Supplementation With ω3 Polyunsaturated Fatty Acids and *all-rac* Alpha-Tocopherol Alone and in Combination Failed to Exert an Anti-inflammatory Effect in Human Volunteers

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There is growing evidence supporting the importance of inflammation in all stages of atherosclerosis. While both ω-3 polyunsaturated fatty acids (n3PUFA) and the lipid-soluble antioxidant alpha-tocopherol (AT) have been shown to independently have significant anti-inflammatory effects, there is paucity of data examining the effect of n3PUFA alone and in combination with AT on markers of inflammation and monocyte function. Therefore, we tested the effect of n3PUFA alone, *all-rac* (synthetic) AT alone, and the combination on markers of inflammation and monocyte function. Healthy nonsmoking volunteers were randomly assigned to 1 of 4 groups (n = 20 per group): 1.5 g/d n3PUFA, 800 IU/d AT, 1.5 g n3PUFA + 800 IU/d AT, or placebo in a parallel double-blinded study. Compared to baseline, 12 weeks of supplementation resulted in no changes in plasma lipids regardless of treatment. Plasma AT was significantly increased only in those groups that received AT ($P < .0001$). Similarly, groups receiving n3PUFA showed a significant increase in plasma docosahexaenoic acid ($P < .0001$). No significant within- or between-group differences were found for plasma levels of high-sensitivity C-reactive protein (hsCRP). Furthermore, there were no differences in monocyte proinflammatory cytokine release (interleukin [IL]-1β, tumor necrosis factor [TNF]-α and IL-6) after activation with monocyte chemotactic protein-1 (MCP-1). In conclusion, supplementation with n3PUFA and *all-rac* AT at these doses is not anti-inflammatory.

FOR YEARS, the main cause of atherosclerosis was considered to be the accumulation of lipids in the arterial wall. However, there is growing evidence supporting the importance of inflammation as a contributor for the initiation and progression of atherosclerosis.[1,2] Several proinflammatory risk factors such as oxidized low-density lipoprotein (LDL) trigger a proinflammatory response that results in the increased secretion of interleukin (IL)-1β and tumor necrosis factor (TNF)-α derived from monocyte-macrophages or adipose tissue. These cytokines promote the release of the messenger cytokine IL-6, which stimulates de novo hepatic synthesis and release of C-reactive protein (CRP), the prototypic marker of inflammation.[2-4] Numerous studies have shown that a high CRP level predicts cardiovascular events.[5,6]

Alpha-tocopherol (AT) is the most abundant and potent lipid-soluble antioxidant present in LDL and tissues.[7] AT has proven to be effective to decrease LDL susceptibility to oxidation in vitro.[8,9] In addition to the antioxidant functions, AT modulates cell signaling and transcription of several genes, processes that might be involved in the antiatherosclerotic and anticarcinogenic properties of AT.[10]

We have previously reported an anti-inflammatory effect of high-dose (1,200 IU/d) natural (RRR)-AT supplementation characterized by decreased IL-1β, TNF-α, and IL-6 secretion and reduced monocyte adhesion to endothelial cells.[11] In addition, AT supplementation has been shown to decrease CRP concentrations in healthy and diabetic individuals at doses of 800 IU/d RRR-AT[12] and 1,200 IU/d RRR-AT.[13]

There is also increasing evidence showing that ω-3 polyunsaturated fatty acids (n3PUFA) may be beneficial against cardiovascular disease. When incorporated into a low-fat diet, n3PUFA have been shown to decrease platelet aggregation and blood pressure.[14] n3PUFA supplementation has also been reported to inhibit mononuclear cell proliferation and monocyte cytokine production.[15-17] Moreover, compared to a control group, mice fed n3PUFA developed significantly smaller atherosclerotic lesions after additional supplementation with saturated fatty acids.[18]

Thus both RRR-AT and n3PUFA have been independently shown to favorably affect monocyte function. Furthermore, in the majority of clinical trials using *all-rac* AT, the primary end point was negative.[19] This could possibly be due to *all-rac* AT not having anti-inflammatory effects. Therefore, the major aims of this study were to test if supplementation with the synthetic form of vitamin E, *all-rac* AT (800 IU/d), would exert similar anti-inflammatory effects as the natural isomer RRR-AT and to test if the addition of n3PUFA (1.5 g/d) to AT supplementation (800 IU/d) would further enhance the anti-inflammatory effects of AT.

MATERIALS AND METHODS

Subjects

Eighty healthy male and premenopausal female volunteers between the ages of 20 and 55 years were included in the study without restriction to race, gender, or socioeconomic status. Volunteers were not accepted in the study if they met with one or more of the following exclusion criteria: smoking; diabetes; antioxidant or vitamin supplementation; consumption of more than 1 meal of fish per week or n3PUFA supplements within the past 5 months; chronic disease or gastrointestinal problems; bleeding diathesis; abnormal complete blood cell count or renal, liver, or thyroid function; alcohol intake greater than 1 oz/d; use of hypolipidemic drugs, thyroid drugs, nonsteroidal anti-

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Table 1. Characteristics of Participants and Plasma Lipids and AT at Baseline

	Group			
	Placebo	n3-PUFA	AT	Combination
Age (yr)	33.2 ± 10.0	29.7 ± 9.3	33.0 ± 7.2	29.3 ± 6.2
BMI (kg/m^2)	24.7 ± 3.5	27.4 ± 8.7	25.7 ± 4.1	24.2 ± 5.3
Total cholesterol (mmol/L)	4.63 ± 0.91	4.60 ± 0.75	4.91 ± 1.06	4.42 ± 0.70
Triglycerides (mmol/L)	0.95 ± 0.46	0.91 ± 0.44	0.96 ± 0.64	0.88 ± 0.37
HDL-cholesterol (mmol/L)	1.47 ± 0.39	1.50 ± 0.34	1.50 ± 0.44	1.29 ± 0.34
LDL-cholesterol (mmol/L)	2.77 ± 0.80	2.66 ± 0.70	2.95 ± 0.93	2.74 ± 0.75
AT (μmol/L)	18.3 ± 5.6	17.8 ± 4.1	20.5 ± 9.9	16.1 ± 3.2

NOTE. Data are presented as mean ± SD.

inflammatory drugs, or anticoagulants; and chronic high-intensity exercise. Postmenopausal women were excluded because estrogen-replacement therapy preserves endothelial function, and inclusion of these subjects may have confounded the results. The Institutional Review Board approved the study protocol and written informed consent was obtained from all participants.

Study Design

Participants were randomly assigned into 1 of 4 supplementation groups (n = 20 per group): (1) placebo alone, (2) n3PUFA alone (0.6 g eicosapentaenoic acid (EPA)/d + 0.9 g docosahexaenoic acid [DHA]/d), (3) AT alone (800 IU/d *all-rac* AT), or (4) combination therapy in a parallel, double-blinded design. Subjects were asked to adhere to their regular diet and activities throughout the study. In addition, participants were advised to avoid consumption of antioxidant-fortified foods. Fasting blood (150 mL) was drawn from subjects at baseline and following 12 weeks of supplementation. Plasma and serum samples were stored at −70°C and analyzed at the end of the study. Before and after supplementation, a complete blood cell count, plasma lipid profile, and serum renal and liver function tests were performed, and serum glucose and serum thyroid-stimulating hormone assayed, as safety measures using standard laboratory techniques in the Clinical Pathology Laboratory.

Plasma Analyses

Plasma AT concentrations were measured by high-performance liquid chromatography (HPLC) as previously described.[20] Plasma fatty acid concentrations were measured by gas chromatography after extraction and transmethylation, as reported elsewhere.[9]

Markers of Inflammation

Mononuclear cells were isolated from fasting heparinized samples of 100 mL of venous blood by centrifugation on a Ficoll gradient by the method of Boyum.[21] Briefly, blood was layered over Histopaque 1077 (Sigma Chemicals, St Louis, MO) and centrifuged at 500 × *g* for 30 minutes. The mononuclear cell interface was removed, and washed with RPMI media and centrifuged at 600 × *g* for 10 minutes twice. The cell pellet was resuspended in RPMI media, plated, and incubated for 2 hours at 37°C, and then activated with monocyte chemoattractant protein-1 (MCP-1; 100 ng/mL) overnight at 37°C. MCP-1 was used due to its chemoattractant properties. The crucial role of MCP-1 in atherosclerosis is evident from studies in which knockout of MCP-1 or its receptor resulted in decreased atherosclerosis in LDL-receptor-deficient mice.[22] The cytokines IL-1β, IL-6, and TNF-α were measured in the supernatant of MCP-1–activated monocytes after a 24-hour incubation at 37°C using a highly sensitive immunoassay (R&D Systems, Minneapolis MN), which uses plates coated with monoclonal antibodies specific for IL-1β, TNF-α, or IL-6, respectively. The intra-assay coefficient of variation of these assays was <4%, and results were expressed per milligram of cell protein.

Serum high-sensitivity (hs)CRP concentrations were determined quantitatively by a high-sensitive particle-enhanced immunonephelometry assay using polystyrene particles coated with monoclonal antibodies to CRP.[23] This immunoassay is sensitive, precise and accurate in the range of 0.2 to 60 mg/L.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using the SAS system (SAS Institute, Cary, NC). Pre- and postsupplementation variables were compared using paired *t* tests for parametric, and Wilcoxon-signed rank tests for nonparametric data. Results are expressed as mean ± SD. Differences were considered significant at $P < .05$.

RESULTS

Characteristics of Participants at Baseline

There were no significant differences in age or body mass index (BMI) between groups at baseline (Table 1). Furthermore, there were no significant differences between treatment groups in plasma total cholesterol, low-density lipoprotein (LDL)-cholesterol, HDL-cholesterol, and triglycerides concentrations at baseline (Table 1). Similarly, plasma AT concentrations were not different among treatment groups prior to supplementation (Table 1), even when lipid-standardized (data not shown). Moreover, there were no significant changes in plasma lipids after supplementation (data not shown).

Plasma Fatty Acids

Plasma fatty acid concentrations were not significantly different between groups prior to supplementation (Table 2). Participants consuming n3PUFA as part of the supplement had a significant increase in plasma DHA concentration after the supplementation period (114% and 143% for n3PUFA alone and combination groups, respectively; $P < .0001$ compared to baseline). Plasma concentrations of EPA were significantly increased only in the n3PUFA alone–supplemented group (109%, $P < .05$, Table 2).

Plasma and Mononuclear Cell AT

As expected, subjects receiving AT as part of the supplement showed a significant 127% increase in plasma AT postsupplementation (Table 3, $P < .0001$ compared to baseline). There were no significant changes in plasma AT after supplementation in participants receiving the placebo or the n3PUFA alone

Table 2. Plasma Fatty Acid Concentrations (μmol/L) Pre- and Postsupplementation

	Group							
	Placebo		n3-PUFA		AT		Combination	
	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
DHA	0.039 ± 0.017	0.046 ± 0.013	0.044 ± 0.015	0.094 ± 0.016*	0.045 ± 0.015	0.044 ± 0.022	0.037 ± 0.01	0.09 ± 0.017*
EPA	0.016 ± 0.01	0.016 ± 0.01	0.033 ± 0.03	0.069 ± 0.03†	0.056 ± 0.07	0.040 ± 0.04	0.079 ± 0.09	0.087 ± 0.05
C16	0.676 ± 0.22	0.66 ± 0.15	0.603 ± 0.23	0.691 ± 0.21	0.722 ± 0.16	0.731 ± 0.18	0.661 ± 0.11	0.64 ± 0.20
C18:0	0.474 ± 0.79	0.504 ± 0.17	0.292 ± 0.09	0.320 ± 0.10	0.326 ± 0.07	0.311 ± 0.11	0.323 ± 0.08	0.30 ± 0.12
C18:1	0.480 ± 0.11	0.538 ± 0.17	0.209 ± 0.006	0.187 ± 0.01	0.536 ± 0.21	0.517 ± 0.19	0.505 ± 0.19	0.59 ± 0.54
C18:2	0.838 ± 0.16	0.901 ± 0.23	0.313 ± 0.25	0.233 ± 0.002	0.894 ± 0.19	0.923 ± 0.18	0.806 ± 0.13	0.79 ± 0.23
C18:3	0.035 ± 0.018	0.036 ± 0.02	0.033 ± 0.009	0.033 ± 0.01	0.045 ± 0.012	0.039 ± 0.018	0.041 ± 0.011	0.04 ± 0.015
C20:0	0.009 ± 0.012	0.010 ± 0.01	0.017 ± 0.01	0.025 ± 0.04	0.012 ± 0.01	0.012 ± 0.01	0.014 ± 0.01	0.012 ± 0.01

NOTE. Data are presented as mean ± SD.

*$P < .0001$ compared to baseline.

†$P < .05$ compared to baseline.

supplements. In addition, mononuclear cell AT concentrations were increased by 140% and 125% post-AT supplementation in the AT and combination groups, respectively (Table 3).

Monocyte Function and Serum hsCRP

Compared to baseline, there were no significant changes in monocyte release of IL-1β TNF-α, or IL-6 (Table 3) after supplementation, regardless of supplementation group. Moreover, serum hsCRP concentrations were not significantly modified after supplementation with AT or n3PUFA compared to placebo (Table 3).

DISCUSSION

The effect of n3PUFA on inflammation, atherosclerosis, and the risk of heart disease is subject to debate. In a population of healthy men, Ascherio et al[24] did not find a reduction on the risk of coronary heart disease as fish intake increased. Furthermore, in the Physicians' Health Study moderate fish consumption was not associated with a reduced risk of cardiovascular disease.[25] In contrast, results from the GISSI-Prevenzione trial suggested that 1 g/d n3PUFA resulted in a reduced risk of death and cardiovascular death in patients surviving a myocardial infarction.[26] Moreover, Mori et al[27] found that after 12 weeks of supplementation, 3.65 g/d n3PUFA significantly reduced platelet aggregation, especially when incorporated into a low-fat diet.

Regarding the anti-inflammatory effects of n3PUFA, Endres et al[16,17] reported the suppression of IL-1β, IL-1α, IL-2, and TNF after a 6-week supplementation of healthy adults with 18 g/d fish oil (about 4.5 g/d n3PUFA). In contrast, Chan et al[28] documented the lack of effect of a 6-week treatment with fish oil (4 g/d) on hsCRP, TNF, and IL-6 in obese individuals, and Holm et al[29] reported an increase in TNF and a decrease in the anti-inflammatory cytokine IL-10 in heart transplant patients supplemented with 3.4 g/d n3PUFA, compared to those placebo-supplemented. Moreover, and in agreement with the failure to decrease monocyte proatherogenic activities with n3PUFA supplementation in the present study, Schmidt et al[30] reported that dietary n3PUFA (0.65 g/d) failed to affect monocyte function. However, it is difficult to compare different studies due to the different doses of n3PUFA used.

The individual pro- or anti-inflammatory effects of EPA or DHA have not been studied in detail. A single study reported that a diet with a higher EPA/DHA ratio (3.4 *v* 1.1) induced greater suppression of inflammation in arthritic Lew/SSN rats.[31] Thies et al[32] found that supplementation with EPA but not DHA, alpha-linoleic acid, or n-6PUFA decreased natural killer cell activity. The supplement used in our study had a

Table 3. Effect of Supplementation on Plasma AT, Monocyte AT, Monocyte Cytokine Release, and Serum hsCRP Concentrations

	Group							
	Placebo		n3-PUFA		AT		Comb	
	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
Plasma AT (μmol/L)	18.3 ± 5.6	17.6 ± 4.8	17.8 ± 4.1	19.1 ± 6.1	20.5 ± 9.9	46.6 ± 24.5*	16.1 ± 3.2	36.6 ± 14.7*
Mononuclear cell AT (μmol/L/10^6 cells)	0.17 (0.10-0.30)	0.17 (0.06-0.26)	0.17 (0.12-0.27)	0.16 (0.13-0.38)	0.20 (0.10-0.53)	0.48 (0.11-0.87)*	0.16 (0.12-0.23)	0.36 (0.12-0.54)
Monocyte cytokine release (pg/mg protein)								
IL-1β	309 (195-2,525)	448 (173-1,828)	330 (182-1,887)	358 (124-5,675)	273 (158-550)	367 (243-2,885)	403 (198-1,440)	346 (184-1,269)
TNF-α	124 (6-1,880)	244 (6-2,350)	109 (27.6-2,236)	110 (1.6-1,716)	134 (5-452)	477 (3-1,352)	200 (2.7-2,190)	132.5 (21-1,203)
IL-6	71 (12-108)	76 (8-99)	79 (9-103)	78 (16-93)	81 (13-97)	80 (10-101)	76 (21-104)	77 (19-100)
Serum hsCRP (mg/L)	1.1 (0.3-5.2)	1.1 (0.2-9.9)	0.7 (0.2-26.5)	1.8 (0.2-40.8)	1.1 (0.02-26.2)	1.2 (0.2-10.3)	1.0 (0.2-14.9)	1.4 (0.2-29.0)

NOTE. Data for plasma AT are presented as mean ± SD. Data for mononuclear cell AT, monocyte cytokine release, and serum hsCRP are presented as median (range).

*$P < .0001$, †$P < .001$ compared to baseline.

EPA/DHA ratio of 0.67. Thus, it is possible that a higher EPA/DHA ratio might have led to more favorable effects.

Information regarding the effect of n3PUFA on CRP is scarce. A report on patients referred for coronary angiography who had not been supplemented with n3PUFA, showed a significant inverse correlation between serum CRP and DHA levels.[33] In contrast, we failed to demonstrate an effect of supplementation with n3PUFA on serum CRP levels using a dose of n3PUFA comparable to that used in the GISSI-Prevenzione trial (1 g/d).[26] However, this is in agreement with recent reports that documented lack of effect of supplementation with n3PUFA on serum hsCRP concentrations even when using higher doses of n3PUFA (2.0 or 6.6 g/d n3PUFA for 12 weeks[34] or 4g/d fish oil for 6 weeks[28]). While several studies have evaluated the effect of AT on the progression of atherosclerosis,[35-37] few have focused on the effect of AT on its inflammatory component. Two trials have shown that supplementation with AT (800 IU/d) is anti-inflammatory in type 2 diabetic patients.[12,13] One of these 2 studies compared tomato juice, vitamin C, and vitamin E supplementation during 4 weeks.[12] The patients supplemented with 800 IU/d RRR-AT experienced a significant 49% decrease in plasma CRP. Similarly, healthy and type 2 diabetic individuals taking 1,200 IU/d RRR-AT for 3 months had a significant reduction in plasma hsCRP concentrations, as well as monocyte IL-6 release.[13] Moreover, in healthy individuals, AT supplementation (1,200 IU/d) has also been associated with anti-inflammatory effects characterized by decreased monocyte IL-1β secretion and reduced monocyte adhesion to endothelial cells.[11] Cannon et al[38] showed that, compared to placebo, *all-rac* AT (800 IU/d) produced a significant lowering of endotoxin-induced IL-1β release. However, this study was conducted in subjects who had increased oxidative stress due to eccentric exercise. Thus, the null effect of *all-rac* AT supplementation in our study may be attributed to the fact that we examined MCP-1–induced secretion of cytokines from healthy subjects.

While the studies reporting an anti-inflammatory effect of AT have used doses similar to that of our study, we used *all-rac* AT, as opposed to the natural form. Synthetic *all-rac* AT consists of equal amounts of the 8 stereoisomers of AT and it has 8 times lower the amount of the natural RRR-AT isomer. Similar to our study, Meydani et al[39] reported that supplementation with 800 IU/d *all-rac* AT did not affect IL-1β. It is possible that the different racemic form of AT in these studies may have accounted for the lack of anti-inflammatory effect. In addition, Traber et al[40] documented 3 to 4 times higher urinary excretion of α-2-[2′-carboxyethyl]hydroxychroman (α-CEHC), a product of the side-chain degradation of AT, from *all-rac* AT compared to RRR-AT. The stereoisomeric form of AT might be crucial for its transfer from the plasma membrane to the cytosol by tocopherol-associated proteins (TAPs) or α-tocopherol transfer protein (α-TTP).[10] We observed similar increases in plasma and monocyte AT concentrations after supplementation with vitamin E. However, it is possible that different stereoisomers of AT are not translocated into the cytosol, therefore not interfering with cell signaling and transduction pathways. Further research regarding this topic will prove instrumental.

The present negative findings accord with our previous report using 400 IU/d *all-rac* AT, which indicated that this lower dose of *all-rac* AT failed to have an effect on inflammatory markers such as hsCRP and release of IL-1β and TNF-α from lipopolysaccharide-activated monocytes.[41] Freedman and Keaney[42] have also reported that RRR-AT significantly decreased protein kinase C (PKC) activity and platelet aggregation, while the *all-rac* isomer failed to decrease PKC activity as well as platelet aggregation in vitro, suggesting differential effects of these 2 forms of AT on intracellular function independent of antioxidant activity. This might in part explain why the majority of prospective clinical trials using *all-rac* AT at different doses (from 50 IU/d to 400 IU/d) have not shown a benefit of supplementation on the primary end point of cardiovascular events.[19] However, Gisinger et al[43] reported a beneficial effect of 400 IU *all-rac* AT on platelet-vessel wall interaction and thromboxane A2 production in type 1 diabetic patients. Therefore, it is possible that populations at higher risk[44] may be more likely to benefit from *all-rac* AT supplementation. Whether the lack of effect of *all-rac* AT in normal individuals is due to the enhanced degradation or decreased bioactivity of *all-rac* AT is subject to research.

In conclusion, the lack of effect of n3PUFA alone, AT alone, or the combination on markers of inflammation and monocyte function suggests that supplementation with n3PUFA and *all-rac* AT at these doses is not anti-inflammatory. Further studies would be instrumental to clarify whether the use of synthetic AT may be responsible for the observed lack of anti-inflammatory effects of AT in the present study.

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