Antioxidant Supplementation Decreases Lipid Peroxidation Biomarker F$_2$-isoprostanes in Plasma of Smokers[1]

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Abstract

Free radicals in cigarette smoke (CS) cause oxidative damage to proteins, DNA, and lipids, contributing to the pathobiology of atherosclerosis, heart disease, and cancer. *In vitro* studies have shown that antioxidants quench free radicals and ameliorate certain aspects of biomolecular damage caused by CS. It is hypothesized that a combination of antioxidants is more effective than a single antioxidant, due to their interactions. To investigate whether supplemental antioxidants reduce CS-related lipid peroxidation *in vivo* and whether they are more effective in combination, we conducted an intervention study in smokers. In a randomized double-blind placebo-controlled trial, we investigated whether vitamin C or an antioxidant mixture containing vitamin C, α-lipoic acid, and vitamin E decreases plasma F$_2$-isoprostane levels, an index of oxidant stress, in smokers. Plasma of 126 smokers (mean age, 46 years; age range, 20–78 years) was analyzed for F$_2$-isoprostanes at baseline and after intervention with antioxidants and placebo. In smokers with a body mass index (BMI) above the median, 2 months of daily supplementation with 500 mg of vitamin C decreased plasma F$_2$-isoprostane levels by 28.8 pmol/liter when compared with the placebo group ($P = 0.001$); levels in the mixture group were 7.45 pmol/liter lower after treatment, but this difference was not statistically significant ($P = 0.14$). There was no treatment effect in smokers with a low BMI. BMI was significantly positively associated with plasma F$_2$-isoprostane levels (trend $P = 0.001$). Antioxidants decrease smoking-related lipid peroxidation markers of oxidative stress in humans with high BMI. Our results do not indicate that an antioxidant combination is more effective than vitamin C alone. The intake of antioxidants may help prevent smoking-related diseases. Smoking cessation should still be considered the most effective way to prevent smoking-related diseases.

Introduction

Cigarette smokers are exposed to reactive free radicals present in CS[3] (1). Free radicals can cause oxidative damage to DNA, proteins, and lipids and may be involved in the development of chronic diseases such as atherosclerosis and cancer (2–5). *In vitro* studies have shown that antioxidants such as vitamin C, vitamin E, carotenoids, and thiols (*e.g.,* glutathione and α-lipoic acid) ameliorate free radical-induced oxidative damage (6–11). *In vitro,* hydrophilic and lipophilic antioxidants work together in a network, recycling each other and thus creating an effective antioxidant defense system (12–15). *In vivo* support for these observations has been elusive (16–19).

There is evidence from epidemiological studies that persons who consume a diet rich in fruits and vegetables have a lower risk of cancer, atherosclerosis, and other diseases (20, 21). Because fruits and vegetables are major sources of antioxidants (as well as other factors), it has been hypothesized that antioxidants in fruits and vegetables are the protective compounds, or major components of the protective effect (5). Thus, there are biochemical data that antioxidants can reduce oxidative damage *in vitro*, and there are epidemiological data implicating micronutrient antioxidants in reduction of disease risk; however, the link between the biochemical and epidemiological data has been difficult to demonstrate.

This situation presents a problem for public health and clinical guidance, as well as for the design of more definitive human studies. Before we can obtain clearer answers on whether antioxidants decrease oxidative stress in humans and ultimately determine whether intervention to decrease oxidative stress will reduce disease, we need answers to several intermediate questions. Some but not all of these questions have been clarified for DNA damage (22), but more research is needed regarding not only DNA damage but also damage to other macromolecules.

Some of those intermediate questions are: (*a*) to what extent are plasma antioxidant concentrations associated inversely with biomarkers of oxidative damage *in vivo*? (*b*) can

[3] The abbreviations used are: CS, cigarette smoke; BMI, body mass index; TG, triglyceride; Tchol, total cholesterol; ANOVA, analysis of variances; ANACOVA, analysis of covariance.

levels of oxidative stress be modified by manipulation of anti-oxidant levels *in vivo*? (*c*) what antioxidants are effective against which biomarkers? (*d*) are combinations of antioxidants more effective than a single antioxidant in reducing the level of a biomarker of oxidative damage? and (*e*) do factors such as gender or obesity modify the effectiveness of antioxidants?

Numerous biomarkers for oxidative damage have been proposed, including biomarkers for DNA damage and for alterations in proteins and lipids (23–25). The F$_2$-isoprostanes, one such biomarker, are products of free radical-catalyzed lipid peroxidation of arachidonic acid (26). They are formed *in situ,* esterified to phospholipids, and subsequently released by phospholipases into the plasma, where they can be measured (27). Increased F$_2$-isoprostane levels in urine or plasma have been found in patients with atherosclerosis (28), severe heart failure (29), diabetes (30), asthma (31), Alzheimer's disease (32), preeclampsia (33), cystic fibrosis (34), chronic obstructive pulmonary disease (35), and unstable angina (36), as well as in smokers (37, 38). Very few studies, however, have investigated whether F$_2$-isoprostane levels can be reduced by administration of antioxidants (39–43).

We conducted a randomized double-blind placebo-controlled trial in smokers to assess several hypotheses: (*a*) that plasma F$_2$-isoprostanes are usable markers of oxidative damage in large studies; (*b*) that antioxidants would reduce this biomarker in smokers; and (*c*) that a combination of antioxidants would be more effective than a single antioxidant. Subjects were randomized to receive either vitamin C alone; a combination of vitamin C, vitamin E, and α-lipoic acid; or a placebo.

Materials and Methods

The protocol was approved by the Committee for the Protection of Human Subjects, University of California (Berkeley, CA) and by the Kaiser Permanente Institutional Review Board (Oakland, CA). Written consent was obtained from all enrolled participants.

Eligibility

Subjects were recruited through letters to members of the Kaiser Permanente Health Plan as well as through fliers and advertisements to the general public and screened over the phone. Eligibility criteria included smoking ≥15 cigarettes/day and willingness to stop consuming vitamin supplements for the duration of the study. Exclusion criteria were as follows: consumption of iron supplements or of vitamin E supplements of 800 IU/day or more; reported consumption of ≥4 servings of fruits and vegetables/day; intake of more than 2 alcoholic drinks/day; history of alcohol problems less than 1 year ago; pregnancy; use of blood-thinning drugs; hemochromatosis; history of kidney stones or any other kidney problems; cancer; stroke; heart attack within the last 5 years; known HIV infection; and any kind of hepatitis or diabetes. Smokers who were taking vitamin C, vitamin E, and/or multivitamin supplements before enrollment in the study were given "washout" periods during which they stopped taking any supplement before their first blood draws. The length of the washout period was dependent on the kind and dosage of vitamin/antioxidant supplements the subjects had been taking before the study. Subjects who took vitamin E (<800 IU) alone or in combination with other antioxidant supplements underwent a minimum 6-week washout period prior to their blood draw before intervention. Subjects taking any dosage of vitamin C and/or multivitamins but no vitamin E were given a minimum 5-week washout

period before their first blood draw. No subjects were taking α-lipoic acid before entering the study.

Clinical Protocol

The study was randomized and double-blinded. Smokers had a total of three clinic visits, and fasting blood samples were obtained at each visit. The first and second visits were 1–4 weeks apart. At the second visit, smokers were randomly assigned to one of three groups based on treatment with: (*a*) vitamin C (vitamin C group); (*b*) a combination of vitamin C, vitamin E, and α-lipoic acid (mixture group); or (*c*) a placebo that was identical in appearance but contained no active ingredients (placebo group). Randomization was stratified on gender, age, and body weight to ensure balance on those factors across the treatment groups. The third blood draw took place after 60 days of study capsule intake.

All blood draws were conducted after an overnight 12-h fast. Subjects refrained from smoking for at least 1 h before their clinic visits (to avoid external nicotine contamination of the plasma samples). At the final clinic visit, the participants were required to bring back their capsule bottles, and the remaining capsules were counted to assess adherence to the protocol and determine the number of missed days.

The participants were instructed to start taking the capsules after the second blood draw on the same day and to take the last capsules the day before their third (final) blood draw. Smokers were on study capsules for an average time of 58 days. The subjects were instructed to take their capsules with a meal.

The capsules were manufactured for this study and provided by Softgel Inc. (Los Angeles, CA). All of the capsules were oil-based. The capsules were assayed to confirm their composition. The vitamin C capsules provided a daily dose of 515 ± 28 mg of vitamin C; the vitamin E/α-lipoic acid capsules provided a daily dose of 371 ± 56 mg of RRR-α-tocopherol, 171 ± 40 mg of RRR-γ-tocopherol, 50 ± 10 mg of α-tocotrienol, 184 ± 39 mg of γ-tocotrienol, 18 ± 2 mg of δ-tocotrienol, and 95 ± 10 mg of α-lipoic acid. The mixture group received both the vitamin C capsules and the vitamin E/α-lipoic acid capsules, whereas the vitamin C group received the vitamin E/α-lipoic acid capsule.

All participants completed a Block98 food frequency questionnaire that produced estimates of macro- and micronutrients including dietary carotenoids. Extensive other data were obtained, including the number of cigarettes smoked/day and numbers of years smoked.

Blood Drawing and Processing. Venous blood was drawn into EDTA vacutainers and centrifuged at 5°C for 10 min at 1200 × *g*. The plasma was removed immediately from the blood cells and aliquoted into cryovials. Plasma aliquots for ascorbic acid measurement were mixed 1:1 with 10% (w/v) meta-phosphoric acid to stabilize ascorbic acid. Meta-phosphoric acid was prepared fresh on a weekly basis and kept refrigerated. The blood and plasma samples were always kept on ice and protected from light during the entire process. All cryovials were stored immediately at −70°C. The time between blood draws and freezer storage of the aliquots did not exceed 1 h.

Laboratory Measurements. With the exception of transferrin saturation, all of the analytes described below were measured both before and after intervention.

Measurement of F$_2$-isoprostanes in Plasma. Free F$_2$-isoprostanes in plasma were quantitated after purification and derivatization by selected ion monitoring gas chromatography negative ion chemical ionization/mass spectrometry using [^{2}H$_4$]8-

Table 1 Baseline characteristics[a]

	Intervention group			All smokers (n = 126)
	Vitamin C (n = 42)	Mixture (n = 39)	Placebo (n = 45)	
Sex (M/F)	19/23	19/20	20/25	58/68
Age (yrs)	46.3 ± 11.3	45.7 ± 11.6	46.1 ± 14.2	46.1 ± 12.4
BMI (kg/m^2)	27.5 ± 5.9	27.1 ± 5.3	27.9 ± 5.6	27.5 ± 5.6
Race (white/black/other)	25/14/3	22/15/2	26/14/5	73/43/10
Years of smoking	27.3 ± 12.7	26.5 ± 11.0	26.9 ± 13.3	26.9 ± 12.3
Cigarettes/day	23.8 ± 8.7	24.0 ± 8.1	21.3 ± 6.1	22.9 ± 7.7
Fruit intake[b] (servings/day)	0.82 ± 0.6	0.85 ± 0.6	0.74 ± 0.7	0.80 ± 0.65
Vegetable intake (servings/day)	2.16 ± 1.4	2.34 ± 1.6	2.39 ± 1.6	2.30 ± 1.5
Dietary vitamin C intake (mg/day)	76.9 ± 51.8	93.7 ± 62.7	78.0 ± 61.4	82.4 ± 58.8
Dietary vitamin E intake (mg/day)	8.9 ± 5.1	11.0 ± 8.4	8.8 ± 3.7	9.5 ± 6.0
Dietary provitamin A intake (mg/day)	3.1 ± 2.7	3.5 ± 2.6	3.7 ± 4.2	3.4 ± 3.3
Baseline plasma				
Cotinine (ng/ml)	265 ± 99	268 ± 110	259 ± 129	264 ± 113
Total carotenes (μg/dl)	89.6 ± 36.0	86.2 ± 33.0	90.8 ± 39.2	88.9 ± 36.1
C-Reactive protein (mg/l)	4.2 ± 4.8	3.0 ± 3.8	3.4 ± 4.2	3.5 ± 4.3
Transferrin saturation (%)	29.2 ± 8.7	26.3 ± 9.0	27.0 ± 9.3	27.5 ± 9.0
TGS (mg/dl)	119.5 ± 74.5	108.5 ± 54.7	121.3 ± 69.6	116.8 ± 66.8
Tchol (mg/dl)	195 ± 40	191 ± 43	204 ± 45	197 ± 43

[a] Data are from visit 2, prior to randomization, except for transferrin saturation, which is from visit 1. Results are reported as means ± SD. No intervention group differences were statistically significant.
[b] Includes fruits and fruit juices.

iso-PGF$_{2\alpha}$ as an internal standard (44). Compounds were analyzed as pentafluorobenzyl ester, trimethylsilyl ether derivates monitoring the M-181 ions, *m/z* 569 for endogenous F$_2$-isoprosanes and *m/z* 573 for [^{2}H$_4$]8-iso-PGF$_{2\alpha}$. The F$_2$-isoprostanes elute as a series of chromatographic peaks over a 20-s interval, and quantitation is based on the primary peak eluting at the same time as the internal standard. Data are expressed in picomoles/liter. The assay is highly precise and accurate with a precision of ±6% and an accuracy of 96%. Interday variability is <6%. The analysis was conducted at the laboratory of J. D. M. (Vanderbilt University, Nashville, TN).

Other Assays. Concentrations of cotinine were determined by gas chromatography with nitrogen-phosphorus detection (45, 46). The plasma samples were analyzed in the laboratory of Dr. Neal Benowitz (University of California, San Francisco, CA). Ascorbic acid was determined spectrophotometrically using 2,4-dinitrophenylhydrazine as chromogen (47). Tocopherols and carotenoids were measured by reversed-phase high-performance liquid chromatography (48). C-reactive protein concentrations were measured using commercially available radial immunodiffusion assay kits (The Binding Site Ltd., San Diego, CA). Tchol and TGs were measured using commercially available analysis kits (Sigma Chemical Co.-Aldrich, St. Louis, MO). Transferrin saturation was analyzed at a commercial clinical laboratory (SmithKline Beecham Clinical Laboratories, Norristown, PA).

Statistical Analysis

Statistical analyses were conducted using SAS Version 6.12. Intervention groups were compared by one-way ANOVA. Intervention effects were assessed by one-way ANACOVA including adjustment for baseline level. Pairwise *post hoc t* tests were used. The presented *P*s have not been corrected for multiple comparisons. All data are expressed as means ± SD.

Covariates examined included age, race, and other demographic characteristics; baseline plasma antioxidant levels including carotenoids, α- and γ-tocopherol and ascorbic acid;

plasma lipids including serum cholesterol and TGs; dietary intake of vegetables and fruits; and C-reactive protein and transferrin saturation. In addition, potential effect modification was examined, including possible modification by BMI and baseline plasma antioxidant level. For some analyses, BMI was dichotomized above and below the median.

Results

Baseline Characteristics. Fifty-eight subjects were male, and 68 were female; subjects ranged in age from 20–78 years (mean age, 46.0 ± 12.4 years). They smoked an average of 23 ± 8 cigarettes/day and had smoked, on average, for 27 ± 12 years. The three treatment groups (vitamin C, mixture, and placebo) were not significantly different with respect to sex, age, or BMI, as expected by design (Table 1). The treatment groups were also not significantly different with respect to race; cigarette consumption/day; years of smoking; daily fruit and vegetable intake; dietary vitamin C, vitamin E, and β-carotene/provitamin A intake; baseline plasma levels of cotinine; total carotenes; C-reactive protein; transferrin saturation; TGs; and Tchol. Baseline ascorbic acid, α-tocopherol, and γ-tocopherol were not significantly different between the three treatment groups (Table 2).

Substantial increases were seen in plasma ascorbic acid in the vitamin C group (69.9% increase) and in plasma ascorbic acid and α-tocopherol in the mixture group (89.7% and 33.8% increase, respectively). There were no increases in vitamin C or vitamin E in the plasma of subjects who were in the placebo group.

Plasma γ-tocopherol levels decreased by 29.6% in the mixture group, significantly different (P < 0.005) from the change in the placebo group (Table 2). In contrast, the change in γ-tocopherol in the vitamin C group was not significantly different from the change in placebo.

Plasma cotinine levels were measured before and after intervention and were not significantly different at those two time points within and between the treatment groups (data not

Table 2   Plasma antioxidant levels before and after intervention by treatment group[a]

	Vitamin C group ($n = 42$)		Mixture group ($n = 39$)		Placebo group ($n = 45$)	
	Before intervention	After intervention	Before intervention	After intervention	Before intervention	After intervention
Ascorbic acid (mg/dl)	0.83 ± 0.41[b]	1.41 ± 0.28	0.68 ± 0.37	1.29 ± 0.49	0.65 ± 0.37	0.65 ± 0.41
α-Tocopherol (mg/dl)	1.31 ± 0.36	1.17 ± 0.29	1.36 ± 0.42	1.82 ± 0.57	1.44 ± 0.47	1.31 ± 0.61
γ-Tocopherol (mg/dl)	0.34 ± 0.20	0.30 ± 0.16	0.27 ± 0.14	0.19 ± 0.13	0.34 ± 0.15	0.30 ± 0.15

[a] Treatment group changes (before/after): ascorbic acid plasma levels increased significantly after intervention in the vitamin C and mixture groups: $P < 0.0001$. α-Tocopherol plasma levels increased significantly in the mixture group ($P < 0.0001$) but not in the vitamin C group. γ-Tocopherol plasma levels decreased significantly only in the mixture group ($P < 0.0001$). No significant changes were seen in the placebo group. Before intervention, no significant treatment group differences were seen. After intervention, differences were as follows. Ascorbic acid: vitamin C group *versus* placebo group and mixture *versus* placebo, $P < 0.0001$; vitamin C group *versus* mixture group, $P = 0.161$. α-Tocopherol: vitamin C group *versus* placebo group, $P = 0.19$; mixture group *versus* placebo group, $P < 0.0001$; vitamin C group *versus* mixture group, $P < 0.0001$. γ-Tocopherol: vitamin c group *versus* placebo group, $P = 0.98$; mixture group *versus* placebo group, $P = 0.0004$; vitamin C group *versus* mixture group, $P = 0.0005$.
[b] Values are the mean ± SD. To convert values to micromoles/liter, divide by 0.01761 for ascorbic acid, 0.043061 for α-tocopherol, and by 0.041666 for γ-tocopherol.

Table 3   Crude change in F$_2$-isoprostane plasma levels before and after intervention, by treatment group[a]

	Free plasma F$_2$-isoprostane (pmol/liter)[a]			
	Vitamin C ($n = 42$)	Mixture ($n = 39$)	Placebo ($n = 45$)	All smokers ($n = 126$)
Before intervention[b]	129.1 ± 67.1	123.9 ± 52.3	164.3 ± 80.2	140.1 ± 70.0
After intervention	114.7 ± 61.9	122.7 ± 43.7	159.7 ± 77.3	
Change (pmol/liter)	-14.4 ± 44.9	-1.2 ± 56.1	-4.6 ± 48.4	
Change (%)	-11.13%	-0.99%	-2.78%	

[a] Unadjusted for baseline F$_2$-isoprostane levels or covariates. Results are reported as means ± SD.
[b] Baseline differences between treatment groups: vitamin C *versus* placebo, $P = 0.018$; Mixture *versus* placebo, $P = 0.008$.

shown). This suggests that the smokers did not change their smoking habits throughout the study period and that changes in F$_2$-isoprostane are not due to a change in the consumption of cigarettes.

The adherence rate, as measured by capsule count, was 92%. Changes in ascorbic acid and α-tocopherol (Table 2) also demonstrate that subjects took their capsules regularly and that the capsule components were absorbed.

Effect of Antioxidant Intervention on Plasma F$_2$-isoprostane Levels. As shown in Table 3, the mean plasma level of free F$_2$-isoprostanes of all smokers at baseline was 140.1 ± 70.0 pmol/liter ($n = 126$). By chance, smokers in the placebo group had significantly higher baseline levels of F$_2$-isoprostanes (164.3 pmol/liter) than those in the vitamin C (129.1 pmol/liter) or mixture (123.9 pmol/liter) groups.

Plasma levels of F$_2$-isoprostanes decreased by 11.1% (14.4 pmol/liter) in the vitamin C group, whereas small and nonsignificant decreases were observed in the mixture group (1.0%, 1.2 pmol/liter) and the placebo group (2.8%, 4.6 pmol/liter; Table 3). These data are unadjusted for baseline F$_2$-isoprostanes levels or covariates.

F$_2$-isoprostane levels were found to be strongly related to adiposity (Fig. 1); those with higher BMI had higher F$_2$-isoprostanes (trend $P = 0.001$). Furthermore, analysis of treatment effects adjusted for baseline F$_2$-isoprostane levels identified a statistically significant interaction between BMI and treatment group, indicating that the effect of treatment depended on the subject's BMI. Consequently, further analysis of treatment effects was conducted separately in individuals above and below the BMI median (26.6 kg/m^2), including adjustment for baseline differences in F$_2$-isoprostanes.

In smokers with a BMI above the median (BMI > 26.6), 2 months of daily supplementation with vitamin C decreased F$_2$-isoprostane levels by 28.83 pmol/liter ($P = 0.001$) when compared with the high BMI placebo group (Table 4). In the
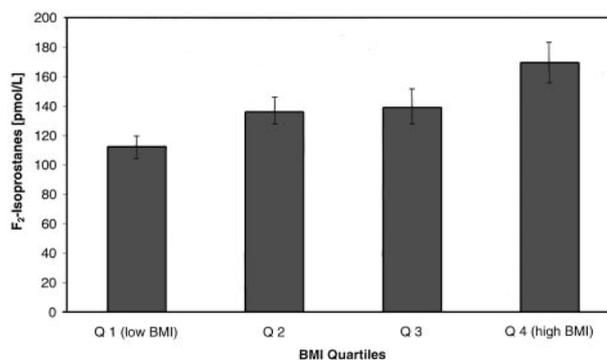

Fig. 1. Mean (±SE) plasma F$_2$-isoprostane levels by BMI quartiles. Quartile 1 (low BMI), 112.0 ± 8.0 pmol/liter; quartile 2, 135.8 ± 10.7 pmol/liter; quartile 3, 138.3 ± 13.7 pmol/liter; quartile 4 (high BMI), 169.1 ± 14.2 pmol/liter. Quartiles 1 and 4 are significantly different from each other ($P = 0.001$). Trend $P = 0.0014$. Means BMIs (±SD) of the quartiles are as follows: quartile 1, 21.9 ± 1.7 kg/m^2; quartile 2, 25.1 ± 0.7 kg/m^2; quartile 3, 28.2 ± 1.1 kg/m^2; and quartile 4, 35.0 ± 5.4 kg/m^2.

high BMI mixture group the F$_2$-isoprostane levels decreased by 7.45 pmol/liter ($P = 0.14$). In smokers with BMI below the median, no significant treatment effects were seen in the vitamin C or mixture groups when compared with the low BMI placebo group. Adjustment for multiple comparisons did not affect these conclusions.

Adjustment for the potential covariates described under "Statistical Analysis," such as age, race, and so forth, also did not alter these results. Plasma Tchol was independently significant but did not alter the treatment effect. The ratio of α-tocopherol:Tchol was not significantly associated with the treatment effect and did not alter the magnitude or significance of it.

Table 4 Adjusted changes in F_2-isoprostane levels by treatment group and by BMI level[a]

	Vitamin C	Mixture	Placebo
High BMI	−28.83[b]	−7.45[c]	13.00
Low BMI	−3.14	3.77	−6.69

[a] Adjusted for baseline F_2-isoprostane levels. Data are in pmol/liter. Covariates were examined, and none were found to alter the results (see text). One subject in the mixture group was an influential data point by regression diagnostics and was removed to satisfy model assumptions. The apparent increase in the placebo group is a result of the ANACOVA adjustment for baseline levels, which were significantly higher in the placebo group (Table 3). Comparisons other than those in [b] and [c], $P > 0.4$.
[b] $P = 0.001$.
[c] $P = 0.14$ for comparison with change in the placebo group.

Discussion

In the present study, daily supplementation for 2 months with vitamin C alone significantly decreased the plasma levels of F_2-isoprostanes in smokers with BMI above the median when compared with those who received placebo. In the mixture group, the treatment effect was smaller and did not achieve statistical significance. Thus, we found that F_2-isoprostanes are usable markers of oxidative damage in reasonably large-scale studies and that at least some antioxidants can reduce this biomarker in smokers. These data do not support the hypothesis that a combination of water- and lipid-soluble antioxidants is superior to a single antioxidant (vitamin C) alone in reducing F_2-isoprostanes.

Strengths of the study include the randomized placebo-controlled design and the sample size. To our knowledge, few other intervention studies have examined the effect of antioxidants on the F_2-isoprostane biomarker (39–43); the largest intervention group sample size was 22 (40), and only two studies (42, 43) were placebo-controlled. Our samples of approximately 40 subjects in each of the two intervention groups and 45 subjects in the placebo group made it possible to minimize possible confounding by stratification or statistical control. Another important strength is the fact that potential covariates or effect modifiers were examined, including plasma lipids, iron status, C-reactive protein, and plasma and dietary antioxidants. In addition, this is the first intervention study to investigate the effect of α-lipoic acid supplementation in smokers.

Data from antioxidant intervention studies have been inconsistent on the question of whether antioxidants can lower biomarkers of lipid peroxidation *in vivo*. Part of the reason for this inconclusive picture may be due to the fact that even among (five) studies using F_2-isoprostanes, different types of subjects were involved [smokers (39, 43), diabetics (41), hypercholesterolemic subjects (40), and coronary artery disease patients (42)], and different antioxidants were used (vitamin C and vitamin E) in different dosages (vitamin C, 500-2000 mg/day) over different time periods (5–30 days).

Two of these five studies reported a reduction in urinary F_2-isoprostane levels with vitamin E supplementation (40, 41), one of these studies found no change in urinary F_2-isoprostane levels with vitamin E supplementation alone, but it found a reduction in urinary F_2-isoprostane levels when vitamin E was administered in combination with vitamin C, and also when vitamin C was administered alone (39). The other two studies found no effect of vitamin C or vitamin E supplementation in plasma or urine, respectively (42, 43).

In addition to the variations in treatments and outcomes, it is possible that some of the inconsistency in the literature

results from an unappreciated role of BMI as an effect modifier in those studies. We have found a highly significant effect modification by BMI on the level of oxidative damage and on the effect of treatment (Table 4; Fig. 1). This will be important for the design of future studies.

The observation of an effect modification by BMI is a new finding. Overweight subjects (subjects with a BMI of ≥ 26.6) had statistically significantly higher baseline F_2-isoprostane levels than did subjects with low BMI (high BMI, 156.0 ± 80.1 pmol/liter; low BMI, 124.1 ± 54.3 pmol/liter; $P = 0.01$) and experienced a statistically significant reduction in F_2-isoprostane levels in the vitamin C treatment group. Low BMI subjects in any of the active treatment groups did not have a significant decrease compared with placebo. It is possible that a certain threshold level of oxidative stress is necessary to see a treatment effect with antioxidants (43).

The more overweight subjects also had statistically significantly higher baseline plasma TG and Tchol levels than the leaner subjects [TG = 136.6 ± 74.6 mg/dl (high BMI) *versus* 96.9 ± 51.3 mg/dl (low BMI), $P < 0.001$; Tchol = 205.9 ± 45.2 mg/dl (high BMI) *versus* 187.8 ± 38.3 mg/dl (low BMI), $P < 0.02$]. It may be that the presence of high levels of body fat or plasma lipids in overweight subjects provides a more substantial substrate susceptible to oxidation by the free radicals in CS. These findings present further evidence for the potentiating effects of serum lipids, smoking habit, and body weight on the incidence of atherosclerosis (40, 43).

The treatment effect in the vitamin C group was considerably greater than that in the mixture group, and only the vitamin C group achieved a statistically significant reduction compared with placebo. This is puzzling because the vitamin C group and the mixture group received identical vitamin C capsules. Increases in plasma ascorbate levels in the two groups were similar (vitamin C group, 0.59 ± 0.47 mg/dl; mixture group, 0.61 ± 0.57 mg/dl; $P = 0.78$). Several explanations are possible. First, the vitamin C or the mixture treatment effects could be due to chance. However, the results are unlikely to be due to chance because $P = 0.001$ for the treatment effect in the vitamin C group. Second, a threshold plasma ascorbic acid level may not have been achieved in the mixture group. However, we examined the possibility of effectiveness only above a threshold and could find no evidence to support such an explanation. Third, vitamin E and/or α-lipoic acid counteracts the effect of vitamin C on F_2-isoprostane. Whereas this cannot be ruled out, most *in vitro* research has found, on the contrary, that lipophilic and hydrophilic antioxidants act synergistically (12–15).

Finally, it is possible that γ-tocopherol is the effective antioxidant against lipid peroxidation and that the mixture was less effective because α-tocopherol suppressed γ-tocopherol. Research on vitamin E has shown that α-tocopherol supplementation results in decreased plasma γ-tocopherol levels (49, 50), and it is notable that in the present study, plasma γ-tocopherol levels in the mixture group decreased by 29.6%. Whereas the mixture capsules contained 171 mg of γ-tocopherol, this dose may not have been sufficient to overcome the γ-tocopherol-depressing effect of α-tocopherol. *In vitro* studies have found that γ-tocopherol was more effective than α-tocopherol in inhibiting lipid hydroperoxide formation in liposomes exposed to NO_x (51). Others, however, found that α-tocopherol alone is sufficient to remove any peroxynitrite-derived reactive nitrogen species (52). If γ-tocopherol were the more effective agent against F_2-isoprostanes, the γ-tocopherol-depressing effect of α-tocopherol could have been responsible for the weaker treatment effect in the mixture group. However, the *in vitro* data are inconsistent, and this is a hypothesis that requires further research.

Only one other study on antioxidant supplementation of smokers examined the effect of single agents and combinations on F$_2$-isoprostanes. Reilly *et al.* (39) reported significant decreases in urinary 8-*epi*-PGF$_{2\alpha}$ excretion after 5-day supplementation with either vitamin C alone (2000 mg/day; $n = 5$) or a combination of vitamin C and vitamin E (2000 mg/day and 800 IU/day, respectively; $n = 4$), whereas those receiving vitamin E alone experienced no significant effect. It is possible that vitamin E supplementation in smokers does not decrease F$_2$-isoprostane levels and that the effect they observed in the smokers receiving a combination of vitamin C and E is due to the high-dose vitamin C alone. In addition, Reilly *et al.* found that F$_2$-isoprostane decreased by 59 pmol/mmol creatinine in the group receiving vitamin C alone, but by only 38 pmol/mmol creatinine in the group receiving both vitamin C and vitamin E, consistent with our observations of a weaker effect of the combination than of vitamin C alone.

Another study on supplementation of smokers with vitamin C and E in combination (53), which measured 8-oxo-7,8-dehydro-2′-deoxyguanosine as biomarker of oxidative stress, also did not find an effect of the combination. Thus, the existing evidence, as well as our study, does not indicate that a combination of antioxidants decreases the levels of these oxidative stress biomarkers more effectively than a single agent, vitamin C. However, the reason for this remains unclear.

Clinical Implications. The results of our study suggest that lipid peroxidation resulting from CS can be reduced by treatment with vitamin C, especially in overweight subjects. This is consistent with epidemiological data indicating a reduced risk of smoking-related cancer among persons with a high fruit and vegetable intake (20) and is also consistent with considerable evidence of cardiovascular benefit associated with higher plasma vitamin C levels (54). Epidemiological data also show that as BMI increases, the risk of a variety of cancers, atherosclerosis, and coronary heart disease increases (55, 56). We observed a strong relationship between the oxidative stress biomarker F$_2$-isoprostanes and BMI (trend $P = 0.001$), thus indicating that obesity is associated with elevated oxidative stress, which in turn may be a possible explanation for increased risk of these diseases in obese human subjects. This finding may have important implications for public health. However, smokers should still be urged to quit smoking to prevent smoking-related diseases. Further research is needed to clarify the lack of effect we observed for the combination of vitamin C, vitamin E, and α-lipoic acid on plasma F$_2$-isoprostanes. Future epidemiological studies and interventions should consider stratifying subjects on BMI, measure plasma ascorbic acid status as well as other antioxidants, and consider markers of oxidative damage in both the design and analysis phases.

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