Dietary α-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects

Guixiang Zhao, Terry D Etherton, Keith R Martin, Peter J Gillies, Sheila G West, and Penny M Kris-Etherton

ABSTRACT

Background: Atherosclerosis is a chronic inflammatory disease. We previously reported that a diet high in α-linolenic acid (ALA) reduces lipid and inflammatory cardiovascular disease risk factors in hypercholesterolemic subjects.

Objective: The objective was to evaluate the effects of a diet high in ALA on serum proinflammatory cytokine concentrations and cytokine production by cultured peripheral blood mononuclear cells (PBMCs) from subjects fed the experimental diets.

Design: A randomized, controlled, 3-diet, 3-period crossover study design was used. Hypercholesterolemic subjects (n = 23) were assigned to 3 experimental diets: a diet high in ALA (ALA diet; 6.5% of energy), a diet high in linoleic acid (LA diet; 12.6% of energy), and an average American diet (AAD) for 6 wk. Serum interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α) concentrations and the production of IL-6, IL-1β, and TNF-α by PBMCs were measured.

Results: IL-6, IL-1β, and TNF-α production by PBMCs and serum TNF-α concentrations were lower (P < 0.05 and P < 0.08, respectively) with the ALA diet than with the LA diet or AAD. PBMC production of TNF-α was inversely correlated with ALA (r = −0.402, P = 0.07) and with eicosapentaenoic acid (r = −0.476, P = 0.03) concentrations in PBMC lipids with the ALA diet. Changes in serum ALA were inversely correlated with changes in TNF-α produced by PBMCs (r = −0.423, P < 0.05).

Conclusions: Increased intakes of dietary ALA elicit antiinflammatory effects by inhibiting IL-6, IL-1β, and TNF-α production in cultured PBMCs. Changes in PBMC ALA and eicosapentaenoic acid (derived from dietary ALA) are associated with beneficial changes in TNF-α release. Thus, the cardioprotective effects of ALA are mediated in part by a reduction in the production of inflammatory cytokines.


KEY WORDS Hypercholesterolemic subjects, α-linolenic acid, eicosapentaenoic acid, interleukin-6, interleukin-1β, tumor necrosis factor-α, peripheral blood mononuclear cells

INTRODUCTION

A Mediterranean-style diet high in α-linolenic acid (ALA) has been shown to markedly reduce the recurrence rate after a first myocardial infarction (1). Epidemiologic studies have also shown that a higher intake of ALA protects against fatal ischemic heart disease in women (2) and is associated with a reduced prevalence of coronary artery disease in both men and women (3). ALA can decrease the risk of cardiovascular disease (CVD) through various biological mechanisms, including reductions in lipids, in lipoproteins, and in the inflammatory markers C-reactive protein (CRP) and cell adhesion molecules (4).

The role of ALA in reducing inflammation is important because inflammatory events are central in the pathogenesis of atherosclerosis (5–7). Activated macrophages and lymphocytes secrete proinflammatory cytokines, including interleukin (IL)-6, IL-1, and tumor necrosis factor-α (TNF-α) (8). These cytokines then activate the endothelial expression of cell adhesion molecules and mediate a series of inflammatory responses such as the up-regulation of acute phase protein expression (9).

Long-chain, marine-derived, n–3 fatty acids [ie, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] elicit antiatherogenic and antiinflammatory effects by modulating endothelial function (10). Fish-oil supplementation suppresses proinflammatory cytokine production in peripheral blood mononuclear cells (PBMCs) (11–15), inhibits lymphocyte proliferation (12, 16), and decreases natural killer cell activity (15, 17). Flaxseed oil consumption (providing 14 g ALA/d) also inhibits IL-1β and TNF-α synthesis in free-living subjects instructed to consume a diet high in n–3 fatty acids (14). However, a similar study reported that an intake of ≤9.5 g ALA/d or ≤1.7 g EPA + DHA/d did not affect cytokine production by PBMCs (18). Moreover, in vitro studies have not consistently shown that n–3 fatty acids reduce inflammation. For example, we have reported that monocyctic THP-1 cells cultured with LA, ALA, and DHA had a reduced secretion of IL-6, IL-1β, and TNF-α (19). However, Toborek et al (20) reported that LA increased the
expression of several genes involved in the inflammatory response, including TNF-α; the effects of ALA paralleled this, albeit to a lesser extent for most markers evaluated.

Dietary recommendations for n-6 fatty acids are 5–10% of total energy (National Academies, 2002; US Dietary Guidelines, 2005) or up to 10% of energy (National Cholesterol Education Program Adult Treatment Panel III). An increase in the LA content over this range in a diet low in saturated fatty acids (SFAs), trans fatty acids, and cholesterol significantly decreases LDL cholesterol. However, there is some concern that an increase in n-6 fatty acids increases inflammation (21–23). Given that n-3 fatty acids exert antiinflammatory effects, an increase in the dietary content of n-3 fatty acids may blunt or attenuate the n-6 response in a diet high in LA. This is important because maximal reduction in LDL cholesterol is a primary treatment goal in clinical practice (24), and lifestyle interventions, such as diet, are the first step in the treatment of hypercholesterolemia. The present controlled clinical study evaluated whether 2 diets high in ALA (3.6% and 6.5% of energy) within the context of diets also high in LA (12.6% and 10.5% of energy) would elicit an antiinflammatory effect as measured by decreases in both serum cytokine concentrations and in the production of proinflammatory cytokines by cultured PBMCs obtained from subjects fed the experimental diets.

SUBJECTS AND METHODS

Subjects and study design

The study design was described previously (4) and is summarized in Figure 1. Briefly, 20 men aged 36–69 y and 3 postmenopausal women aged 55–65 y (who had not received hormone replacement therapy in the previous 6 mo) with moderate hypercholesterolemia [serum total cholesterol (TC) between 4.81 and 7.11 mmol/L, LDL cholesterol between 3.03 and 5.07 mmol/L] and who were overweight or obese [body mass index (BMI; kg/m²) between 25 and 35] were enrolled in the study. All subjects were otherwise healthy, not taking any lipid-lowering or antiinflammatory medications, and not taking nutritional supplements. The study protocol was approved by the Institutional Review Board of The Pennsylvania State University. Written informed consent was obtained from each subject.

The subjects were assigned to a sequence of 3 experimental diets: an average American diet (AAD), a diet high in polyunsaturated fatty acids (PUFAs) and ALA (ALA diet), and a diet high in PUFAs and linoleic acid (LA diet) for 6 wk with a break between diet periods of ≤3 wk, the intent of which was to improve diet compliance (not for washout). The nutrient composition of the experimental diets is presented in Figure 1. For a diet that provided ≈2400 kcal/d, 36.9 g/d walnuts and ≈13.5 g/d walnut oil were used to increase dietary ALA and LA intakes in the experimental diets. In addition, flaxseed oil (≈13.5–20.3 g/d) was used to increase the ALA content of the LA diet. The amounts of ALA and LA in the AAD, ALA diet, and LA diet were 2.3 and 22.1 g, 19.1 and 30.8 g, and 10.6 and 37.3 g, respectively. At the end of each diet period, blood samples were collected on 2 consecutive days, after the subjects had fasted for 12 h, for subsequent endpoint assays.

Materials and reagents

The human recombinant cytokines IL-1β, IL-6, and TNF-α; mouse anti-human monoclonal antibodies; and goat anti-human polyclonal antibodies against IL-1β, IL-6, and TNF-α were purchased from R&D Systems (Minneapolis, MN). Escherichia coli lipopolysaccharide (LPS, strain 055:B5) was obtained from Sigma (St Louis, MO). RPMI-1640 medium (without l-glutamine and phenol red) was obtained from Gibco Life-Technologies (Grand Island, NY). TCH serum replacement was purchased from CELOX Laboratories Inc (St Paul, MN).

Endpoint assays

PBMC isolation and culture

At the end of each diet period, 25 mL blood was collected into heparinized tubes after the subjects had fasted for 12 h. PBMCs were isolated by using a Ficoll-histopaque-1077 gradient following the protocol previously described (11, 12). Briefly, 25 mL blood was first diluted with an equal volume of sterile, pyrogen-free saline and then loaded onto 10 mL Ficoll histopaque-1077. After centrifugation at 600 × g for 45 min at 18 °C, the interface mononuclear cell layer was drawn out and washed with sterile saline 3 times. The cells were suspended at 5 × 10⁶ cells/mL in RPMI-1640 medium supplemented with 2% TCH serum replacement; 0.5 mL cell suspension (ie, 2.5 × 10⁶ cells/well) was added to each well on a 24-well plate. The cell suspension (0.5...
mL) was added to either 0.5 mL RPMI-1640 medium or RPMI-1640 medium containing LPS at 1 ng/mL and 10 ng/mL. Cells were cultured for 24 h at 37 °C in a humid atmosphere containing 5% CO₂.

**Fatty acid profile of PBMCs**

Total lipids were extracted from PBMCs with the use of a chloroform:methanol mixture (1:1, by vol) containing 250 μg butylated hydroxyl toluene (used as an antioxidant; Sigma) and 240 nmol heptadecanoic acid (used as an internal standard; Nu-Chek Prep Inc, Elysian, MN). Lipid extracts were dried under nitrogen at 50 °C and resuspended in 150 μL of the chloroform: methanol mixture (2:1, by vol).

Methylation of fatty acids and the separation of fatty acid methyl esters was performed on an SP-2330 capillary column (30 m × 0.25 mm with 0.2 μm film; Supelco, Bellefonte, PA), installed in a Hewlett-Packard 5890 II gas chromatograph, following the protocol described previously (4, 19). Peak areas were integrated as relative weight by using Hewlett-Packard ChemStation software. Percentages of individual fatty acids were calculated according to the peak areas relative to the total area (total fatty acid was set at 100%).

**Statistical analyses**

Statistical analyses were performed by using SAS software (version 8.2; SAS Institute Inc, Cary, NC). Data from the male and female participants were pooled because the 3 female subjects were postmenopausal. Results were expressed as least-squares means ± SEM. Data for serum IL-6, IL-1β, and TNF-α were logarithmically transformed (base 10) before conducting analyses because of skewed distributions. The mixed procedure (PROC MIXED) was used to test for effects of diet, order (the sequence of the 3 diets given to each subject), and their interactive effects on each outcome variable. When significant diet effects were detected, a Tukey’s least-significant difference tests was performed to detect individual differences between 2 diets. Pearson correlation analyses were performed to test the associations between individual fatty acids and cytokines. Probability values ≤0.05 were considered statistically different, and probability values ≤0.1 denoted a trend for significance.

**RESULTS**

**Effect of diets on serum cytokines**

In subjects who had serum concentrations of the proinflammatory cytokines below the limit of detection (6 subjects who had serum IL-6 concentrations below the detection limit; 2 for IL-1β and 7 for TNF-α), the values of a half of the detection limits were used for these individuals during data analyses. Serum concentrations of IL-6 and IL-1β did not change after subjects consumed the 3 diets (Table 1). However, serum TNF-α concentrations were lower when the subjects were consuming the ALA diet than when consuming the LA diet and the AAD (P < 0.08). None of the order effects or the interactions of diets with orders were statistically significant.

**Effect of diets on cytokine production in PBMCs**

When PBMCs were cultured in the absence of LPS, the production of IL-6, IL-1β, and TNF-α was low and for the most part not detectable (data not shown). Incubation of PBMCs with LPS at 1 ng/mL for 24 h significantly induced cytokine production (data not shown). The production of IL-6, IL-1β, and TNF-α by PBMCs was lower by PBMCs was lower after consumption of the ALA diet than after the AAD (P < 0.05), and the production of IL-1β and TNF-α was even lower when compared with the LA diet (P < 0.05). The decrease in cytokine production was not associated with changes in the total count or in the composition of white blood cells in the systemic circulation; these measurements were not affected by the 3 diets (data not shown).

Changes in serum ALA were significantly inversely correlated (r = −0.423, P < 0.05) with changes in PBMC TNF-α concentrations when the subjects were consuming the ALA diet (Figure 2). However, similar correlations were not observed for PBMC IL-6 and IL-1β concentrations.

### TABLE 1

Cytokine concentrations in serum and peripheral blood mononuclear cell (PBMC) cultures

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>AAD (ng/L)</th>
<th>LA diet (ng/L)</th>
<th>ALA diet (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>239.9 (4.8–7320.3)</td>
<td>169.6 (4.8–7626.6)</td>
<td>107.9 (4.8–7875.1)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>29.8 (2.4–12048.8)</td>
<td>42.5 (2.4–9417.7)</td>
<td>20.0 (2.4–12072.7)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>18.2 (2.4–1232.5)</td>
<td>13.3 (2.4–1513.0)</td>
<td>10.3 (2.4–1192.6)</td>
</tr>
</tbody>
</table>

1 AAD, average American diet; LA, linoleic acid; ALA, α-linolenic acid; IL, interleukin; TNF-α, tumor necrosis factor-α.
2 All values are medians; ranges in parentheses.
3 Significantly different from the AA and LA diets, P < 0.08 (PROC MIXED model and Tukey’s least-squares-means test).
4 All values are x ± SEM; n = 23. Values in a row with different superscript letters are significantly different, P < 0.05 (PROC MIXED model and Tukey’s least-squares-means test).
The PBMC production of TNF-α was inversely correlated with ALA and EPA in PBMC lipids \( (P = 0.071 \) and \( P = 0.029 \), respectively; Figure 3) when subjects were consuming the ALA diet. However, similar correlations were not observed for PBMC IL-6 and IL-1β concentrations. The data suggest that n-3 fatty acid enrichment, notably ALA and EPA, in PBMC lipids inhibits TNF-α production.

**DISCUSSION**

In the present study, changes in serum ALA were inversely correlated with changes in PBMC TNF-α concentrations, and PBMC ALA and EPA concentrations also were inversely correlated with the production of TNF-α. These data suggest that the increased incorporation of ALA and EPA into PBMC lipids may, in part, account for the observed differences in proinflammatory cytokine release in response to a diet high in ALA. Importantly, the decrease in proinflammatory cytokines occurred in a cell environment high in LA. Of significance is that the amount of LA in the PBMC lipids was similar in subjects consuming the LA and ALA test diets. Given that the amount of n-6 fatty acids was comparable in PBMC lipids with the LA and ALA diets, the cytokine response observed in subjects consuming the ALA diet appears to reflect differences in ALA, EPA, or both in PBMC lipids. Moreover, we did not see an increase (and, in fact, a trend for a decrease) in the proinflammatory cytokines in subjects consuming the LA diet. This might be explained by the higher n-3 fatty acid concentration, particularly ALA, in PBMCs with the LA diet than with the AAD. Whereas other investigators have reported an inflammatory response of n-6 fatty acids (20–23), our results suggest that a diet high in n-3 fatty acids can attenuate or blunt this response.

The proinflammatory cytokines IL-6, IL-1, and TNF-α are central mediators of chronic inflammation associated with atherogenesis (25–27). Several studies have shown that a diet supplemented with fish oil (high in EPA and DHA) and flaxseed...
oil (high in ALA) significantly decreased serum IL-1β and TNF-α concentrations and other inflammatory mediators such as prostaglandin E₂ and leukotriene B₄ (11–15). Fish-oil supplementation has been shown to be associated with a decreased production of inflammatory cytokines in both healthy subjects and rheumatoid arthritis patients (11–14). A recent study reported that the intake of EPA and DHA was inversely correlated with the plasma soluble TNF receptors TNF-R1 and TNF-R2, which are markers of TNF-α activity (28). In a controlled clinical study our group conducted to evaluate the effects of diets high in LA and ALA (derived from walnuts, walnut oil, and flaxseed oil) on multiple cardiovascular disease risk factors, we observed a decrease in the inflammatory marker CRP and cell adhesion molecules in addition to lipid-lowering effects elicited by test diets high in LA and ALA (4). Moreover, our in vitro study also showed that ALA, LA, and DHA reduced the LPS-stimulated production of IL-6, IL-1β, and TNF-α in a dose-dependent manner in THP-1 cells (19). Consistent with these findings, the present study showed that a diet high in ALA (providing 19.1 g ALA/d) inhibits the PBMC production of IL-6, IL-1β, and TNF-α and decreases serum TNF-α concentrations. These results agree with the findings from the study conducted by Caughey et al (14) in free-living subjects who consumed ALA at 14 g/d. The lack of a beneficial effect of ALA on the production of cytokines by PBMCs, as reported by Kew et al (18), may result from a lower intake of ALA (≤9.5 g/d), which is similar to what we found in subjects who consumed the LA diet (providing 10.6 g ALA/d) in the present study. Because ALA is the most commonly consumed n-3 fatty acid in Western diets, these results suggest that dietary ALA, specifically at higher intakes than currently consumed, could be a simple-to-implement anti-inflammatory intervention. This strategy, in combination with low intakes of dietary saturated fat and adequate intakes of n-6 fatty acids to achieve a significant cholesterol-lowering response, will likely further reduce the risk of CVD beyond that achieved by reducing LDL cholesterol alone.

The mechanisms by which dietary fatty acids inhibit cytokine production are not clear. Induction of cytokine gene expression is regulated by a common pathway, ie, activation of nuclear factor κB (NF-κB) signaling (29, 30). The presence of NF-κB binding sites has been found in the promoter regions of the IL-6, IL-1, and TNF-α genes (31). Studies have shown that statins and oleic acid inhibit inflammatory responses within the atherosclerotic lesions by inhibiting NF-κB activity in vascular smooth muscle cells and mononuclear cells (32, 33). In our in vitro study also showed that ALA, LA, and DHA inhibited the NF-κB DNA binding activity in THP-1 cells (19), which has been shown to be associated with activation of peroxisome-proliferator activated receptor γ (PPAR-γ) (19, 34, 35). Thus, dietary ALA appears to elicit antiinflammatory effects via activation of PPAR-γ.

In addition to the favorable effects of ALA on cytokine production, we also found that the diet high in PUFAs and ALA significantly decreased serum CRP and cell adhesion molecules, including vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1, and E-selectin (4). The present study provides important information about a potential mechanism that mediates the favorable effects of ALA on these inflammatory markers. It has been shown that the proinflammatory cytokines play a key role in regulating CRP production and cell adhesion molecule expression (33, 36–40). One recent study showed that TNF-α was a better predictor of cell adhesion molecule expression than was IL-6 in patients with coronary heart disease (41). Thus, our finding that PBMC TNF-α concentrations decreased with the ALA diet may be important in the prevention or treatment of heart disease. The lack of a significant decrease in serum IL-6 and IL-1β in subjects consuming the LA and ALA diets could be explained, in part, by the marked variability in these endpoints. Importantly, there was a trend for serum TNF-α to decrease with the ALA diet. Nonetheless, the PBMC culture data consistently showed a beneficial effect of a diet high in LA and ALA on cytokine production. Collectively, the data from the present study and the results from our previous studies (4, 19) showed that ALA beneficially affects multiple CVD risk factors.

In conclusion, a diet high in ALA confers cardiovascular benefits, in part by exerting an antiinflammatory effect. Evidence from the present study and our previous report (4) indicates that the antiinflammatory effects of ALA involve multiple markers of inflammation. Given that inflammation is an important component of all steps in the progression of atherosclerosis, it appears that ALA exerts its cardioprotective effect in a manner that involves many components of the inflammatory response. Importantly, the responses we observed occurred in a high n-6 fatty acid environment. Thus, strategies to increase ALA in the diet are

![FIGURE 3. Inverse correlations between the contents of α-linolenic acid (ALA) and eicosapentaenoic acid (EPA) in peripheral blood mononuclear cell (PBMC) lipids and tumor necrosis factor-α (TNF-α) concentrations produced by PBMCs when subjects (n = 23) were consuming the diet high in ALA (Pearson correlation analysis).](image-url)
important from the perspective of vascular inflammation to reduce CVD risk. Maintaining an adequate intake of n–6 fatty acids is important for lowering LDL-cholesterol concentrations (42). Our evidence suggests that n–6 fatty acid intake can be increased without any adverse inflammatory response if sufficient n–3 fatty acids are provided. Thus, an adequate intake of n–3 and n–6 PUFAs is central to achieving CVD risk benefits. Consequently, incorporating food sources of ALA in the diet, such as walnuts, walnut oil, and flaxseed oil, is important for the next generation of dietary strategies to reduce CVD risk. We believe that a diet high in ALA is an important prerequisite for the evolving diet strategies that further reduce the risk of CVD.

GZ, TDE, SGW, PIG, and PMK-E were responsible for the study design, endpoint analyses, data analyses and interpretation, and writing of the manuscript. KRM assisted in establishing the fatty acid assays and provided valuable expertise in the conduct of the experiments. All authors reviewed the manuscript and provided scientific and editorial input. None of the authors had a conflict of interest.

REFERENCES


