time of d4-15-F2t-isoprostane identifies isoprostanes similar to 15-F2t-isoprostane. In contrast, quantification of F1-phytoprostanes utilizes a number of peaks corresponding to authentic F1-phytoprostanes (mixture of all isomers) between the retention times of 11.8 min and 13 min and relates that area to the d4-15-F2t IsoP internal standard.

F1-phytoprostane content of the oils. Both of the oils contained F1-phytoprostanes; flaxseed oil contained 25.6 mg/L and olive oil contained 12.9 mg/L.

Discussion

This is the first study, to our knowledge, showing that supplementation with 9 g/d of flaxseed oil for 4 wk leads to significant increases in oxidation products of ALA (F1-phytoprostanes) in plasma compared with olive oil. The increase in plasma F1-phytoprostanes in the flaxseed oil group appears to be due to an increase in ALA and/or intake of F1-phytoprostanes present in the flaxseed oil. Flaxseed oil did not affect oxidation products of AA (F2-isoprostanes) in plasma or urine, indicating that flaxseed oil supplementation had no effect on plasma or whole-body lipid peroxidation. This finding is in contrast to the effects of long-chain (n-3) fatty acids derived from fish that we have shown to reduce urinary F2-isoprostanes in healthy (16) as well as in diabetic and hyperlipidemic (11,12) individuals and plasma F2-isoprostanes in neonates (14).

In this study, supplementation for 4 wk with a relatively high dose of ALA (5.4 g/d) led to increases of ~2- and 0.5-fold in plasma phospholipid ALA and EPA levels, respectively. This observation is consistent with findings from a randomized trial comparing similar doses of ALA and fish oil supplementation for 4 wk (21). The incorporation of ALA into plasma phospholipids after flaxseed oil supplementation was at the expense of 20:3(n-6) and 22:3(n-6), but not AA. This is in contrast to studies supplementing with EPA, DHA, or fish oil (11,12,14) that consistently and significantly reduce AA concentrations.

In an open trial comparing supplementation of food with 20 g/d of ALA as flaxseed or an equivalent amount of monounsaturated fat as sunola, there was a 9-fold increase in total plasma fatty acid ALA from 0.4 to 3.5% that did not alter total cholesterol or triglycerides (26). We did not observe any differences in serum triglycerides or total or HDL cholesterol after supplementation with ALA for 4 wk. This is not a surprising result given that the men in our study were healthy.
with a normal lipid profile. Our study agrees with previous findings of a lack of effect of ALA on plasma lipid profiles (26), the exception being when ALA equivalent to 3.8 g/d was given to hypercholesterolemic individuals as ground flaxseed for 10 wk as a part of a low-fat diet (27). In that study, the decrease in LDL cholesterol observed after 5 wk, and the reduction in HDL cholesterol in men, could have been due to the flaxseed lignans and dietary fiber rather than the (n-3) fatty acids (27).

We measured total F1-phytoprostanes in plasma after base hydrolysis, because F1-phytoprostanes are esterified in plasma (18). We found that flaxseed oil supplementation significantly increased the concentration of plasma F1-phytoprostanes relative to olive oil. However, when we adjusted for changes in plasma ALA composition, the difference in plasma F1-phytoprostanes between the 2 groups was no longer significant. This finding, and the significant correlation between changes in ALA content and changes in plasma F1-phytoprostanes after supplementation with flaxseed oil but not olive oil, suggests that the increase in plasma ALA (the substrate for F1-phytoprostanes) in the flaxseed oil group was responsible for increased plasma F1-phytoprostanes. Furthermore, the change in ALA in the flaxseed oil group accounted for 38% of the variance in plasma F1-phytoprostanes in this group. Urinary excretion of F1-phytoprostanes also tended to be elevated after flaxseed oil supplementation (P = 0.06).

Both the encapsulated olive oil and flaxseed oils contained measurable concentrations of F1-phytoprostanes. This confirms a previous report that plant-derived oils contain quantities of F1-phytoprostanes (18). Therefore, it is possible that raised concentrations of F1-phytoprostanes in the flaxseed oil may have contributed to elevated concentrations of F1-phytoprostanes in plasma and urine after supplementation. It is of interest that prior to the intervention, plasma F1-phytoprostanes were significantly correlated with the percentage of ALA in plasma phospholipids. The baseline concentration of F1-phytoprostanes in plasma and urine were also relatively high, suggesting that F1-phytoprostanes in the diet may make a substantial contribution to the concentration in plasma and urine.

Our study confirms a previous report that flaxseed oil does not alter markers of AA-derived lipid peroxidation. Bloedon et al. (27) showed that 3.8 g/d of ALA given to hypercholesterolemic individuals for 10 wk did not alter urinary 8,12-iso-PF2α-VI. Similarly, we showed that plasma F2-iso prostanes were not altered by 4-wk supplementation with flaxseed oil regardless of whether or not values were corrected for AA substrate. Urinary F2-iso prostanes were also not altered by flaxseed oil supplementation.

The lack of effect of ALA on plasma and urinary F2-iso prostanes contrasts studies of supplementation with long-chain fatty acids EPA or DHA or fish oils in healthy individuals (16) and those under oxidative stress (12–14) that have consistently shown that F2-iso prostanes were reduced by these oils independent of changes in AA substrate concentration (12). Our study was powered to show a 25% difference in plasma and urinary F2-iso prostanes and we cannot exclude that a smaller change may have been detected if larger numbers of individuals had been studied. We have suggested that the antioxidative effects of EPA and DHA manifested by a reduction in F2-iso prostanes are likely related to their antiinflammatory effects and their ability to suppress production of reactive oxygen species, including superoxide and hydrogen peroxide, by stimulated leukocytes. Although a recent study in the LDL knockout mouse suggests that ALA has antiinflammatory actions (28), in vivo studies in humans have yet to confirm this finding. In addition, it is unlikely that lipid peroxidation is redirected toward oxidation of EPA and DHA, as we did not detect F3 or F4-isoprostanes in our studies (14).

The role of ALA in prevention of CVD is controversial (1,2). However, a recent large case control study from Costa Rica showed that increased ALA consumption, assessed by adipose tissue composition or questionnaire, was associated with lower risk of myocardial infarction (29). Furthermore, fish or EPA and DHA intake did not modify the effect of ALA consumption on myocardial infarction (29). The mechanism by which ALA might reduce the risk of myocardial infarction is not clear, but it does not appear to be via conversion to EPA or DHA. Increased consumption of ALA is widely advocated for health benefit; the AHA recommends the inclusion of vegetable oils and food sources high in ALA to achieve an intake of ALA intake between 1.5 and 3 g/d (30). Given that this study shows that ALA supplementation results in significant increases in F1-phytoprostanes, further research into the biological actions of F1-phytoprostanes is needed.

Our study was powered to show a 25% difference in plasma and urine F2-isoprostanes derived from AA. This suggests that supplementation with large doses of flaxseed oil, similar to those used in this study, are unlikely to produce beneficial effects on oxidative stress in a manner similar to those for fish oils (11–14). However, the possibility exists that increased ALA intake may attenuate inflammatory responses by forming F1-phytoprostanes. Further studies are required to comprehensively study the bioavailability and physiological effects of the F1-phytoprostanes. Studies are also required to determine the doses of ALA that increase F1-phytoprostane concentration.

**Literature Cited**


