Long-Term Combined Supplementations with α-Tocopherol and Vitamin C Have No Detectable Anti-Inflammatory Effects in Healthy Men[1]

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ABSTRACT Inflammatory and oxidative stresses play a pivotal role in atherogenesis. Vitamin E and vitamin C are the two most important dietary antioxidants; moreover, vitamin E has anti-inflammatory effects. Combined supplementations with vitamin E and vitamin C twice daily for 3 y reduced lipid peroxidation and retarded the progression of common carotid atherosclerosis in healthy men in the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study. To further elucidate the underlying mechanisms that retarded the progression of atherosclerosis in the ASAP study, we investigated the effect of a combined intake of vitamin E and vitamin C on inflammatory markers in vivo. Circulating levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and C reactive protein (CRP) were measured in 45- to 69-y-old men from the ASAP study with cholesterol >5.0 mmol/L before and after treatment with either placebo (*n* = 52) or a combined supplementation with 91 mg (136 IU) α-tocopherol and 250 mg of slow-release vitamin C twice a day (*n* = 55) for 3 y. Antioxidant treatment for 36 mo had no effect on circulating levels of TNF-α, IL-6 or CRP. In conclusion, long-term combined supplementations with α-tocopherol and vitamin C in reasonable doses have no detectable systemic anti-inflammatory effects in a healthy population of men with slight hypercholesterolemia and no overt signs of inflammation. J. Nutr. 133: 1170–1173, 2003.

KEY WORDS: • inflammation • antioxidants • atherosclerosis • cytokines • humans

Increasing evidence suggests that inflammatory and oxidative stress play a pivotal role in atherogenesis. Thus, lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that can be described as an age-related inflammatory disease (1), and atherosclerosis is now considered in part to be the consequence of chronic, low-grade inflammatory activity (2). Furthermore, enhanced lipid peroxidation is associated with accelerated atherogenesis (3,4). Activation of monocytes induces LDL oxidation in vitro but importantly, modified LDL themselves are also able to induce inflammation because they induce the adhesion and influx of monocytes and influence cytokine release by monocytes (1).

Vitamin E and vitamin C are considered two of the most important dietary antioxidants; moreover, vitamin E has anti-inflammatory effects (5). It was shown previously that short-term supplementations with high doses of vitamin E decrease the in vitro production of cytokines such as tumor necrosis factor (TNF)[3]-α (6–8), interleukin (IL)-1β (7–10) and IL-6 (11) by peripheral mononuclear cells (PMNC) and reduce levels of C reactive protein (CRP) (11). TNF-α and IL-1β are classical proinflammatory cytokines; together, they initiate a second wave of cytokines, including IL-6, whose activities include stimulating liver production of acute phase proteins such as CRP (12). Endothelial dysfunction is considered to be one of the first steps in atherosclerosis (1), and it has been demonstrated that TNF-α and IL-1β impair endothelium-dependent relaxation in humans (13) and are a direct cause of endothelial up-regulation of cellular adhesion molecules, facilitating the migration of leukocytes across the endothelium (14). TNF-α and IL-6 also cause dyslipidemia (15,16) and IL-6 promotes procoagulant changes (17). Moreover, circulating levels of IL-6 (18) and CRP (19) are strong independent predictors of the risk of thromboembolic complications. Elevated plasma levels of TNF-α have been associated with carotid artery intima-media thickness (IMT) in healthy middle-aged men (20).

α-Tocopherol (AT) is the best-characterized component of the vitamin E family. When AT works as an antioxidant, it is oxidized to α-tocopheroxyl radicals, which vitamin C is able to regenerate to AT, suggesting the synergistic effect of a "cocktail" approach (5). In accordance with this, combined supplementation with 91 mg (136 IU) of AT and 250 mg of slow-release vitamin C twice a day for 3 y retarded the progression of common carotid atherosclerosis in healthy men aged 45–69 y with hypercholesterolemia compared with a placebo group in the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study; the "cocktail" was more effective than single supplementations with AT or vitamin C (21). Subsequently, it was demonstrated in subsets of men from the ASAP study that AT, but not vitamin C, reduced lipid peroxidation both in vitro and in vivo (22,23).

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[3] Abbreviations used: ASAP, Antioxidant Supplementation in Atherosclerosis Prevention; AT, α -tocopherol; CRP, C reactive protein; IL, interleukin; IMT, intima-media thickness; PMNC, peripheral mononuclear cells; TNF, tumor necrosis factor.

TABLE 1

Baseline characteristics of men in the ASAP study[1]

	Placebo $n = 52$	Vitamin E + C $n = 55$
Age, *y*	59 (47–70)	61 (45–69)
Carotid artery IMT,[2] *mm*	1.00 (0.62–1.79)	0.99 (0.65–2.11)
Fibrinogen, *g/L*	3.6 (2.1–5.5)	3.6 (2.2–5.4)
Total leucocytes, 10^9*/L*	6.5 (3.2–12.5)	6.5 (3.2–10.9)
Total cholesterol, *mmol/L*	6.1 (4.2–8.3)	6.4 (4.4–8.3)
Triglycerides, *mmol/L*	1.4 (0.4–4.4)	1.5 (0.5–4.7)
Body fat, *g/100 g*	23.0 (10.3–35.6)	23.6 (12.6–32.9)

[1] Values are medians and range. Treatment groups did not differ, $P > 0.1$.
[2] IMT, intima-media thickness.

We hypothesized that long-term supplementations with antioxidants inhibit inflammation and thereby exert beneficial effects on health. The purpose of the present study was to investigate whether ingesting a cocktail of AT and slow-release vitamin C for 3 y affected plasma levels of TNF-α, IL-6 and CRP compared with a placebo group in men from the ASAP study. The group of men with combined antioxidant supplementation was chosen because it had been demonstrated previously that this particular group had the most retarded progression of common carotid atherosclerosis in the ASAP study and, accordingly, a possible reduction in inflammatory mediators would most likely be detected in these subjects (21). The men in the placebo group provided the natural control group. We evaluated plasma levels of cytokines and CRP because these are markers of cardiovascular diseases and provide strong predictors of the clinical outcome (18–20).

SUBJCTS AND METHODS

Study design and supplements. The ASAP study was designed to test the main study hypothesis that supplementations twice a day with either 91 mg of AT or 250 mg of slow-release ascorbic acid or AT and vitamin C in combination retarded the progression of common carotid atherosclerosis in 520 men and postmenopausal women aged 45–69 y as previously described (22). All subjects had serum cholesterol >5.0 mmol/L at the screening visit. The study consisted of an 8-wk placebo lead-in phase and a 3-y double-masked phase, for which the subjects were randomly allocated to one of the following: *1*) 100 mg of AT acetate twice a day corresponding to 272 IU of vitamin E and 182 mg of *RRR*-α-tocopherol; *2*) 250 mg slow-release ascorbic acid twice a day; *3*) 100 mg of AT acetate + 250 mg slow-release ascorbic acid twice a day; or *4*) placebo. The Research Ethics Committee of the University of Kuopio approved the study protocol and all subjects gave a written informed consent. Participants came for baseline visits and were randomly assigned to groups between October 1994 and October 1995. In the present study, plasma levels of TNF-α, IL-6, and CRP were measured in a subset that consisted of all men who received vitamin E+C ($n = 55$) as well as all men who received placebo ($n = 52$). The number of smokers was equal in the intervention (48%) and placebo groups (43%).

Plasma TNF-α, IL-6 and CRP. Circulating levels of TNF-α and IL-6 were measured in EDTA plasma that had been stored at −80°C until analyzed by ELISA (kits HSTA50 and HS600, R&D Systems, Minneapolis, MN). The immunoassays measure the total amount of free TNF-α or IL-6 plus the amount bound to soluble receptors. All samples and standards were analyzed in duplicate and the mean of the duplicates was used in the statistical analyses. Detection limits were 0.1–0.2 μg/L. For the actual intra-assay variation, the CV was 15.7% for TNF-α and 8.7% for IL-6. CRP was also measured by a commercial high sensitivity ELISA kit (Alpha Diagnostic, San Antonio, TX).

Other measurements. The common carotid artery mean IMT was measured by ultrasonographic scanning, and atherosclerotic progression was defined as the linear regression slope calculated over semiannual assessments as previously described (21). The percentage of body fat was estimated using a near infrared interactance technique (Futrex-500, Futrex, Gaithersburg, MD). Total cholesterol and triglycerides were measured in serum by enzymatic colorimetric methods (Kone Specific, Thermo Clinical Labsystems, Vantaa, Finland). Blood samples for insulin determinations were cooled on ice, and serum was separated at 4°C. Levels of insulin were determined by RIA (Phadeseph Insulin RIA, Pharmacia&Upjohn, Uppsala, Sweden). Fibrinogen was determined with a coagulometer (KC4, Heinrich Amelung GMBH, Lemco, Germany) using reagents from Dade AG (Duedingen, Switzerland).

Statistics. SPSS version 10.0 (Chicago, IL) was used for all analyses. Cytokine and CRP data did not show normal distributions; accordingly, nonparametric analyses were used. Independent groups were compared by the Mann-Whitney U test. Differences between values at baseline and after 36 mo were compared by the Wilcoxon matched pairs signed rank sum test. The absolute difference calculated between baseline and 36 mo later was compared in the placebo vs. the intervention group by the Mann-Whitney U test. Associations between continuous variables were evaluated by Spearman's rank correlation coefficient (r_S). In all analyses, $P < 0.05$ was considered significant.

RESULTS

There were no differences in measured risk factors for atherosclerosis between the placebo and the intervention group at baseline (**Table 1**). Plasma levels of TNF-α and CRP at baseline were positively and significantly correlated with age, fibrinogen, the percentage of body fat, the total number of leukocytes and levels of insulin. TNF-α was correlated with age (**Table 2**). No similar correlations were found for IL-6 (data not shown). Smokers ($n = 49$) had higher levels of CRP than the 49 nonsmokers: medians (25th-75th percentile ranges); 1.8 mg/L (0.9–3.5) vs. 0.6 mg/L (0.3–2.5); $P = 0.007$). No similar differences were detected in concentrations of TNF-α or IL-6 in relation to smoking status: TNF-α, 1.5 μg/L (1.0–2.2) vs. 1.5 μg/L (0.9–2.4); $P = 0.9$; IL-6, 1.5 μg/L (1.2–2.1) vs. 1.9 μg/L (1.2–2.6), $P = 0.1$. There was no effect of AT + vitamin C treatment for 3 y on plasma concentrations of TNF-α, IL-6 or CRP (**Table 3**). In addition, the cytokines and CRP did not differ when the intervention

TABLE 2

Correlations between TNF-α, CRP and risk markers of atherosclerotic cardiovascular diseases at baseline in men in the ASAP study[1,2]

	TNF-α		CRP	
	r_S	*P*	r_S	*P*
Age	0.27	0.006	0.05	0.6
Carotid artery IMT	0.18	0.06	0.13	0.2
Fibrinogen	0.33	0.001	0.69	<0.0005
Leukocytes total	0.21	0.04	0.44	<0.0005
Total cholesterol	0.06	0.5	0.05	0.6
Triglycerides	0.19	0.06	0.13	0.2
Body fat	0.29	0.003	0.27	0.005
Insulin	0.24	0.02	0.19	0.05

[1] Abbreviations: TNF, tumor necrosis factor; CRP, C reactive protein; ASAP, Antioxidant Supplementation in Atherosclerosis Prevention; r_S, Spearman correlation coefficient; IMT, intima-media thickness.
[2] TNF-α, $n = 105$; CRP, $n = 107$.

TABLE 3

Plasma TNF-α, IL-6 and CRP in healthy men before and after 3 y of antioxidant supplementation or placebo[1,2]

	n	Treatment	Baseline	After 3 y
TNF-α, *ng/L*	54	Vitamin E + C	1.6 (1.0–2.5)	1.4 (0.8–2.0)*
	51	Placebo	1.3 (0.8–2.2)	1.1 (0.8–1.8)*
IL-6, *ng/L*	54	Vitamin E + C	1.8 (1.2–2.6)	1.5 (1.1–2.8)
	51	Placebo	1.6 (1.12–2.5)	1.5 (1.0–2.3)
CRP, *mg/L*	50	Vitamin E + C	1.0 (0.4–2.7)	1.2 (0.3–2.9)
	52	Placebo	1.5 (0.4–3.3)	1.7 (0.5–3.6)

[1] Values are medians and interquartile range (25th–75th percentile). * Different from baseline, $P < 0.05$. Treatment groups did not differ, $P > 0.10$.

[2] Abbreviations: TNF, tumor necrosis factor; IL, interleukin; CRP, C reactive protein.

group was compared with the placebo group at the end of the study. When the statistical analyses were performed separately for smokers and nonsmokers, there still were no detectable effects of antioxidant supplementation on concentrations of the two cytokines and CRP (data not shown).

DISCUSSION

Circulating levels of TNF-α, IL-6 and CRP were not influenced by 3 y of a combined supplementation with AT and vitamin C in healthy men with high cholesterol from the ASAP study. Accordingly, the retarded progression of common carotid atherosclerosis in relation to supplementation with a cocktail of AT and vitamin C is not related to systemic anti-inflammatory activities, but is due rather to an antioxidative effect (22,23). Consistent with this conclusion, it has been demonstrated that men, and smokers in particular, have enhanced oxidative stress and lipid peroxidation (4,24); the benefit of the supplementation in the ASAP study was indeed detected only in men and the treatment effect was more pronounced among smoking men than nonsmoking men (21).

It is very likely that AT doses in the present study were too low to induce an anti-inflammatory effect even in the combination with vitamin C. It was shown previously that supplementation with 600-1200 IU/d of AT for 1–3 mo reduced the levels of CRP, the in vitro production of TNF-α, IL-1β and IL-6 by PMNC, and circulating levels of several adhesions molecules (6–9,11), whereas 6–8 wk of supplementation with 400 IU/d of AT did not influence these variables (25). To our knowledge, this is the first study to evaluate circulating levels of cytokines. Baseline levels of inflammatory markers in the present study were low compared with some population-based cohort studies (26); accordingly, it is possible that the combined supplementation of AT and vitamin C would have an effect in populations with low-grade inflammation such as older population-based cohorts or populations with preexisting cardiovascular diseases. These considerations illustrate that dose-response studies of antioxidant therapy are desirable in different populations, under different conditions and in different combinations, but such studies are likely impossible to put into practice. It is possible that the antioxidant therapy in the present study affected local production of cytokines and that this was undetectable in the circulation. However, given that systemic low-grade inflammatory activity has strong prognostic value in cardiovascular diseases, we thought that circulating levels of TNF-α, IL-6, and CRP would be appropriate markers of a possible anti-inflammatory effect in relation to antioxidant supplementation. In contrast, the cytokine production in cultures of PMNC has not demonstrated similar clinical relevance to our knowledge. We cannot exclude that protein degradation during the 3 y of storage may have affected our results. However, the existence of a placebo group offsets such an effect. Furthermore, we found no difference in levels of TNF-α between 40 healthy people aged 18–30 y whose plasma was stored for 6 y compared with 40 sex- and age-matched controls with freshly isolated plasma (Bruunsgaard et al., unpublished data)

Circulating levels of TNF-α were correlated with age, body fat and levels of insulin in the present study. In accordance with this, age-related increases in TNF have been reported in other population-based studies (26), and basal studies have demonstrated that fat tissue produces TNF-α, leading to insulin resistance and elevating the production of free fatty acids that inhibit the breakdown of insulin in the liver (27). Accordingly, plasma levels of TNF-α in healthy populations may largely be controlled by variables that are not subject to modulation by antioxidant supplementation. Circulating levels of IL-6 are probably a better marker of the sum of ongoing inflammation in the body than systemic levels of TNF-α because a local production of TNF-α may not escape into the circulation, although it can induce a strong systemic IL-6 response. Circulating levels of IL-6 increase also with age but, in contrast to TNF-α, this elevation is already well established in middle-aged people and is not expanded further in octogenarians (26); this may explain why we did not detect a correlation between IL-6 and age in the present study. Furthermore, we found no association between body fat and IL-6 in contrast to other epidemiologic studies. This discrepancy may be because previous investigations focused on obese individuals with high amounts of body fat (28). One of the biological activities of IL-6 may be to regulate the size of the body fat compartment. Thus, IL-6 knock-out mice develop obesity (29), mice with IL-6–secreting tumors have reduced fat mass (16) and chronic centrally administered IL-6 decreases body fat in rats (30). CRP production is controlled by TNF-α and/or IL-6 (12) and correlations between CRP and risk factors in the present study may simply reflect the activities of these two cytokines.

In conclusion, combined supplementation with AT and vitamin C for 3 y had no detectable systemic anti-inflammatory effect in a healthy population of men with slight hypercholesterolemia but no overt signs of inflammation.

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