Effect of Combined Supplementation With α-Tocopherol, Ascorbate, and Beta Carotene on Low-Density Lipoprotein Oxidation

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Background. **Data continue to accumulate supporting a proatherogenic role for oxidized low-density lipoprotein (Ox-LDL). Antioxidant micronutrients such as ascorbate, α-tocopherol, and beta carotene, levels of which can be favorably manipulated by dietary measures without side effects, could be a safe approach in inhibiting LDL oxidation. In fact, in vitro studies have shown that all three antioxidants can inhibit LDL oxidation. The present study was undertaken to ascertain both the safety and antioxidant effect of combined supplementation with α-tocopherol, ascorbate, and beta carotene on LDL oxidation.**

Methods and Results. **The effect of combined supplementation with α-tocopherol (800 IU/d) plus ascorbate (1.0 g/d) and beta carotene (30 mg/d) on copper-catalyzed LDL oxidation was tested in a randomized, placebo-controlled study in two groups of 12 male subjects over a 3-month period. Blood samples for the lipoprotein profile, antioxidant levels, and LDL isolation were obtained at baseline and at 3 months. Neither placebo nor combined antioxidant therapy resulted in any side effects or exerted an adverse effect on the plasma lipoprotein profile. Compared with placebo, combined antioxidant therapy resulted in a significant increase in plasma ascorbate and lipid standardized α-tocopherol and beta carotene levels (2.6-, 4.1-, and 16.3-fold, respectively). At baseline, there were no significant differences in the time course curves and kinetics of LDL oxidation as evidenced by the thiobarbituric acid reacting substances (TBARS) assay and the formation of conjugated dienes. However, at 3 months, combined supplementation resulted in a twofold prolongation of the lag phase and a 40% decrease in the oxidation rate. The combined antioxidant group was also compared with a group that received 800 IU of α-tocopherol only. Although the combined antioxidant group had significantly higher ascorbate and beta carotene levels than the group supplemented with α-tocopherol alone, there were no significant differences between the two groups with respect to LDL oxidation kinetics.**

Conclusions. **Combined supplementation with ascorbate, beta carotene, and α-tocopherol is not superior to high-dose α-tocopherol alone in inhibiting LDL oxidation. Hence, α-tocopherol therapy should be favored in future coronary prevention trials involving antioxidants. (*Circulation*. 1993;88:2780-2786.)**

KEY WORDS • lipoproteins • lipids • antioxidants

There is a growing body of data to support the concepts that oxidatively modified low-density lipoprotein (LDL) is proatherogenic[1-4] and exists in vivo.[5-8] Additional support for this concept comes from the finding that antioxidant supplementation will inhibit the progression of atherosclerosis in animal models.[9-12] A clinical trial with antioxidants in humans is crucial in validating the oxidized LDL hypothesis. However, most of the antioxidants tested in animal models to date are chemical compounds—probucol, butylated hydroxytoluene (BHT), and diphenylphenylenediamine (DPPD)—and they carry potential side effects; therefore, their utility for prevention of atherosclerosis in humans is probably limited.[13,14] On the other hand, the dietary micronutrients with antioxidant properties—ascorbate, α-tocopherol, and beta carotene—may provide an alternate approach to protection of LDL against oxidative modification and hence, prevention of atherosclerosis.[13,14] Previous investigations[15-19] have shown that these antioxidant micronutrients can inhibit LDL oxidation when added in vitro. Also, supplementation of the diet with α-tocopherol or ascorbate has been reported to decrease susceptibility of LDL to oxidation.[20-23] Whether these effects are additive when given in combined supplementation, however, has not been determined in depth. Recently, a report appeared using high doses of combined antioxidant supplementation and using a nonrandomized, sequential design. In this report, the authors failed to show an added benefit on LDL oxidation of combined ascorbate, beta carotene, and vitamin E to high-dose vitamin E alone.[24] The present study was carried out to ascertain both the safety and antioxidant effect of combined supplementation with α-tocopherol, ascorbate, and beta carotene on LDL oxidation, as assessed in a randomized, placebo-controlled study.

Methods

This study was approved by the Institutional Review Board, University of Texas Southwestern Medical Center. The design was that of a placebo-controlled ran-

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domized, single-blind study. A total of 24 volunteers were recruited. Participants had to fulfill all of the following criteria to be entered into the study: nonsmoking male subject, not on any vitamin supplements for at least 6 months, alcohol intake <1 oz/d, normal plasma glucose hepatic and renal function, no evidence of malabsorption, pancreatic, or biliary disease, and no acute medical condition for at least 3 months. Fasting blood samples were obtained at baseline for assessment of the complete blood count, plasma glucose, plasma proteins, hepatic function, and renal function; all of these were assayed by standard laboratory techniques.

Blood also was obtained for the lipid and lipoprotein profile, plasma α-tocopherol, ascorbate, and beta carotene levels and for LDL isolation. The samples for LDL isolation were collected in tubes containing EDTA (1 mg/mL). All blood samples were collected on ice, and the plasma was separated by low-speed centrifugation at 4°C. Thereafter, the participants were prescribed either the placebo capsules (soy bean oil) or combined antioxidant supplements comprising ascorbate 1.0 g/d, dl-α-tocopherol 800 IU (727.3 mg) per day, and beta carotene 30 mg/day for 3 months. The doses of antioxidant micronutrients used were based on the published literature to ascertain if safe but high doses will inhibit LDL oxidation. The capsules were provided by Hoffman-La Roche Inc (Nutley, NJ). The two groups were studied in parallel with a third group, which received vitamin E alone (800 IU/d). They were advised to adhere to their usual diet and exercise activity throughout the 12 weeks and to immediately report any side effects. The study subjects returned to the clinic at 12 weeks. At each visit, a clinical examination was performed and blood samples were obtained as described above for the baseline period. Samples for plasma ascorbate were deproteinized with ice-cold 10% metaphosphoric acid, centrifuged, and the supernatant was purged with nitrogen and stored below −20°C in tubes covered with foil. Plasma samples for α-tocopherol and beta carotene were also purged with nitrogen and stored below −20°C in tubes covered with foil.

The lipid and lipoprotein levels were assayed using Lipid Research Clinic methodology except that cholesterol and triglycerides were determined enzymatically.[25] The concentrations of α-tocopherol and beta carotene were measured in plasma, following extraction, by reversed-phase, high-performance liquid chromatography.[26] The levels of both α-tocopherol and beta carotene were standardized to total plasma lipids. The sum of cholesterol and triglyceride levels (milligrams per deciliter) was taken as an estimate of total lipid.[27] Plasma ascorbate levels were determined spectrophotometrically after derivatization with 2,4-dinitrophenylhydrazine.[28]

LDL (density, 1.019 to 1.063 g/mL) was isolated by preparative ultracentrifugation in salt solutions (NaBr, NaCl) containing 1 mg/mL EDTA, as previously described.[15] The isolated LDL was exhaustively dialyzed against 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4, filtered, and stored at 4°C under nitrogen. Protein was measured by the method of Lowry et al,[29] using bovine serum albumin as standard. Stock LDL solutions obtained at 12 weeks were diluted with the saline-EDTA dialysis buffer such that the protein concentration did not vary from the baseline levels by more than 0.5 mg/mL. Oxidation studies were performed within 48 hours after LDL isolation. The oxidation of LDL was undertaken after an overnight dialysis against 1 L of phosphate-buffered saline (PBS). LDL (200 μg protein/mL) was oxidized in a cell-free system using 5 μmol/L copper in PBS at 37°C.[22] The time course of oxidation was studied over an 8-hour period. Each time point was set up in triplicate. At 0, 1, 3, 5, and 8 hours, oxidation was arrested by refrigeration and the addition of 200 μmol/L EDTA and 40 μmol/L BHT.

Two indices of oxidation were used in this study. The lipid peroxide content of oxidized LDL (Ox-LDL) was measured by a modification of the thiobaribituric acid reacting substances (TBARS) assay of Buege and Aust, as described previously.[30] TBARS activity was expressed as malondialdehyde (MDA) equivalents using freshly diluted 1,1,3,3-tetramethoxypropane as the standard. The amount of conjugated dienes formed was determined by monitoring the absorbance of the oxidized LDL against a PBS blank at a wave length of 234 nm.[17] The data are expressed as the increase in conjugated dienes over baseline (ΔA234). The rate of LDL oxidation was determined from the propagation phase of the time course curve using a spline function.[22] The lag phase was obtained by drawing a tangent to the slope of the propagation phase and extrapolating it to the horizontal axis.[14] The lag time constitutes the interval from zero time to the intersection point.[14]

All results are expressed as mean±SEM of multiple determinations unless stated otherwise. A preliminary two-factor repeated-measures ANOVA was used to assess differences in parameters within groups (baseline, 12 weeks) and between groups (placebo versus combined antioxidants). Assumptions of homogeneity of variance and normality were tested using Levene's test and the Wilk-Shapiro test, respectively. Comparisons between groups were made using the Mann-Whitney *U* test. Significance was defined at the 5% level using the two-tailed test of significance. Within-group comparisons were made using the Wilcoxon signed rank test. Pearson correlations were performed on log-transformed data.

Results

There were no significant differences between the placebo and combined antioxidant supplemented groups with respect to age (47.9±5.4 and 49.3±5.0 years, P=.95) and body mass index (27.4±1.2 and 26.1±0.9 kg/m^2, P=.45). None of the participants in either group experienced any side effects as detected by clinical examination and standard laboratory techniques. Also, there were no significant differences between the two groups with respect to plasma lipid and lipoprotein levels. Furthermore, neither placebo nor combined antioxidant supplementation had a significant effect on the plasma lipid and lipoprotein profile (Table 1).

Plasma levels of all three micronutrient antioxidants were similar between the two groups at baseline (Table 2). Also, there were no significant changes in levels in the placebo groups at 3 months. Absolute levels and lipid standardized levels of the lipid-soluble antioxidants are shown in Table 2. Levels of all three antioxidants were significantly higher in the supplemented group at 3 months, compared with placebo. Levels of ascorbate and lipid-standardized α-tocopherol and beta carotene were 2.6-, 4.1-, and 16.3-fold higher in the supplemented group at 3 months compared with base-

TABLE 1. Effect of Placebo and Combined Antioxidant Supplementation on Plasma Lipid and Lipoprotein Profiles

	Time	
Group	**Baseline**	**12 Weeks**
Placebo, mg/100 mL		
Total cholesterol	200.3±11.2	201.5±15.2
Triglycerides	148.1±18.0	139.8±18.8
LDL cholesterol	130.3±10.1	133.9±12.7
HDL cholesterol	45.3±2.2	44.2±2.5
Combined antioxidants, mg/100 mL		
Total cholesterol	217.0±14.1	218.3±10.2
Triglycerides	145.8±22.1	130.8±16.2
LDL cholesterol	147.3±11.8	153.3±9.7
HDL cholesterol	45.1±2.0	43.8±1.5

LDL indicates low-density lipoprotein and HDL, high-density lipoprotein.

line. Although the increase in beta carotene levels were the greatest, the maximum levels of beta carotene (0.84±0.3 mg/g lipid; 0.29±0.12 mg/dL) were substantially lower than the maximum levels of α-tocopherol (9.7±1.4 mg/g lipid; 3.5±0.7 mg/dL) and ascorbate (2.5±0.17 mg/dL).

There were no significant differences in the time course curves of TBARS activity or conjugated dienes formed in the placebo group at baseline and 3 months (data not shown). Also, when the time course curves of oxidation at baseline between the placebo and combined antioxidant groups were compared, no significant differences were noted (Fig 1). However, at 3 months, the mean levels of both TBARS activity and conjugated dienes formed were lower in the antioxidant-supplemented group, as depicted in Fig 2 (asterisks denote significantly lower mean levels).

TABLE 2. Plasma Levels of Ascorbate, α-Tocopherol, and Beta Carotene in the Placebo and Combined Antioxidant Groups

	Groups		
	Placebo		**Combined Antioxidant**
Ascorbate, mg/100 mL			
Baseline	1.16±0.13		1.17±0.10
3 Months	0.97±0.13	*	2.5±0.17
α-Tocopherol, mg/100 mL			
Baseline	0.70±0.07		0.87±0.09
3 Months	0.76±0.13	*	3.5±0.66
α-Tocopherol, mg/g lipid			
Baseline	2.0±0.16		2.4±0.15
3 Months	2.1±0.27	*	9.7±1.4
Beta carotene, mg/100 mL			
Baseline	0.01±0.003		0.02±0.004
3 Months	0.01±0.003	*	0.294±0.12
Beta carotene, mg/g lipid			
Baseline	0.03±0.01		0.05±0.01
3 Months	0.02±0.01	*	0.84±0.30

*$P<.001$.

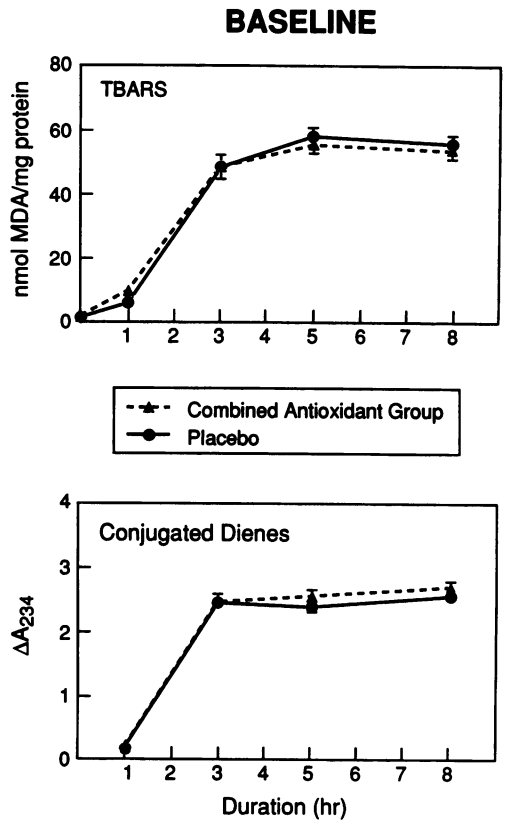

FIG 1. Graphs show comparison of the time course curves of low-density lipoprotein (LDL) oxidation at baseline between the placebo and combined antioxidant group. LDL was isolated and subjected to copper-catalyzed oxidation as described in "Methods." At the indicated times, oxidation was arrested and the samples were assayed for thiobaribituric acid reacting substances (TBARS) activity and the amount of conjugated dienes formed.

Fig 3 shows the kinetics of LDL oxidation as quantitated by the lag time and rate of oxidation. At baseline there were no significant differences between the two groups with respect to both the lag time and rate of oxidation. However, at 12 weeks, the combined antioxidant group had significantly increased lag times and significantly decreased oxidation rates. Similar data were obtained when the lag time and oxidation rates of the time course curves of conjugated diene formation were analyzed; for example, the prolongation in the lag phase was 2.1-fold in the TBARS assay and 1.8-fold in the conjugated diene assay. Compared with placebo, the oxidation rate was decreased 43.9% in the TBARS assay and 35.3% in the conjugated diene assay.

Since this study was done in parallel with our previously reported α-tocopherol study[22] and the subjects were recruited from the same population, we compared the combined antioxidant group with the α-tocopherol group to determine if combined supplementation re-

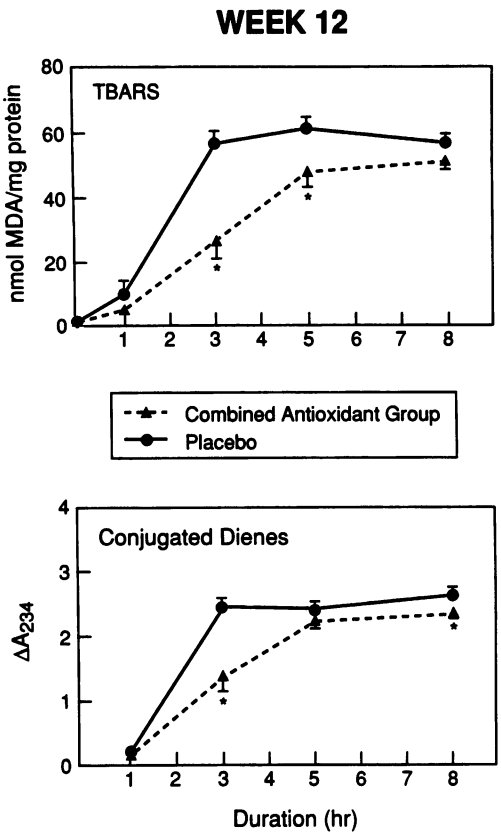

FIG 2. Graphs show comparison of the time course curves of low-density lipoprotein (LDL) oxidation at 12 weeks between the placebo and combined antioxidant groups. LDL samples were subjected to oxidation and the amount of thiobaribituric acid reacting substances (TBARS) activity and conjugated dienes formed were assayed as described in "Methods." Asterisks denote significantly lower levels in the combined antioxidant group: TBARS panel (3 hours, $P=.0002$; 5 hours, $P=.03$). Conjugated diene panel (3 hours, $P=.003$; 8 hours, $P=.04$).

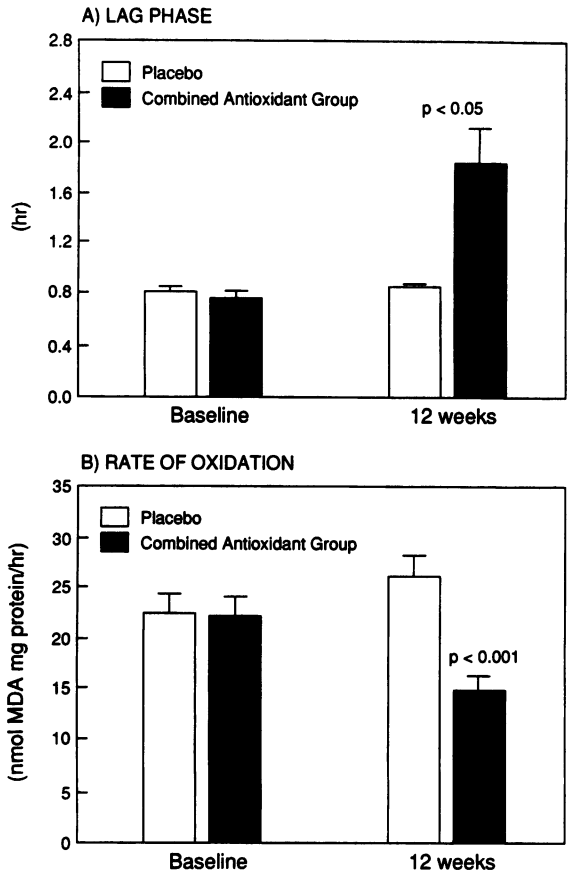

FIG 3. Bar graphs show effect of combined antioxidant supplementation on the kinetics of low-density lipoprotein (LDL) oxidation. The rate of LDL oxidation was determined from the propagation phase of the time course curve using a cubic spline. The lag phase was obtained by drawing a tangent to the slope and extrapolating it to the horizontal axis.

sulted in a greater inhibition of LDL oxidation. As shown in Table 3, the groups were matched for age and body mass index; also, their plasma lipoprotein profiles were similar. Furthermore, there were no significant differences between the two groups at baseline with respect to the levels of micronutrients and parameters of LDL oxidation kinetics. At 3 months, as expected, both plasma ascorbate and beta carotene levels were significantly increased in the combined antioxidant group (Table 3). However, when the LDL oxidation kinetics were compared, there were no significant differences in the lag phase and oxidation rates between the two groups. Also, there were no significant differences in the time course curves of TBARS activity or conjugated diene formation between the α-tocopherol and the combined antioxidant groups (data not shown).

Discussion

Although several lines of evidence support the in vivo existence of Ox-LDL, the most persuasive data on the role of Ox-LDL in atherogenesis derives from studies showing that antioxidants prevent atherosclerosis in animal models.[9-12]

While some antioxidants such as DPPD and BHT prevented atherosclerosis in animal models, their side effects preclude their use in human subjects.[14] A better alternative may be to use dietary micronutrients with antioxidant properties such as ascorbate, α-tocopherol, and beta carotene. Their plasma levels can be manipulated by dietary measures presumably without causing side effects.[13,31,32] Previous investigations have revealed that ascorbate, α-tocopherol, and beta carotene interfere with oxidation of LDL in in vitro systems.[13] In addition, it has been reported that α-tocopherol decreases the susceptibility of LDL to oxidation when given as supplements to humans.[21-24] Few studies, however, have tested the effect of combinations of nutrient antioxidants on LDL oxidation.[24] Accordingly, the major goal of the present study was to determine both the safety and antioxidant effect of combined supplementation of these three micronutrients on LDL oxidation in a placebo-controlled, randomized study.

The present study records at least two significant observations. First, combined supplementation with high doses of ascorbate, α-tocopherol, and beta carotene does not appear to cause any significant side effects over the 3-month study period. Also, unlike other chemical antioxidants such as probucol and BHT, the current antioxidant

TABLE 3. Comparison of Low-Density Lipoprotein Oxidation Kinetics Between α-Tocopherol and Combined Antioxidant Groups

	α-Tocopherol		Combined Antioxidants
Age, y	47.4±4.5		49.3±5.0
Body mass index, kg/m²	25.3±0.9		26.1±0.9
Baseline			
Ascorbate, mg/100 mL	1.2±0.1		1.2±0.1
α-Tocopherol, mg/g lipid	2.3±0.2		2.4±0.2
Beta carotene, mg/g lipid	0.06±0.01		0.05±0.01
Lag phase, h	0.86±0.03		0.76±0.05
Oxidation rate, nmol MDA/mg protein/h	22.0±2.0		22.0±1.9
3 Months			
Ascorbate, mg/100 mL	1.1±0.1	*	2.5±0.2
α-Tocopherol, mg/g lipid	9.9±1.4		9.7±1.4
Beta carotene, mg/g lipid	0.08±0.02	*	0.84±0.30
Lag phase, h	1.90±0.28		1.82±0.28
Oxidation rate, nmol MDA/mg protein/h	15.3±2.4		14.7±1.4

*$P<.001$.

"cocktail" did not have a deleterious effect on the plasma lipoprotein profile. These findings support the recommendation of a recent consensus conference that these micronutrient antioxidants should be the first choice of any clinical trial evaluating the role of antioxidants for prevention of atherosclerosis.[33]

Among the antioxidants studied, beta carotene levels increased about 16-fold during dietary supplementation; however, the absolute level (milligrams per gram of lipid) on supplementation was only about one-twelfth that achieved by α-tocopherol. The latter increased approximately fourfold during supplementation. Of particular interest was the finding that ascorbate levels increased by only 2.6-fold despite an intake of 1000 mg/d. These increments in plasma levels of each micronutrient during combined supplementation are similar to those reported previously for micronutrients given individually.[20,21-23,34,35] Thus combined supplementation does not appear to affect the absorption and plasma levels of each of these antioxidant micronutrients and hence result in a negative interaction.

The current results indicate clearly that combined supplementation with antioxidants resulted in a significant inhibition of LDL oxidation. This was most clear when the kinetics of oxidation were compared. In addition to prolonging the lag phase approximately twofold, combined supplementation decreased the oxidation rate by 40%. To ascertain if the combined antioxidant supplementation was superior to α-tocopherol alone, the two groups were compared, since they were matched. Despite combined supplementation resulting in a substantial and significant increase in plasma ascorbate (2.3-fold) and beta carotene levels (10.8-fold) compared with the α-tocopherol group, there were no significant differences between the two groups when LDL oxidation kinetic parameters such as lag phase and oxidation rates were compared at 3 months. This finding of no added benefit of combined supplementation compared with α-tocopherol alone is in agreement with a recent report by Reaven et al.[24] In their study using twofold higher doses of all three micronutrients compared with the present study, the authors also failed to show a superior antioxidant effect of combined supplementation compared with α-tocopherol alone. Although both studies arrive at a similar conclusion, it should be pointed out that the designs were very dissimilar. While the present study was a randomized, placebo-controlled study, these investigators used a nonrandomized sequential design. Also, since the authors administered beta carotene during the α-tocopherol phase, they cannot completely rule out a synergistic effect of these two antioxidants with beta carotene potentiating the effect of α-tocopherol. More important, these two studies clearly show that high doses of α-tocopherol, ranging from 727 to 1600 mg, are safe and significantly inhibit LDL oxidation.

While it would appear from these studies that ascorbate and beta carotene do not confer an added antioxidant effect, it should be pointed out that the doses of α-tocopherol administered are very high (greater than 20-fold the RDA) and in this type of antioxidant environment, the milder antioxidant effects of beta carotene and ascorbate are probably lost.

More recently we re-examined the effects of beta carotene on oxidative modification of LDL both in a cell-free system and a cellular system.[19] Beta carotene inhibited LDL oxidation in both systems and prevented its subsequent uptake by macrophages. Also Navab et al[36] showed that preincubation of cocultures of aortic endothelial cells and smooth muscle cells with beta carotene prevented LDL modification and its induction of monocyte transmigration. Furthermore, in a recent report it was demonstrated that beta carotene in vitro inhibited the oxidation of both LDL and Lp(a).[37] In the only other study that investigated the effect of beta carotene supplementation on LDL oxidation, the authors showed in a population of smokers that beta carotene had a mild but significant effect on the lag phase of oxidation but did not alter the oxidation rate.[23] This effect was substantially lower than that obtained with α-tocopherol.[23] However, it should be noted that at the doses given during supplementation in that study and in the present report, the concentrations of α-tocopherol were much higher (at least 5.7-fold) than those of beta carotene. Thus, the lower concentrations of beta carotene may have lessened its contribution in preventing LDL oxidation.

It also must be noted that the test system used may not fully determine the efficacy of beta carotene for prevention of atherosclerosis. For example, a recent preliminary report of a clinical trial in a group of male subjects in which beta carotene was used suggested that supplementation reduced not only major coronary events but all major vascular events.[38] In view of the possibility of its direct protection against atherogenesis and since in vitro studies indicate that beta carotene can interfere with LDL oxidation, further investigation on the effect of beta carotene is warranted before the role of beta carotene as a cardioprotective antioxidant can be established or dismissed.

In vitro studies from our laboratory[15,16] have shown that ascorbate is a potent inhibitor of LDL oxidation. A potentially important finding of our in vitro investigations[16] was that ascorbate preserves the other antioxidants in LDL (α-tocopherol and beta carotene). This preservation was not observed with a chemical antioxidant, probucol. This preservation of tocopherol in LDL by ascorbate has been confirmed by Kagan et al.[39] Previous studies in experimental animals have shown that ascorbate supplementation leads to increased plasma and tissue concentrations of α-tocopherol.[40] Thus, ascorbate could indirectly protect LDL against oxidation by preventing loss of α-tocopherol and beta carotene.[16] The higher ascorbate levels during supplementation may have raised concentrations of lipid-soluble antioxidants in both plasma and the LDL particle and thus added to LDL stability. This was recently shown by Reaven et al[24] in their sequential study. At the same time, higher tissue concentrations of ascorbate resulting from supplementation may moderate the peroxide tone of cells and thereby protect LDL against cellular oxidation. This additional protective effect could not be examined in the current study, but it could be an important action of ascorbate that would enhance its benefit in combined supplementation. While the present study and Reaven et al[24] failed to show an added antioxidant effect of ascorbate in combination with other micronutrient antioxidants, Harats et al[20] showed that supplementation with ascorbate alone decreased the susceptibility of LDL to oxidation after an acute episode of smoking in a placebo-controlled study. Also, a recent study suggests that there is an inverse relation between vitamin C intake and both cardiovascular disease and total mortality.[41]

While ascorbate and beta carotene did not result in greater inhibition of LDL oxidation in the report of Reaven et al[24] and the present study, it is possible that these antioxidants could have effects on cellular oxidation and also could mediate their antiatherogenic effect via other potential mechanisms.[42] However, the current results support the use of α-tocopherol supplementation in at least one arm of future clinical prevention trials examining the effect of antioxidants on cardiovascular events and mortality in human subjects. These studies are crucial in validating the oxidized LDL hypothesis further. In fact, two recent reports show that in both men and women, there was an association between a high intake of vitamin E and a lower risk of coronary heart disease.[43,44]

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